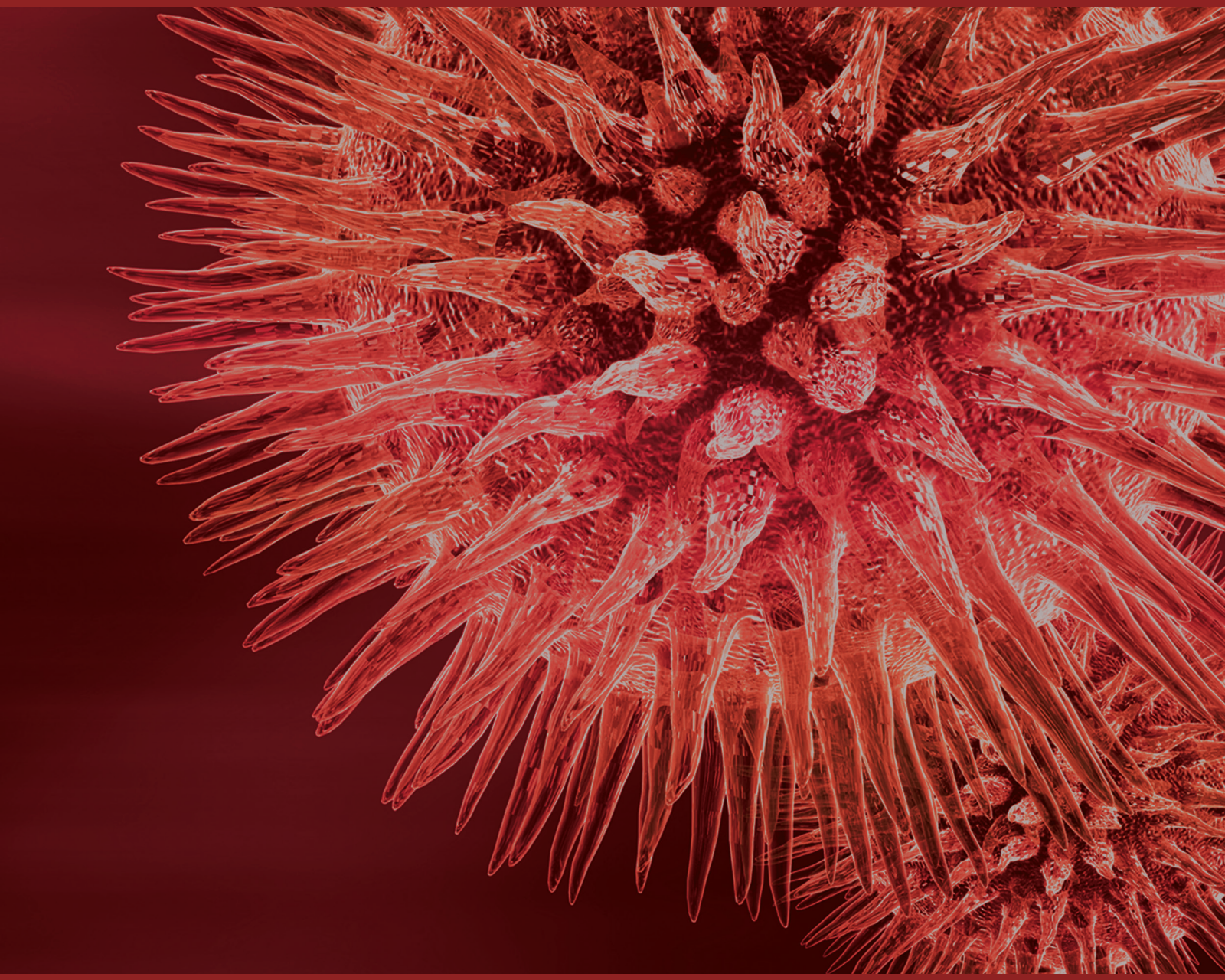
BioMed Research International

Antimicrobial Peptides: Current and Potential Applications in Biomedical Therapies

Guest Editors: Joel E. López-Meza, Alejandra Ochoa-Zarzosa, José E. Barboza-Corona, and Dennis K. Bideshi



Antimicrobial Peptides: Current and Potential Applications in Biomedical Therapies

BioMed Research International

Antimicrobial Peptides: Current and Potential Applications in Biomedical Therapies

Guest Editors: Joel E. López-Meza, Alejandra Ochoa-Zarzosa, José E. Barboza-Corona, and Dennis K. Bideshi

Contents

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 367243, 2 pages
http://dx.doi.org/10.1155/2015/367243

Editorial

Antimicrobial Peptides: Current and Potential Applications in Biomedical Therapies

Joel E. López-Meza,[1] Alejandra Ochoa-Zarzosa,[1] José E. Barboza-Corona,[2] and Dennis K. Bideshi[3,4]

[1] *Centro Multidisciplinario de Estudios en Biotecnología, Universidad Michoacana de San Nicolás de Hidalgo, km 9.5 Carretera Morelia-Zinapécuaro, 58893 Morelia, MICH, Mexico*
[2] *División de Ciencias de la Vida, Departamento de Alimentos, Posgrado en Biociencias, Universidad de Guanajuato, Campus Irapuato-Salamanca, 36500 Irapuato, GTO, Mexico*
[3] *Department of Entomology, University of California Riverside, Riverside, CA 92521, USA*
[4] *California Baptist University, Riverside, CA 92504, USA*

Correspondence should be addressed to Joel E. López-Meza; elmeza@umich.mx

Received 18 December 2014; Accepted 18 December 2014

The evolution of pathogenic bacteria has allowed many microbes to develop resistance mechanisms against conventional antibiotics, leading to the search for new therapeutic alternatives. As such, the clinical uses of antimicrobial peptides (AMPs) are among the most promising alternative options to circumvent the proliferation of antibiotic resistant pathogens. AMPs are produced by a wide variety of organisms and have a broad and largely nonspecific activity, a characteristic that strongly qualifies them as potent candidates for pharmacological applications. Indeed, the continuous discovery of new AMP groups in diverse microorganisms has expanded their potential as a new generation of antimicrobial agents for treating bacterial diseases in humans and also in animals. Intriguingly, the broad spectrum of biological activities reported for many of these molecules suggests that AMPs could also be incorporated in integrative regimen strategies against viral, fungal, and parasitic diseases and cancer, as well as in modulation of the immune system. These possibilities of uses reinforce the importance of studying the biological and applied properties of AMPs.

The articles contained in the present issue include both reviews and basic scientific studies focused on characterizing AMPs from different sources to evaluate their numerous biological activities. This issue comprises the description of effects of naturally occurring AMPs from bacteria, plants, humans, and pigs as well as the effect of synthetic AMPs derived from bovines and humans. In addition, work related to the regulation of AMP expression is also included.

AMPs are part of the innate response elicited by most living forms. In plants, they are produced ubiquitously in roots, seeds, flowers, stems, and leaves, highlighting their physiological importance. The contribution by C. E. Salas et al. "Biologically Active and Antimicrobial Peptides from Plants," provides an overview of what is currently known about bioactive peptides from plants, focusing on their antimicrobial activity and their role in the plant-signaling network and offering perspectives on their potential application.

The wide-ranging functionality of AMPs against infection and disease of the urinary tract expands the list of effects beyond the "antimicrobial effects" originally assigned to them. In the paper by J. Lo and D. Lange "Current and Potential Applications of Host-Defense Peptides and Proteins in Urology," the authors discuss the existing and possible applications of these host-defense peptides in the field of urology.

Transkingdom signaling is a mechanism in which molecules produced by bacteria, such as cyclodipeptides (CDPs), positively or negatively affect the development, growth, or differentiation of eukaryotic organisms. The paper by D. Vázquez-Rivera et al. "Cytotoxicity of Cyclodipeptides from *Pseudomonas aeruginosa* PAO1 Leads to Apoptosis in Human

Cancer Cell Lines" describes that a CDP mixture promoted apoptosis in cultures of HeLa and Caco-2 cell lines in a dose-dependent manner, with 50% inhibitory concentration (IC_{50}) of 0.53 and 0.66 mg/mL, respectively.

Bacteriocins are AMPs synthesized by prokaryotes that inhibit or kill phylogenetically related and/or unrelated microorganisms that share the same microbial niche. These peptides have a potential for diversified use in the food and pharmaceutical industries, agriculture, and apiculture. In the work by M. F. León-Galvan et al. entitled "Molecular Detection and Sensitivity to Antibiotics and Bacteriocins of Pathogens Isolated from Bovine Mastitis in Family Dairy Herds of Central Mexico," the authors showed that bacteriocins synthesized by *Bacillus thuringiensis*, a Gram-positive bacterium, inhibited the growth of multiantibiotic resistance bacteria responsible of mastitis, a very important disease in bovines.

The anticancer activity elicited by AMPs has stimulated intriguing prospects for their use in chemotherapy as cancer remains a cause of high morbidity and mortality worldwide of utmost interest. J. Guzmán-Rodríguez et al. in the paper entitled "Plant Antimicrobial Peptides as Potentials Anticancer Agents" provide an overview of plant AMPs (thionins, defensins, and cyclotides) with anticancer activities with particular emphasis on their mode of action, their selectivity, and their efficacy.

Innate immunity defense is upregulated by antimicrobial peptide elicitors, which are defined as physical, chemical, and biological agents that promote upregulation of endogenous AMPs. The paper by L. A. C. Díaz et al. entitled "Ascorbic Acid, Ultraviolet C Rays, and Glucose but Not Hyperthermia Are Elicitors of Human β-Defensin 1 mRNA in Normal Keratinocytes" investigates the effects of hyperthermia, ultraviolet A rays, and ultraviolet C rays, as well as glucose and ascorbic acid, on the regulation of human β-defensin 1 (*DEFB1*), cathelicidin (*CAMP*), and interferon-γ (*IFNG*) genes in normal human keratinocytes.

The effects of AMPs on bacteria have been extensively studied. However, fewer reports exist regarding their effects on protozoa. The work by J. L. Hernández-Flores entitled "Effect of Recombinant Prophenin 2 on the Integrity and Viability of *Trichomonas vaginalis*" reports that the propeptide and the processed peptide of prophenin 2 affect the integrity and growth of *T. vaginalis* and that proprophenin displays some resistance to proteolysis by *T. vaginalis* proteinases. Its effect on *T. vaginalis*, as well as its low hemolytic activity and short-time stability to parasite proteinases, makes prophenin an interesting candidate for synergistic or alternative treatment against *T. vaginalis*.

The development of novel synthetic analogs of AMPs could enhance their activities, facilitating the development of new drugs. In their paper "Antibacterial Activity of Synthetic Peptides Derived from Lactoferricin against *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212", M. A. León et al. demonstrate that peptides derived from human and bovine lactoferricin exhibit higher or similar activity against *E. coli* (MIC 4–33 μM) and *E. faecalis* (MIC 10–33 μM) compared with lactoferricin protein. Their work shows that it is possible to design and obtain synthetic peptides that exhibit enhanced antibacterial activity.

The low amount of bacteriocins obtained from direct purification from natural producers and the elevated production costs of chemical synthesis have stimulated interests in producing these proteins in heterologous microbial hosts through recombinant genetic manipulations. In the paper by S. Arbulu et al. entitled "Cloning and Expression of Synthetic Genes Encoding the Broad Antimicrobial Spectrum Bacteriocins SRCAM 602, OR-7, E-760, and L-1077, by Recombinant *Pichia pastoris*", the authors evaluated the cloning and functional expression and production of several broad spectrum bacteriocins in recombinant *Pichia pastoris* and assayed these antimicrobials against *Listeria monocytogenes* CECT4032, *E. coli* O157:H7, *Yersinia ruckeri* LMG3279, *Campylobacter jejuni* ATCC33560, and *C. jejuni* NCTC11168.

We hope that this special issue would shed light on major developments in the area of AMPs and attract attention by the scientific community to pursue further investigations leading to the rapid implementation of these molecules in chemotherapeutics.

Acknowledgments

We would like to express our appreciation to all the authors for their informative contributions and the reviewers for their support and constructive critiques in making this special issue possible.

Joel E. López-Meza
Alejandra Ochoa-Zarzosa
José E. Barboza-Corona
Dennis K. Bideshi

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 197608, 9 pages
http://dx.doi.org/10.1155/2015/197608

Research Article

Cytotoxicity of Cyclodipeptides from *Pseudomonas aeruginosa* PAO1 Leads to Apoptosis in Human Cancer Cell Lines

Dolores Vázquez-Rivera,[1] Omar González,[1] Jaquelina Guzmán-Rodríguez,[2] Alma L. Díaz-Pérez,[1] Alejandra Ochoa-Zarzosa,[2] José López-Bucio,[1] Víctor Meza-Carmen,[1] and Jesús Campos-García[1]

[1] *Instituto de Investigaciones Químico Biológicas, Universidad Michoacana de San Nicolás de Hidalgo (UMSNH), Edificio B-3, Ciudad Universitaria, 58030 Morelia, MICH, Mexico*
[2] *Centro Multidisciplinario de Estudios en Biotecnología, Facultad de Medicina Veterinaria y Zootécnia, UMSNH, 58893 Morelia, MICH, Mexico*

Correspondence should be addressed to Víctor Meza-Carmen; victor_meza2004@yahoo.com.mx and Jesús Campos-García; jcgarcia@umich.mx

Received 14 August 2014; Revised 18 October 2014; Accepted 19 October 2014

Academic Editor: J. Eleazar Barboza-Corona

Pseudomonas aeruginosa is an opportunistic pathogen of plants and animals, which produces virulence factors in order to infect or colonize its eukaryotic hosts. Cyclodipeptides (CDPs) produced by *P. aeruginosa* exhibit cytotoxic properties toward human tumor cells. In this study, we evaluated the effect of a CDP mix, comprised of cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Pro-L-Phe) that were isolated from *P. aeruginosa*, on two human cancer cell lines. Our results demonstrated that the CDP mix promoted cell death in cultures of the HeLa cervical adenocarcinoma and Caco-2 colorectal adenocarcinoma cell lines in a dose-dependent manner, with a 50% inhibitory concentration (IC_{50}) of 0.53 and 0.66 mg/mL, for HeLa and Caco-2 cells, respectively. Flow cytometric analysis, using annexin V and propidium iodide as apoptosis and necrosis indicators, respectively, clearly showed that HeLa and Caco-2 cells exhibited apoptotic characteristics when treated with the CDP mix at a concentration <0.001 mg/mL. IC_{50} values for apoptotic cells in HeLa and Caco-2 cells were 6.5×10^{-5} and 1.8×10^{-4} mg/mL, respectively. Our results indicate that an apoptotic pathway is involved in the inhibition of cell proliferation caused by the *P. aeruginosa* CDP mix.

1. Introduction

Pseudomonas aeruginosa colonizes many biological environments, such as soil, plants, and animal tissue, being an important pathogen involved in opportunistic infections in humans [1] and a major cause of nosocomial infections [2]. Several mechanisms for driving infection in the host have been attributed to *P. aeruginosa*, and, among these, the production of toxins, adhesins, pyocyanin, and other virulence factors plays an important role in infecting different hosts, from plants to animals [3, 4]. *P. aeruginosa* produces and senses N-acyl-L-homoserine lactones (AHLs) for cell-to-cell communication via a regulatory mechanism known as quorum sensing (QS), which links the perception of bacterial cell density to gene expression. QS coordinates many physiological processes, such as symbiosis, production of virulence factors, resistance to oxidative stress, antibiotic resistance, motility, biofilm formation, and the progression of *P. aeruginosa* infection in animals [5, 6].

The cyclodipeptides (CDPs) cyclo(D-Ala-L-Val) and cyclo(L-Pro-L-Tyr) have been identified in *P. aeruginosa* cultures, which led to the proposition that CDPs have the ability to inhibit the activity of regulatory LuxR-type proteins that are involved in AHL-dependent QS signaling. This in turn led to the proposition that CDPs and their derivatives, the diketopiperazines (DKPs), represent a new class of QS signals and that they could potentially act as interkingdom

signals. However, the mechanism of action and physiological relevance of CDPs are poorly understood [7, 8].

DKPs are cyclized molecules comprising two amino acids bound by two peptide bonds; they are produced by a wide range of organisms, from bacteria to fungi and animals (Figure 1(a)) [9, 10]. DKPs belong to the nonribosomal peptides that are synthesized in microorganisms by a multifunctional assembly of enzymes known as nonribosomal peptide synthases [10] and by CDP synthases, another kind of enzymes that utilizes aminoacylated transfer RNAs as substrates instead of free amino acids [11].

CDPs are structurally diverse, and they have been implicated in multiple functions; the CDPs cyclo(D-Ala-L-Val) and cyclo(L-Pro-L-Tyr) have been identified as a new class of QS autoinducers in *Pseudomonas* strains, based on their ability to activate AHL-dependent biosensors [12–14]. The CDP cyclo(L-Phe-L-Pro) isolated from *Lactobacillus plantarum* exhibited an antifungal effect against *Fusarium sporotrichioides* and *Aspergillus fumigatus* [15], while the CDPs cyclo(L-Leu-L-Pro), cyclo(L-Phe-L-Pro), cyclo(L-Val-L-Pro), cyclo(L-Trp-L-Pro), and cyclo(L-Leu-L-Val) isolated from the deep-sea bacterium *Streptomyces fungicidicus* showed antifouling effects [16]. Moreover, synthetic CDPs such as cyclo(Phe-Pro) induced apoptosis in the HT-29 colon cancer cell line [17], and cyclo(L-Cys-L-Leu) exhibited potential for scavenging free radicals [18]. Recently, it was reported that *P. aeruginosa* is capable of interacting with the plant *Arabidopsis thaliana* via the secretion of CDPs such as cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Pro-L-Phe), appearing to mimic the biological role of auxin, a natural plant hormone [12] (Figure 1(b)). In *Staphylococcus aureus*, the aureusimines A/B, comprised of the CDP cyclo(L-Val-L-Tyr) and cyclo(L-Val-L-Phe), respectively, are involved in the regulation of bacterial virulence factors in a murine host [19]; similarly, the CDP cyclo(L-Phe-L-Pro) in *Vibrio cholerae*, *V. parahaemolyticus*, and *V. harveyi* is involved in controlling the expression of genes that are important in pathogenicity [20]. Moreover, it was reported that CDPs and DKPs may induce cell death in several cancer cell lines [21], by affecting biological processes such as microtubule polymerization; for example, cyclo(D-Tyr-D-Phe), isolated from *Bacillus* species, induced apoptosis via caspase-3 activation in the A549 pulmonary adenocarcinoma cell line [22]. In addition, it was reported that the CDPs cyclo(L-Leu-L-Pro) and *cis*-cyclo(L-Phe-L-Pro) isolated from *Lactobacillus* exhibited antiviral activity against the influenza A (H3N2) virus [23].

Although, in the context of bacteria-mammalian interaction, it has been suggested that CDPs could play an important role in bacterial pathogenesis, bacteria-host signaling, or mammalian cell growth, the mechanisms involved are unknown. Therefore, in this study, we focused on investigating the cellular effect of CDPs produced from *P. aeruginosa* strain PAO1, a pathogenic bacterium in humans that is capable of secreting the CDPs, cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Pro-L-Phe) into the culture medium (Figure 1(b)). The biological effects of these CDPs on the growth and/or pathogenesis of mammalian cells remain unknown; the *P. aeruginosa* CDPs could be involved in bacterial host colonization phenomena during disease episodes, where antiproliferative or anti-immune properties of these compounds could affect the host organism. In this regard, we employed the HeLa cervical adenocarcinoma and Caco-2 colorectal adenocarcinoma cell lines as host models in this study.

2. Materials and Methods

2.1. Chemicals and Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotic antimycotic solution (100X) penicillin, streptomycin, and amphotericin B were purchased from Sigma-Aldrich Co. 4,6-diamidino-2-phenylindole (DAPI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. Alexa Fluor 488 annexin V and the PI/dead cell apoptosis kit were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Tissue-culture plastic ware was acquired from Corning (Tewksbury, MA, USA).

The *P. aeruginosa* CDP mix was characterized as described previously [12]. Briefly, the *P. aeruginosa* WT strain was placed in 100 mL of Luria Bertani (LB) medium and incubated for 24 h at 30°C for bacterial growth. Cell-free supernatants were prepared by centrifugation (10,000 ×g, 25°C for 10 min). The resulting supernatant was extracted twice with ethyl acetate supplied with acetic acid (0.1 mL/L). The extracts were evaporated to dryness using a rotavapor at 60°C (Buchi Co., Lawil, Switzerland). The residue was solubilized in methanol : acetonitrile (1 : 1) and analyzed by GC-MS as described [12]. The CDP mix is constituted by the cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Pro-L-Phe) in a 1 : 1 : 1 molar ratio. For dose-response assays, the CDP mix was evaporated to dryness, weighed out, and dissolved with DMSO to prepare a 100 mg/mL concentration as stock solution.

2.2. Cell Line Growth. The human cancer cell lines HeLa and Caco-2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell procedures were performed under class II biological safety cabinets. Cells were cultured in DMEM supplemented with 10% (v/v) FBS (complete medium) and 1% antibiotic (10,000 units of penicillin, 10 mg streptomycin, and 25 μg of amphotericin B per mL) solution. The cultures were fed twice a week and maintained at 37°C under 80% humidity and incubated in an atmosphere of 5% CO_2. HeLa and Caco-2 cells were collected by trypsinization using trypsin/EDTA buffered solution for 5 min at room temperature, followed by the addition of 5 mL of serum-enriched medium (CM) to stop trypsin action. After trypsinization the cells were collected and washed with CM. Finally, cells were counted in a hemocytometer chamber and incubated in fresh CM media.

2.3. Cell Viability Assay. Cell viability was determined by the MTT colorimetric method using thiazolyl blue tetrazolium bromide (Sigma-Aldrich Co). Briefly, HeLa and Caco-2 cells were seeded in 96-well flat-bottomed plates at a density of

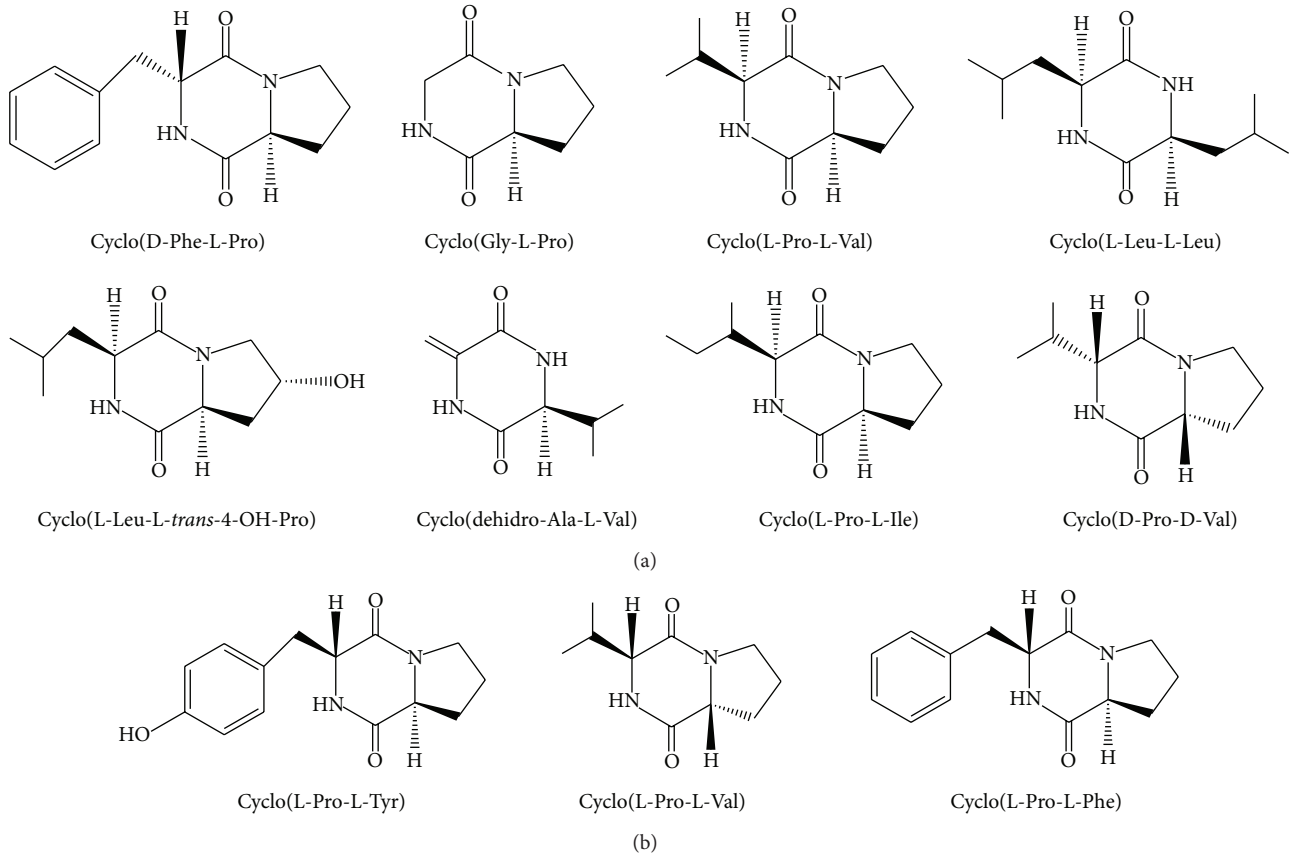


Figure 1: Chemical structures of cyclodipeptides (CDPs) from bacteria. (a) Structures of CDPs synthesized by some bacterial species, modified from [9, 10]. (b) CDPs isolated from *Pseudomonas aeruginosa* strain PAO1. Isolation of CDPs was carried out according to the previously reported protocol [12]. A mixture of CDPs, mainly comprised of cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Pro-L-Phe) in a 1 : 1 : 1 molar ratio, was used in this study.

3×10^4 cells per well in 200 μL of CM and incubated for 24 h at 37°C as described above. Then, the medium was removed and replaced with new CM or serum-free medium (SS). Then, cells were incubated with CDP mix solution at indicated concentration. Cells were incubated for another 24 h at 37°C. To determine cell viability, 10 μL of 5 mg MTT per mL in PBS was added to each well and incubated for 4 h at 37°C. Finally, 100 μL of 2-propanol/1 M HCl (19 : 1 v/v) was added to dissolve the formazan crystals. Absorbance measurements were conducted utilizing a microplate spectrophotometer (IMarK Microplate Reader, BIO-RAD, Hercules, CA, USA) at 595 nm.

2.4. Necrosis and Apoptosis Assay. HeLa and Caco-2 cell lines were seeded in 96-well flat-bottomed plates at a density of 3×10^4 cells per well in 200 μL of CM and incubated for 24 h at 37°C. Then, cells were synchronized with SS medium for 12 h at 37°C and were incubated with different concentrations of CDPs mixture. DMSO was used as control at same concentration used to dissolve the CDP mix. To determinate the apoptotic effect, cells were collected by centrifugation at 2,000 ×g for 10 min. The pellet was suspended in 20 μL of SS medium and treated with annexin V and propidium iodide (PI) (Dead Cell Apoptosis Kit; Molecular Probes, Invitrogen Life Technologies, Carlsbad, CA, USA) following the indications recommended by the manufacturer. Fluorescence was immediately quantified by flow cytometry (FC) using a BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA). The populations of cells for each of the treatments were gated in forward scatter and side scatter dot plots to eliminate cell debris. Populations corresponding to auto- or basal-fluorescence were located in the left quadrant and cells with emission of fluorescence increasing at least one log unit value were located in the right quadrant of the dot plots. In addition, the percentage of fluorescent cells (PFC) and median fluorescence intensity (FI) were determined in monoparametric histograms of fluorescence emission obtained from the dot plots and labeled as PFC and as relative units of fluorescence. The equipment was calibrated using Spherotech 8-peak (FL1–FL3) and 6-peak (FL-4) validation beads (BD Accuri, San Jose, CA, USA). For apoptosis and necrosis assays, fluorescence for annexin V in emission fluorescence channel FL1 at 495/519 nm and for propidium iodide in the FL2 channel at 535/617 nm was monitored. A minimum of 20,000 cellular events were analyzed.

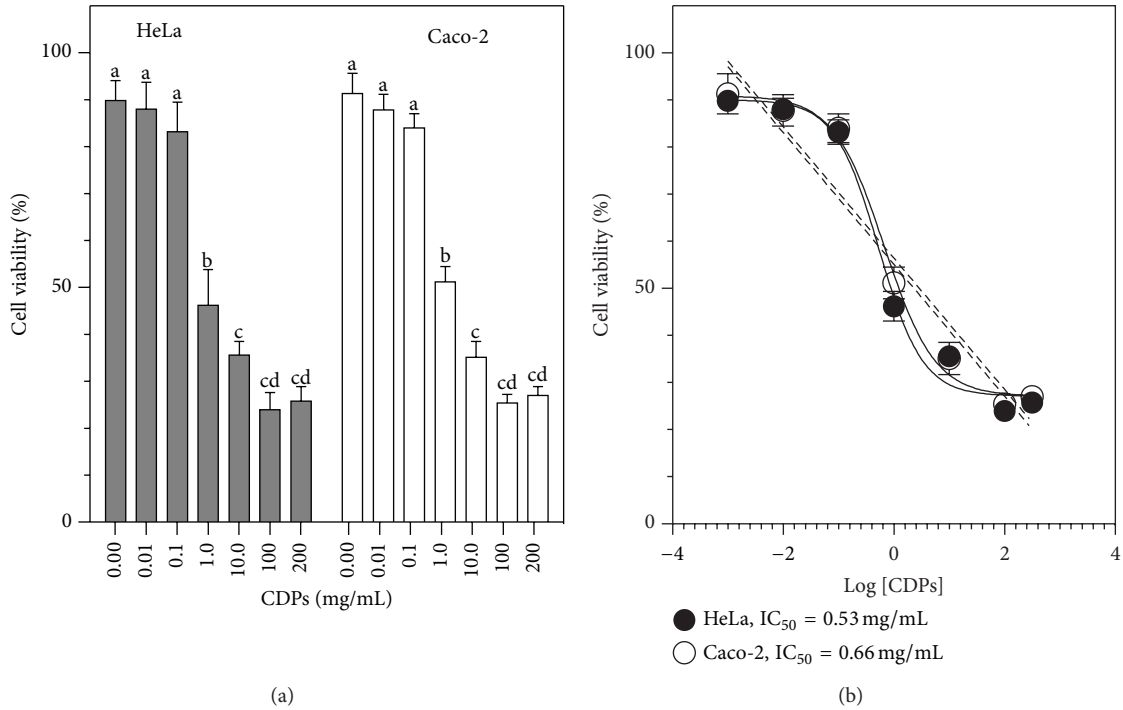


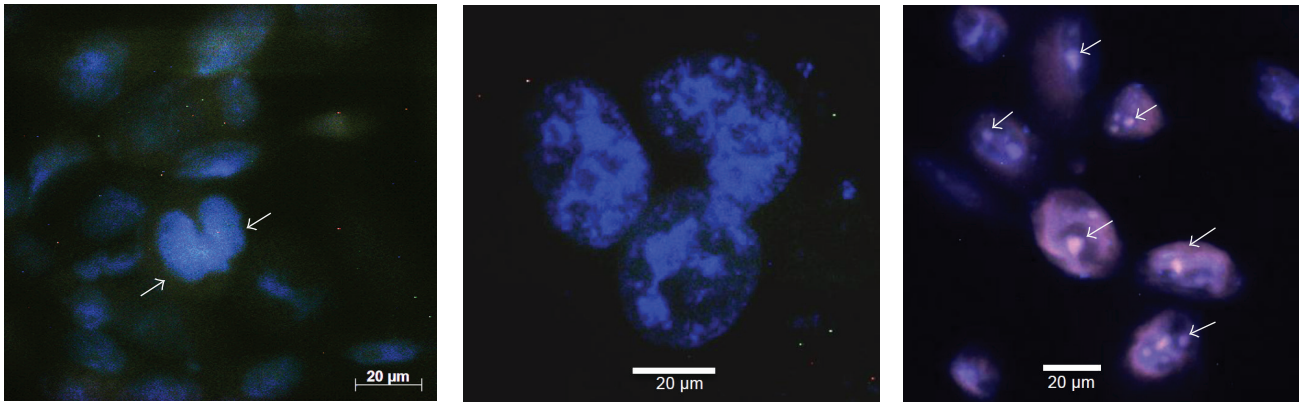
Figure 2: Effect of cyclodipeptides from *Pseudomonas aeruginosa* on HeLa and Caco-2 cell viability. HeLa and Caco-2 cells were incubated in CM medium containing the CDP mix for 24 h. (a) Viability was determined by the MTT assay and quantitation of fluorescence. Bars represent the mean value ± the standard error (SE) of three independent experiments. One-way analysis of variance was carried out, with Tukey's post hoc test; $n = 6$. Values for SE ($P < 0.05$) are shown in lowercase letters. (b) Nonlinear regression analysis of dose-response for the inhibition of viability by the CDP mix; 95% confidence interval, $P < 0.001$. HeLa: 50% inhibitory concentration (IC_{50}) = 0.53 mg/mL; R^2 = 0.96. Caco-2: IC_{50} = 0.66 mg/mL, R^2 = 0.93.

2.5. Cell Image Captures. HeLa and Caco-2 cells were seeded in 12-well flat-bottomed plates with sterile-covered round objects covered with collagenase at a density of 1×10^4 cells per well with one mL of CM and incubated for 24 h at 37°C. Cells were incubated with serum-free medium (SS) for 12 h at 37°C and an atmosphere of 5% CO_2 and incubated with different concentrations of the CDP mix. After 12 h of treatment, the cells were washed with PBS. Cells were fixed with paraformaldehyde (PFA at 4%) for 10 min on ice. Then, cells were incubated with DAPI (1 : 1,000) for 10 min at room temperature. Finally, cells were washed with PBS, and the cover glass was removed and placed into a holder with a drop of PBS and glycerol 1 : 1. Cultured cells were photographed using an inverted phase-contrast microscope (Carl-Zeiss HB0-50, San Diego, CA, USA) equipped with an AxioCam/Cc1 digital camera. Cultures of HeLa and Caco-2 cells were grown in CM and incubated with DAPI and visualized using a confocal microscope (Olympus FV1000, Center Valley, PA, USA). The cells were observed by fluorescence emission between 405 and 505 nm.

3. Results and Discussion

3.1. CDPs from P. aeruginosa Cultures Affect the Viability of Human Cancer Cell Lines. In order to test the effect of CDPs from *P. aeruginosa* on mammalian cell growth, we used the HeLa and the Caco-2 cell lines as models in this study. The HeLa cell line has been extensively employed to test anticancer drugs [24], while the Caco-2 cell line has been used to evaluate the ability of chemicals to cross the intestinal barrier and to study their transport mechanisms [25]. A mixture of CDPs, mainly comprised of cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Pro-L-Phe) in a 1 : 1 : 1 molar ratio, was isolated from the *P. aeruginosa* PAO1 strain, grown on Luria Bertani broth. The CDP mix was applied in a dose-dependent manner to human cells grown in CM medium. The results obtained showed that CDPs caused a decrease in the viability of HeLa and Caco-2 cells in a dose-dependent manner, cell cultures exhibiting 75% dead cells following treatment with the CDP mix at 100 mg/mL (Figure 2(a)). The 50% inhibitory concentration (IC_{50}) for the CDP mix from the PAO1 strain was 0.53 and 0.66 mg/mL, for HeLa and Caco-2 cells, respectively (Figure 2(b)). Although CDPs incubated in serum-free (SS) medium showed slight differences in activity compared to those incubated in serum-containing (CM) medium, these differences were not significant (see Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/197608). These findings indicate that the CDP mix from *P. aeruginosa* inhibited the viability of HeLa and Caco-2 cells and that this effect was independent of the presence or absence of serum.

Microscopic observation of cells following treatment with the CDP mix and staining with DAPI showed that while HeLa cells treated with DMSO solvent alone did not exhibit nuclear



HeLa (control) HeLa + CDPs (10 mg/mL) Hela + CDPs (100 mg/mL)

(a) (b) (c)

Figure 3: Morphological changes in HeLa cells, induced by cyclodipeptides from *Pseudomonas aeruginosa*. ((a), (b)) Images of HeLa cells were taken under phase-contrast confocal microscopy following treatment with the *P. aeruginosa* CDP mix for 24 h and staining with DAPI. Images of cells were taken at 20x magnification (a) or 40x magnification (b). (c) Assessment of nuclear condensation by DAPI staining of cells treated with *P. aeruginosa* PAO1 CDPs (20x magnification). After treatment, the number of apoptotic nuclei was increased and more nuclear condensation was observed in cultures of both cell lines, in comparison with untreated controls. Arrows indicate apoptotic nuclei.

DNA fragmentation (Figure 3(a)), HeLa cells did exhibit nuclear DNA fragmentation after treatment with the CDP mix at a concentration of 10 mg/mL for 24 h (Figure 3(b)); in addition, apoptotic bodies were clearly visible in cells treated with CDPs (Figure 3(c)). These results indicate that the CDP mix from *P. aeruginosa* produced a decrease in cell viability by means of a mechanism of DNA fragmentation.

3.2. Inhibition of HeLa and Caco-2 Cell Viability by CDPs from P. aeruginosa Involves an Apoptotic Pathway. In order to identify the mechanism underlying the decrease in viability of HeLa and Caco-2 cells due to CDP treatment, flow cytometric analysis was carried out using markers for apoptosis (annexin V) or necrosis (propidium iodide; PI), with cultures of both cell lines, after their treatment with the CDP mix. The value for the percentage of fluorescent cells (PFC), corresponding to cells that were positive for the annexin V marker, was ≤14% for negative controls of both HeLa and Caco-2 cells (Figures 4(a) and 4(e)), but ≥97% for both cell lines when actinomycin D was used as an apoptosis inducer (Figures 4(b) and 4(f)). Importantly, HeLa and Caco-2 cells treated with the *P. aeruginosa* CDP mix at a concentration of 0.01 mg/mL showed PFC values ≥50% for the annexin V marker (Figures 4(c) and 4(g); lower-right quadrants), increasing to ≥90% when treated with 1 mg/mL and 0.1 mg/mL CDP mix (Figures 4(d) and 4(h); resp.); however, at higher concentrations of the CDP mix, the PFC values did not show a further significant increase (Figure 4(i)). With respect to PI staining, to identify necrosis in HeLa and Caco-2 cell cultures, positive cells were not detected under the same CDP-treatment conditions (Figures 4(c), 4(d), 4(g), and 4(h); upper-right quadrants). The IC_{50} CDP-mix doses for apoptosis of HeLa and Caco-2 cells, after treatment for 24 h, were calculated as 6.5×10^{-5} mg/mL and 1.8×10^{-4} mg/mL, respectively (Figure 4(j)). These findings indicate that the CDP mix from *P. aeruginosa* caused inhibition of the viability of these two cancer cell lines via an apoptotic mechanism, in a dose-dependent manner. Interestingly, the CDP concentration for apoptosis induction in HeLa cells was 30-fold lower than for Caco-2 cells (Figure 4(j)). In addition, the CDP concentration for apoptosis induction was two log units lower than that for nonapoptotic cell death in both the cancer cell lines tested. These findings were confirmed by microscopic observation of cells; HeLa and Caco-2 cells exhibited apoptotic cell morphology following treatment with the CDP mix, at a concentration of 0.1 mg/mL (Figure 5), and similar morphology was observed following treatment with actinomycin D (apoptosis control; Figures 5(b) and 5(h)). These results indicate that CDPs from *P. aeruginosa* caused a decrease in the cell viability of HeLa and Caco-2 cell lines by means of a mechanism that involves apoptotic pathways.

These two cell lines are the most commonly used for testing drugs, for which details of molecular mechanisms of transport and cell signaling, among other biological processes, have been described. It is noteworthy that similar compounds from other organisms induce apoptosis in different cell lines, for example, cyclo(L-Pro-L-Tyr) and cyclo(L-Pro-L-Phe) isolated from *Bacillus* species. Although these CDPs were not tested for their effects on mammalian cell viability, they provoked a slight decrement in the phosphorylation of the AKT1 serine/threonine kinase at a concentration of 0.01 mg/mL in U-87 MG cells from human glioblastoma, where AKT1 inactivation is an important event that leads to apoptosis [26, 27]. The quest for novel molecules with properties involved in the control of cancer cell growth is a scientific field in growing demand [28]. Natural molecules with antiproliferative activity are considered more specific for their target than synthetic molecules; one of the probable reasons for this is that molecules produced from biological organisms such as *P. aeruginosa* tend to have more chiral

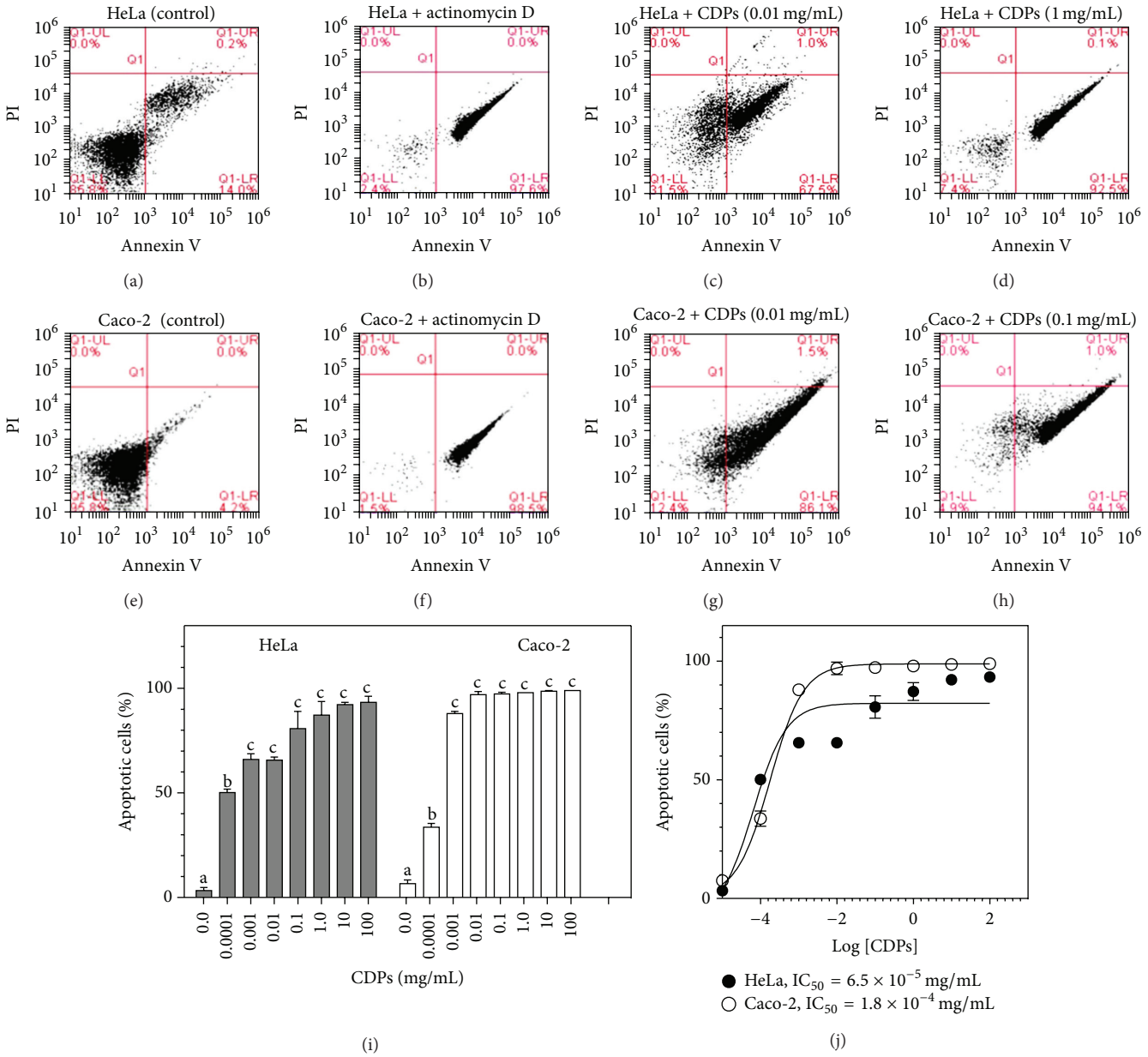


FIGURE 4: Induction of apoptosis in HeLa and Caco-2 cells by cyclodipeptides from *Pseudomonas aeruginosa*. HeLa and Caco-2 cells were incubated in CM medium after treatment with the CDP mix for 24 h. HeLa cells were stained with annexin V and propidium iodide and analyzed by flow cytometry. ((a)–(h)) Schematic diagrams of dot plots, showing the quadrant divisions for the determination of apoptosis using the annexin V and PI probes. The populations of cells for each of the treatments were gated in the forward scatter and side scatter analyses, in order to eliminate dead cells and cell debris. Populations corresponding to autofluorescence or basal fluorescence are located in the lower-left quadrants. Cells with increased fluorescence emission of at least one log unit are located in the lower-right quadrants. The percentage of fluorescent cells is indicated in the dot plots. HeLa cell treatment: (a) DMSO (0.05%; negative control); (b) actinomycin D (50 mg/mL; positive control); (c) 0.01 mg/mL CDP mix; (d) 1.0 mg/mL CDP mix. Caco-2 cell treatment: (e) DMSO (0.05%; negative control); (f) actinomycin D (50 mg/mL; positive control); (g) 0.01 mg/mL CDP mix; (h) 0.1 mg/mL CDP mix. (i) Dose-response plot of apoptotic cell induction by CDP treatment. Percentages of fluorescent cells, determined from dot plots, were used in this analysis. Bars represent the mean value ± the standard error (SE) of three independent experiments. One-way analysis of variance was carried out, with Tukey's post hoc test; $n = 6$. Values for SE ($P < 0.05$) are shown in lowercase letters. (j) Nonlinear regression analysis of dose-response for the induction of apoptosis by the CDP mix; 95% confidence interval, $P < 0.001$. HeLa: 50% inhibitory concentration (IC_{50}) = 6.5×10^{-5} mg/mL; $R^2 = 0.92$. Caco-2: IC_{50} = 1.8×10^{-4} mg/mL; $R^2 = 0.99$.

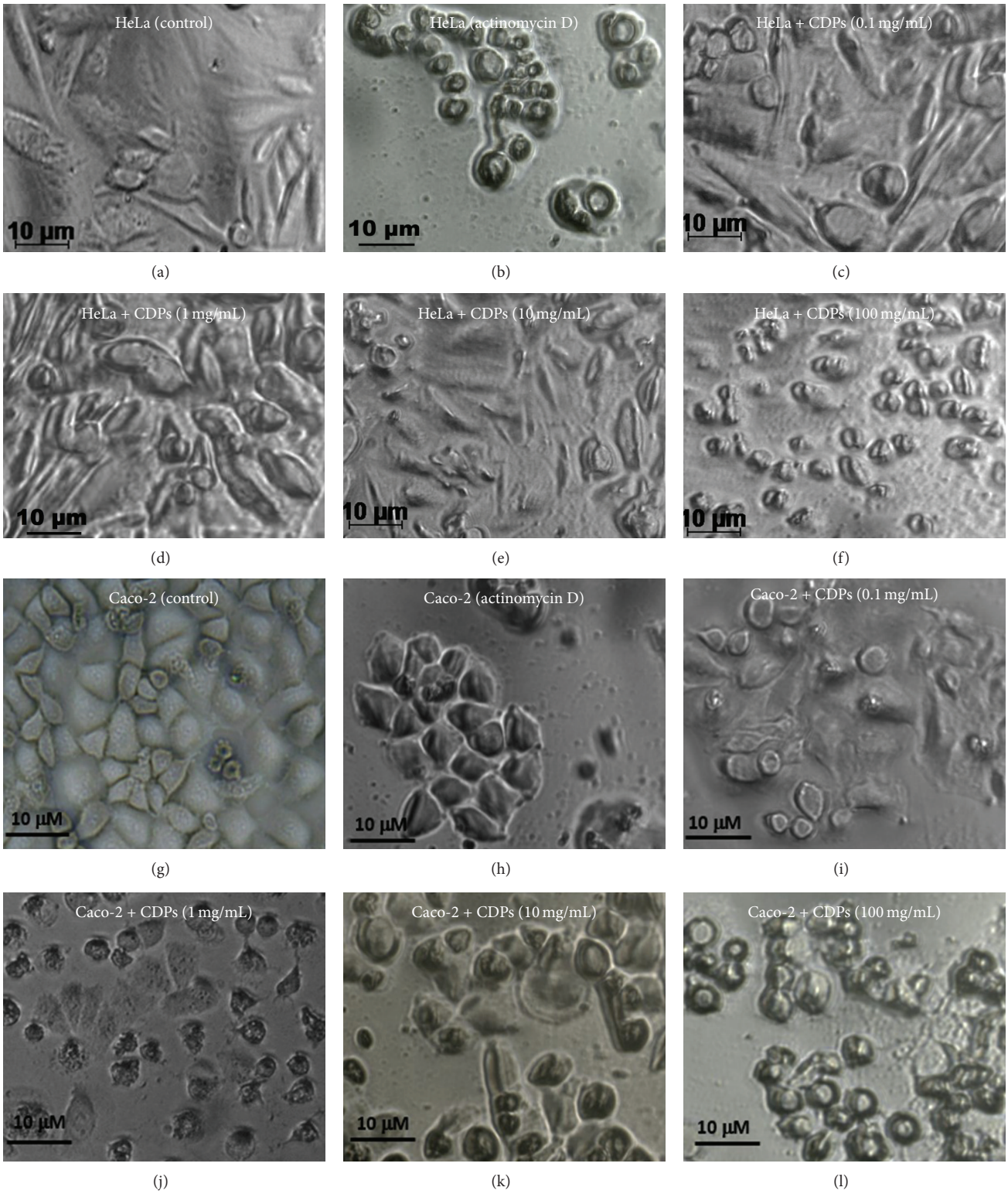


Figure 5: Apoptotic morphological changes in HeLa and Caco-2 cells induced by cyclodipeptides from *Pseudomonas aeruginosa*. Images of cells were taken under phase-contrast inverted microscopy after treatment with the *P. aeruginosa* CDP mix for 24 h. HeLa cell treatment: (a) DMSO (0.05%; negative control); (b) actinomycin D (50 mg/mL; positive apoptosis control); ((c)–(f)) CDP mix at a concentration of 0.1, 1.0, 10, and 100 mg/mL, respectively. Caco-2 cell treatment: (g) DMSO (0.05%; negative control); (h) actinomycin D (50 mg/mL; positive apoptosis control); ((i)–(l)) CDP mix at a concentration of 0.1, 1.0, 10, and 100 mg/mL, respectively.

centers, which give them stereochemical specificity [29]. The synthetic CDP cyclo(L-Phe-L-Pro) is capable of inducing apoptosis via cleavage of poly(ADP-ribose) polymerase in a caspase-dependent manner; however, treatment with 5 mM CDP led to apoptosis in only ≤18% of cells after 72 h [17]. In contrast, in this study, CDPs from *P. aeruginosa* induced apoptosis at a lower concentration (~0.04 mM CDP mix), in 67% and 86% of HeLa and Caco-2 cells, respectively, after 24 h. Our results indicate that the CDP mix from *P. aeruginosa* is more active in inducing cell death, leading us to suggest that the CDPs from *P. aeruginosa* could constitute a novel factor that plays important roles in the steps leading to colonization by *P. aeruginosa* of its host; these roles probably result in the inhibition of cellular growth or affect the viability of immunoprotective cells. Further investigations are necessary in order to determine the molecular target(s) of the CDPs from *P. aeruginosa* that induces apoptosis in mammalian cells.

In conclusion, this study indicates that a CDP mix composed of cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Pro-L-Phe), isolated from the *P. aeruginosa* PAO1 strain, promotes cell death of HeLa and Caco-2 cell cultures in a dose-dependent manner and suggesting an apoptotic pathway as the mechanism underlying the inhibition of cell proliferation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This research was partially funded by CONACYT (106567), FOMIX-C01-117130, and C.I.C. 2.14/UMSNH grants. VR-D received a scholarship from CONACYT.

References

[1] S. E. Battle, F. Meyer, J. Rello, V. L. Kung, and A. R. Hauser, "Hybrid pathogenicity island PAGI-5 contributes to the highly virulent phenotype of a Pseudomonas aeruginosa isolate in mammals," *Journal of Bacteriology*, vol. 190, no. 21, pp. 7130–7140, 2008.

[2] P. M. de Abreu, P. G. Farías, G. S. Paiva, A. M. Almeida, and P. V. Morais, "Persistence of microbial communities including *Pseudomonas aeruginosa* in a hospital environment: a potential health hazard," *BMC Microbiology*, vol. 14, article 118, 2014.

[3] C. K. Stover, X. Q. Pham, A. L. Erwin et al., "Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen," *Nature*, vol. 406, no. 6799, pp. 959–964, 2000.

[4] A. Fajardo, N. Martínez-Martín, M. Mercadillo et al., "The neglected intrinsic resistome of bacterial pathogens," *PLoS ONE*, vol. 3, no. 2, Article ID e1619, 2008.

[5] M. B. Miller and B. L. Bassler, "Quorum sensing in bacteria," *Annual Review of Microbiology*, vol. 55, pp. 165–199, 2001.

[6] U. Müh, B. J. Hare, B. A. Duerkop et al., "A structurally unrelated mimic of a *Pseudomonas aeruginosa* acyl-homoserine lactone quorum-sensing signal," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 45, pp. 16948–16952, 2006.

[7] M. T. G. Holden, S. R. Chhabra, R. de Nys et al., "Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria," *Molecular Microbiology*, vol. 33, no. 6, pp. 1254–1266, 1999.

[8] J. Campbell, Q. Lin, G. D. Geske, and H. E. Blackwell, "New and unexpected insights into the modulation of LuxR-type quorum sensing by cyclic dipeptides," *ACS Chemical Biology*, vol. 4, no. 12, pp. 1051–1059, 2009.

[9] J. Seguin, M. Moutiez, Y. Li et al., "Nonribosomal peptide synthesis in animals: the cyclodipeptide synthase of Nematostella," *Chemistry and Biology*, vol. 18, no. 11, pp. 1362–1368, 2011.

[10] L. Bonnefond, T. Arai, Y. Sakaguchi, T. Suzuki, R. Ishitani, and O. Nureki, "Structural basis for nonribosomal peptide synthesis by an aminoacyl-tRNA synthetase paralog," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 10, pp. 3912–3917, 2011.

[11] S. Lautru, M. Gondry, R. Genet, and J.-L. Pernodet, "The albonoursin gene cluster of *S. noursei*: biosynthesis of diketopiperazine metabolites independent of nonribosomal peptide synthetases," *Chemistry and Biology*, vol. 9, no. 12, pp. 1355–1364, 2002.

[12] R. Ortiz-Castro, C. Díaz-Pérez, M. Martínez-Trujillo, R. E. del Río, J. Campos-García, and J. López-Bucio, "Transkingdom signaling based on bacterial cyclodipeptides with auxin activity in plants," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 17, pp. 7253–7258, 2011.

[13] J. E. González and N. D. Keshavan, "Messing with bacterial quorum sensing," *Microbiology and Molecular Biology Reviews*, vol. 70, no. 4, pp. 859–875, 2006.

[14] G. Degrassi, C. Aguilar, M. Bosco, S. Zahariev, S. Pongor, and V. Venturi, "Plant growth-promoting *Pseudomonas putida* WCS358 produces and secretes four cyclic dipeptides: cross-talk with quorum sensing bacterial sensors," *Current Microbiology*, vol. 45, no. 4, pp. 250–254, 2002.

[15] K. Ström, J. Sjögren, A. Broberg, and J. Schnürer, "*Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(*L*-Phe-*L*-Pro) and cyclo(*L*-Phe-*trans*-4-OH-*L*-Pro) and 3-phenyllactic acid," *Applied and Environmental Microbiology*, vol. 68, no. 9, pp. 4322–4327, 2002.

[16] X. Li, S. Dobretsov, Y. Xu, X. Xiao, O. Hung, and P.-Y. Qian, "Antifouling diketopiperazines produced by a deep-sea bacterium, *Streptomyces fungicidicus*," *Biofouling*, vol. 22, no. 3, pp. 201–208, 2006.

[17] S. C. Brauns, P. Milne, R. Naudé, and M. van de Venter, "Selected cyclic dipeptides inhibit cancer cell growth and indace apoptosis in HT-29 colon cancer cells," *Anticancer Research*, vol. 24, no. 3, pp. 1713–1719, 2004.

[18] T. Furukawa, T. Akutagawa, H. Funatani et al., "Cyclic dipeptides exhibit potency for scavenging radicals," *Bioorganic & Medicinal Chemistry*, vol. 20, no. 6, pp. 2002–2009, 2012.

[19] M. A. Wyatt, W. Wang, C. M. Roux et al., "*Staphylococcus aureus* nonribosomal peptide secondary metabolites regulate virulence," *Science*, vol. 329, no. 5989, pp. 294–296, 2010.

[20] D. K. Park, K. E. Lee, C. H. Baek et al., "Cyclo(Phe-Pro) modulates the expression of ompU in *Vibrio* spp," *Journal of Bacteriology*, vol. 188, no. 6, pp. 2214–2221, 2006.

[21] N. Boyer, K. C. Morrison, J. Kim, P. J. Hergenrother, and M. Movassaghi, "Synthesis and anticancer activity of epipolythiodiketopiperazine alkaloids," *Chemical Science*, vol. 4, no. 4, pp. 1646–1657, 2013.

[22] S. N. Kumar, C. Dileep, C. Mohandas, B. Nambisan, and J. Ca, "Cyclo(D-Tyr-D-Phe): a new antibacterial, anticancer, and antioxidant cyclic dipeptide from *Bacillus* sp. N strain associated with a rhabditid entomopathogenic nematode," *Journal of Peptide Science*, vol. 20, no. 3, pp. 173–185, 2014.

[23] M. K. Kwak, R. Liu, J. O. Kwon, M. K. Kim, A. H. Kim, and S. O. Kang, "Cyclic dipeptides from lactic acid bacteria inhibit proliferation of the influenza a virus," *Journal of Microbiology*, vol. 51, no. 6, pp. 836–843, 2013.

[24] L. J. Jing, M. F. A. Bakar, M. Mohamed, and A. Rahmat, "Effect of selected *Biosenbergia* species on the proliferation of several cancer cell lines," *Journal of Pharmacology and Toxicology*, vol. 6, no. 3, pp. 272–282, 2011.

[25] A. M. Nauli and S. M. Nauli, "Intestinal transport as a potential determinant of drug bioavailability," *Current Clinical Pharmacology*, vol. 8, no. 3, pp. 247–255, 2013.

[26] S. Hong, B.-H. Moon, Y. Yong, S. Y. Shin, Y. H. Lee, and Y. Lim, "Inhibitory effect against Akt by cyclic dipeptides isolated from *Bacillus* sp," *Journal of Microbiology and Biotechnology*, vol. 18, no. 4, pp. 682–685, 2008.

[27] R. Würth, F. Barbieri, and T. Florio, "New molecules and old drugs as emerging approaches to selectively target human glioblastoma cancer stem cells," *BioMed Research International*, vol. 2014, Article ID 126586, 11 pages, 2014.

[28] C. Basmadjian, Q. Zhao, E. Bentouhami et al., "Cancer wars: natural products strike back," *Frontiers in Chemistry*, vol. 2, article 20, 2014.

[29] M. P. de Carvalho and W.-R. Abraham, "Antimicrobial and biofilm inhibiting diketopiperazines," *Current Medicinal Chemistry*, vol. 19, no. 21, pp. 3564–3577, 2012.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 767183, 11 pages
http://dx.doi.org/10.1155/2015/767183

Research Article

Cloning and Expression of Synthetic Genes Encoding the Broad Antimicrobial Spectrum Bacteriocins SRCAM 602, OR-7, E-760, and L-1077, by Recombinant *Pichia pastoris*

Sara Arbulu, Juan J. Jiménez, Loreto Gútiez, Luis M. Cintas, Carmen Herranz, and Pablo E. Hernández

Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid (UCM), Avenida Puerta de Hierro, s/n, 28040 Madrid, Spain

Correspondence should be addressed to Pablo E. Hernández; ehernan@vet.ucm.es

Received 30 July 2014; Accepted 2 November 2014

Academic Editor: J. Eleazar Barboza-Corona

We have evaluated the cloning and functional expression of previously described broad antimicrobial spectrum bacteriocins SRCAM 602, OR-7, E-760, and L-1077, by recombinant *Pichia pastoris*. Synthetic genes, matching the codon usage of *P. pastoris*, were designed from the known mature amino acid sequence of these bacteriocins and cloned into the protein expression vector pPICZαA. The recombinant derived plasmids were linearized and transformed into competent *P. pastoris* X-33, and the presence of integrated plasmids into the transformed cells was confirmed by PCR and sequencing of the inserts. The antimicrobial activity, expected in supernatants of the recombinant *P. pastoris* producers, was purified using a multistep chromatographic procedure including ammonium sulfate precipitation, desalting by gel filtration, cation exchange-, hydrophobic interaction-, and reverse phase-chromatography (RP-FPLC). However, a measurable antimicrobial activity was only detected after the hydrophobic interaction and RP-FPLC steps of the purified supernatants. MALDI-TOF MS analysis of the antimicrobial fractions eluted from RP-FPLC revealed the existence of peptide fragments of lower and higher molecular mass than expected. MALDI-TOF/TOF MS analysis of selected peptides from eluted RP-FPLC samples with antimicrobial activity indicated the presence of peptide fragments not related to the amino acid sequence of the cloned bacteriocins.

1. Introduction

The antimicrobial peptides (AMPs) are broad spectrum small molecular weight compounds with antagonistic activity against bacteria, viruses, and fungi. Among them, bacteriocins form a widely studied and well-characterized group of ribosomally synthesized peptides produced by bacteria, and those produced by lactic acid bacteria (LAB) attract considerable interest primarily as natural food preservatives but also with great interest in exploring their application as therapeutic antimicrobial agents [1–3]. Most LAB bacteriocins are synthesized as biologically inactive precursors or prepropeptides containing an N-terminal extension that is cleaved off during export to generate their biologically active or mature form. The mature peptides are often cationic, amphiphilic molecules that are generally classified into two main classes: the lantibiotics or class I bacteriocins that consist of modified bacteriocins and the class II or nonmodified bacteriocins [4, 5]. The class II bacteriocins have been further divided into several subgroups, from which the class IIa or pediocin-like bacteriocins show a strong antilisterial activity and the N-terminal consensus sequence YGNGV(X)C [5].

Considering the low amount of AMPs obtained from their direct purification from natural producers and the elevated production costs of chemical synthesis, the biological production of bacteriocins by heterologous microbial hosts may provide an opportunity for their production in large amounts and with higher specific antimicrobial activity [6–8]. Furthermore, the production of bacteriocins by yeasts may have some advantages over bacterial cells regarding specific posttranscriptional and posttranslational modifications [9]. Yeasts are also cost-effective producers with large

production and high yields of the desired protein [10]. *Pichia pastoris* (currently reclassified as *Komagataella pastoris*) is being used as heterologous producer of bacteriocins because of its ability to produce large amounts of properly folded and biologically active bacteriocins [8, 11, 12]. *P. pastoris* also produces disulfide-bonded and glycosylated proteins, which are crucial features for functionality [13]. The use of synthetic genes may also constitute a successful approach for heterologous production and functional expression of bacteriocins by recombinant yeasts when the DNA sequence encoding the bacteriocin is not available or difficult to obtain [12]. Furthermore, the use of synthetic genes matching the codon usage of the host microorganism can have a significant impact on gene expression levels and protein folding [14].

Several bacteriocins with broad antimicrobial spectrum have been identified from chicken commensal bacteria including bacteriocin SRCAM 602 produced by *Paenibacillus polymyxa* [15, 16], bacteriocins OR-7 and L-1077 produced by *Lactobacillus salivarius* [17, 18], and bacteriocins E-760 and E 50-52 produced by *Enterococcus* spp. [19, 20]. All these bacteriocins have been reported to be active against Gram-positive and Gram-negative bacteria including *Campylobacter* spp., reducing *Campylobacter* colonization in poultry and considered potentially useful towards on-farm control of this foodborne human pathogen [21]. Most animal studies suggest that these bacteriocins considerably reduce *C. jejuni* colonization in chicken intestine and thus may reduce *Campylobacter* spp. infections in humans [17, 19, 20]. However, to our knowledge, none of the genes encoding these bacteriocins have been sequenced so far. Accordingly, in this study we report the use of synthetic genes designed from the published amino acid sequence of the mature bacteriocins SRCAM 602, OR-7, E-760, and L-1077 and with adapted codon usage for expression by *P. pastoris*, their cloning into the protein expression vector pPICZαA, and their expression by recombinant *P. pastoris* X-33.

2. Materials and Methods

2.1. Microbial Strains and Plasmids. Microbial strains and plasmids used in this study are listed in Table 1. *Enterococcus faecium* T136 and *Pediococcus damnosus* CECT4797 were grown in MRS broth (Oxoid Ltd., Basingstoke, UK) at 32°C. *P. pastoris* X-33 (Invitrogen S.A., Barcelona, Spain) was cultured in YPD medium (Sigma-Aldrich Inc., St. Louis, MO, USA) at 30°C with shaking (200–250 rpm). *Escherichia coli* JM109 (Promega, WI, USA) was grown in LB broth (Sigma-Aldrich) at 37°C with shaking (250 rpm). *Listeria monocytogenes* CECT4032 was grown in LB at 37°C and *Salmonella typhimurium* CECT443 was grown in TSB (Oxoid) at 37°C. *Campylobacter jejuni* ATCC33560 and *C. jejuni* NCTC11168 were grown in BHI supplemented with 1% defibrinated horse serum (BD Bioscience, CA, USA) at 37°C in microaerophilic conditions. *E. coli* O157:H7 was grown in LB at 37°C with shaking (250 rpm). *Yersinia ruckeri* LMG3279 was grown in TSB at 28°C. Zeocin (Invitrogen) was added when needed at concentrations of 25, 100, or 1000 μg/mL. Strains cited as CECT belong to the Colección Española de Cultivos Tipo (Valencia, Spain), ATCC to the American Type Culture Collection (Rockville, MD, USA), and NCTC to the National Collection of Type Cultures (London, UK).

2.2. Basic Genetic Techniques and Enzymes. The published amino acid sequences of mature bacteriocins SRCAM 602, OR-7, E-760, and L-1077 were used as templates to design the nucleotide sequence of the synthetic genes *srcam602, or-7, e-760,* and *l-1077* matching the codon usage of *P. pastoris* X-33. These synthetic genes contained a 5′-nucleotide appendix including a *Xho*I restriction site and a 3′-nucleotide appendix including the termination of translation codon (TAA) and the *Not*I restriction site. All synthetic genes were supplied by GeneArt (Life Technologies, Paisley, UK). DNA restriction enzymes were supplied by New England BioLabs (Ipswich, MA, USA). Ligations were performed with the T4 DNA ligase (Roche Molecular Biochemicals, Mannheim, Germany). *E. coli* JM109 cells were transformed as described by the supplier. Competent *P. pastoris* X-33 cells were obtained as recommended by the supplier and electroporation of competent cells was performed as previously described [22]. Electrocompetent cells were transformed with a Gene Pulser and Pulse Controller apparatus (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. PCR Amplification and Nucleotide Sequencing. Oligonucleotide primers were obtained from Sigma-Genosys Ltd. (Cambridge, UK). PCR amplifications were performed in 50 μL reaction mixtures containing 1 μL of purified DNA, 70 pmol of each primer, and 1 U of Platinum Pfx DNA Polymerase (Invitrogen) in a DNA thermal cycler Techgene (Techne, Cambridge, UK). The PCR-generated fragments were purified by a NucleoSpin Extract II Kit (Macherey-Nagel GmbH & Co., Düren, Germany) for cloning and nucleotide sequencing. Nucleotide sequencing of the purified PCR products was performed using the ABI PRISM BigDye Terminator cycle sequence reaction kit and the automatic DNA sequencer ABI PRISM, model 377 (Applied Biosystems, Foster City, CA, USA), at the Unidad de Genómica, Facultad de Ciencias Biológicas, Universidad Complutense de Madrid (UCM), Madrid, Spain.

2.4. Cloning of the srcam602, or-7, e-760, and l-1077 Synthetic Genes in P. pastoris X-33 and Antimicrobial Activity of the Transformants. The primers and inserts used for construction of the recombinant plasmids are listed in Table 2. Derivatives of plasmid pPICZαA were constructed as follows: primers S602-F, S071-F, and SARP-R were used for PCR amplification from plasmids pMATSRCAM602, pMATOR-7, pMATE-760, and pMATL-1077 of nucleotide fragments in frame with the *S. cerevisiae* α-factor secretion signal, without the Glu-Ala spacer adjacent to the Kex2 protease cleavage site, fused to the *srcam602*, *or-7*, *e-760,* and *l-1077* synthetic genes. Digestion of the above cited fragments with the *Xho*I-*Not*I restriction enzymes permitted ligation of the resulting R-SRCAM602, R-OR7, R-E760, and R-L1077 nucleotide fragments of 136-, 181-, 242-, and 167-bp, respectively, into pPICZαA digested with the same enzymes to generate the plasmid-derived vectors pSRCAM602, pOR-7, pE-760, and pL-1077, respectively. Competent *E. coli* JM109 cells were used

Table 1: Bacterial strains and plasmids used in this study.

Strain or plasmid	Description[a]	Source and/or reference[b]
Strains		
Enterococcus faecium T136	Enterocin A and B producer; MPA positive control	DNBTA
Pediococcus damnosus CECT4797	MPA indicator microorganism	CECT
Escherichia coli JM109	Selection of recombinant plasmids	Promega
Pichia pastoris X-33	Yeast producer	Invitrogen Life Technologies
Plasmids		
pMA-T	Amp^r; carrier of synthetic genes	GeneArt Life Technologies
pPICZαA	Zeo^r; integrative plasmid carrying the secretion signal sequence from the *S. cerevisiae* α-factor prepropeptide and functional sites for integration at the 5′ AOX1 locus of *P. pastoris* X-33	Invitrogen Life Technologies
pMATSRCAM602	Amp^r; pMA-T plasmid carrying the *srcam602* synthetic gene with the *P. pastoris* codon usage	GeneArt Life Technologies
pMATOR-7	Amp^r; pMA-T plasmid carrying the *or-7* synthetic gene with the *P. pastoris* codon usage	GeneArt Life Technologies
pMATE-760	Amp^r; pMA-T plasmid carrying the *e-760* synthetic gene with the *P. pastoris* codon usage	GeneArt Life Technologies
pMATL-1077	Amp^r; pMA-T plasmid carrying the *l-1077* synthetic gene with the *P. pastoris* codon usage	GeneArt Life Technologies
pSRCAM602	pPICZαA derivative with the *srcam602* synthetic gene	This work
pOR-7	pPICZαA derivative with the *or-7* synthetic gene	This work
pE-760	pPICZαA derivative with the *e-760* synthetic gene	This work
pL-1077	pPICZαA derivative with the *l-1077* synthetic gene	This work

[a] Amp^r: ampicillin resistance; Zeo^r: zeocin resistance.

[b] DNBTA: Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid (Madrid, Spain); CECT: Colección Española de Cultivos Tipo Valencia, Spain).

Table 2: Primers and PCR products used in this study.

Primers, PCR products, or bacteriocins	Nucleotide sequence (5′-3′) or description	Amplifications
Primers		
S602-F	GCCATGAGCTCGAATTCTCGAGAAAAG	R-SRCAM602, R-E760, R-L1077
S071-F	GTCCAGAGCTCGAATTCTCGAGAAAAG	R-SRCAM 602, R-E760, R-L1077, R-OR7
SARP-R	AGGTACCATAAGTTGCGGCCGC	R-OR7
ALFA-F	TACTATTGCCAGCATTGCTGC	pPICZαA amplification fragment including the cloned synthetic gene
3AOX1-R	GCAAATGGCATTCTGACATCC	pPICZαA amplification fragment including the cloned synthetic gene
PCR products		
R-SRCAM602	136-bp *Xho*I/*Not*I fragment containing the α-factor Kex2 signal cleavage fused to the mature synthetic *srcam602* gene with the *P. pastoris* codon usage	
R-OR7	181-bp *Xho*I/*Not*I fragment containing the α-factor Kex2 signal cleavage fused to the mature synthetic *or-7* gene with the *P. pastoris* codon usage	
R-E760	242-bp *Xho*I/*Not*I fragment containing the α-factor Kex2 signal cleavage fused to the mature synthetic *e-760* gene with the *P. pastoris* codon usage	
R-L1077	167-bp *Xho*I/*Not*I fragment containing the α-factor Kex2 signal cleavage fused to the mature synthetic *l-1077* gene with the *P. pastoris* codon usage	
Bacteriocins		
BacSRCAM602 (amino acid sequence)	ATYYGNGLYCNKQKHYTWVDWNKASR EIGKITVNGWVQH	
BacSRCAM602 (*P. pastoris* codon usage)	gctacttactacggtaacggtctttactgtaacaagcagaagcact acacttgggttgactggaacaaggcttccagagagatcggtaag atcactgttaacggttgggttcaaca	
BacOR-7 (amino acid sequence)	KTYYGTNGVHCTKNSLWGKVRLKNMK YDQNTTYMGRLQDILLGWATGAFGKTFH	
BacOR-7 (*P. pastoris* codon usage)	aagacttactacggaactaacggtgttcactgtactaagaattcctt gtggggtaaggttagattgaagaacatgaagtacgaccagaaca ctacttacatgggtagattgcaggacatcttgttgggttgggctact ggtgctttcggtaagacttttcat	
BacE-760 (amino acid sequence)	NRWYCNSAAGGVGGAAVCGLAGYVGE AKENIAGEVRKGWGMAGGFTHNKACKS FPGSGWASG	
BacE-760 (*P. pastoris* codon usage)	aacagatggtactgtaactccgctgctggtggtgttggtggtgct gctgtttgtggtttggctggttatgttggtgaggctaaagaaaacat tgctggtgaggttagaaagggttggggtatggctggtggtttcac tcataacaaggcttgtaagtccttcccaggttctggttgggcttctggt	
BacL-1077 (amino acid sequence)	TNYGNGVGVPDAIMAGIIKLIFIFNIRQGY NFGKKAT	
BacL-1077 (*P. pastoris* codon usage)	actaactacggtaacggtgttggtgttccagacgctattatggctg gtatcatcaagttgatcttcatcttcaacatcagacagggttacaac ttcggtaagaaggctact	

for cloning and vector propagation and the resulting transformants were confirmed by PCR amplification and sequencing of the inserts. Subsequently, the *Sac*I-linearized pSRCAM602, pOR-7, pE-760, and pL-1077 vectors were transformed into competent *P. pastoris* X-33 cells yielding zeocin resistant derivatives on YPD agar supplemented with zeocin (100 and 1,000 μg/mL) and sorbitol (1 M). The presence of the integrated synthetic genes in the transformed yeast cells was confirmed by PCR and DNA sequencing of the inserts.

The antimicrobial activity of individual *P. pastoris* X-33SRCAM602, *P. pastoris* X-33OR-7, *P. pastoris* X-33E-760, and *P. pastoris* X-33L-1077 was screened by a streak-on-agar test (SOAT). Briefly, the *P. pastoris* transformants were streaked onto BMMY buffered methanol complex medium (1% yeast, 2% peptone, 100 mM potassium phosphate (pH 6), 1,34% yeast nitrogen base (YNB) without amino acids, 4×10^{-5}% biotin, 0.5% methanol) agar and grown at 30°C to induce production of the bacteriocins. After incubation of the plates at 30°C during 24 h, 40 mL of MRS soft-agar containing 10^5 cfu/mL of the indicator microorganism *Pediococcus damnosus* CECT4797 was added to the plates that were further incubated at 32°C for 24 h inhibition halos visualization.

2.5. Purification of the Antimicrobial Activity of Supernatants from the Recombinant Yeasts. The antimicrobial activity of supernatants from *P. pastoris* X-33 and *P. pastoris* X-33 (pPICZαA) and the recombinant *P. pastoris* X-33SRCAM602, *P. pastoris* X-33OR-7, *P. pastoris* X-33E-760, and *P. pastoris* X-33L-1077 was purified using a previously described procedure [12]. Briefly, supernatants from early stationary phase 0.5 L cultures of the recombinant yeasts, grown in BMMY buffered methanol complex medium at 30°C, were precipitated with ammonium sulfate, desalted by gel filtration, and subjected to cation exchange-chromatography, followed by hydrophobic interaction-chromatography and reverse phase-chromatography in a fast protein liquid chromatography system (RP-FPLC) (GE Healthcare, Barcelona, Spain). The antimicrobial activity of the purified fractions was evaluated against *Pediococcus damnosus* CECT4797 by the microtiter plate assay (MPA).

2.6. Mass Spectrometry Analysis of Purified Supernatants. For determination of the molecular mass of peptides in supernatants of the recombinant yeasts, the eluted purified fractions with antimicrobial activity were subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Briefly, 1 μL samples were spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 μL of a 3 mg/mL of α-cyano-4-hydroxy-transcinnamic acid matrix (Sigma) in 50% acetonitrile were added to the dried peptide to digest spots and allowed again to air-dry at room temperature. MALDI-TOF MS analyses were performed in a 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA), operated in 1 KV reflector mode. All mass spectra were calibrated externally using a standard peptide mixture (AB Sciex, MA, USA).

To determine the amino acid sequence of the purified peptides, the eluted purified fractions with antimicrobial activity were further subjected to MALDI-TOF/TOF tandem mass spectrometry. The samples were reduced, alkylated, digested with trypsin [23], and analysed in a 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems). The acquisition method for MS analysis was 1 KV reflector positive mode. Peptides from the MS spectra were manually selected for fragmentation analysis. The acquisition method for MS/MS analysis was MS/MS-1 KV in reflector positive mode with CID for fragmentation. The collision gas was atmospheric and the precursor mass window was ±5 Da. The plate model and default calibration were optimized for the MS/MS spectra processing. The parameters used to analyze the data were signal to noise = 20, resolution >6000. For protein identification, the *de novo* amino acid sequence from the fragmentation spectra of selected peptides was performed using the De Novo tool software (Applied Biosystems), and tentative sequences were manually checked and validated. Homology searches of the deduced amino acid sequences were performed through the NCBI/Blast. All mass spectrometry (MS) analyses were performed at the Unidad de Proteómica, Facultad de Farmacia, Universidad Complutense de Madrid (UCM), Madrid, Spain.

2.7. Antimicrobial Activity of Purified Supernatants from the Recombinant Yeasts. The antimicrobial activity of purified supernatants from the recombinant *P. pastoris* X-33SRCAM602, *P. pastoris* X-33OR-7, *P. pastoris* X-33E-760, and *P. pastoris* X-33L-1022 was evaluated against *Listeria monocytogenes* CECT4032, *E. coli* O157:H7, *Yersinia ruckeri* LMG3279, *Campylobacter jejuni* ATCC33560, and *C. jejuni* NCTC11168 by using a MPA, as previously described [12].

3. Results

3.1. Cloning of Synthetic Genes Encoding Bacteriocins and Their Expression by Recombinant P. pastoris. The cloning of PCR-amplified fragments from plasmids encoding synthetic genes designed from the published amino acid sequence of the mature bacteriocins SRCAM 602, OR-7, E-760, and L-1077 into the protein expression vector pPICZαA resulted in the recombinant plasmids pSRCAM602, pOR-7, pE-760, and pL-1077 (Table 1). Similarly, transformation of the linearized plasmids into competent *P. pastoris* X-33 permitted isolation of the *P. pastoris* X-33SRCAM602 (*srcam602*), *P. pastoris* X-33OR-7 (*or-7*), *P. pastoris* X-33E-760 (*e-760*), and *P. pastoris* X-33L-1077 (*l-1077*) recombinants. However, none of the recombinant yeasts showed direct antimicrobial activity against *P. damnosus* CECT4797, even those recombinant yeasts selected for their high zeocin resistance (1,000 μg/mL). Colonies of *P. pastoris* X-33 and *P. pastoris* X-33 (pPICZαA) were used as bacteriocin-negative controls to discard the possibility that the antimicrobial activity possibly observed was due to metabolites other than bacteriocins.

3.2. Purification of the Antimicrobial Activity of Supernatants from the Recombinant P. pastoris and Mass Spectrometry Analysis. When supernatants from *P. pastoris* X-33SRCAM602, *P.*

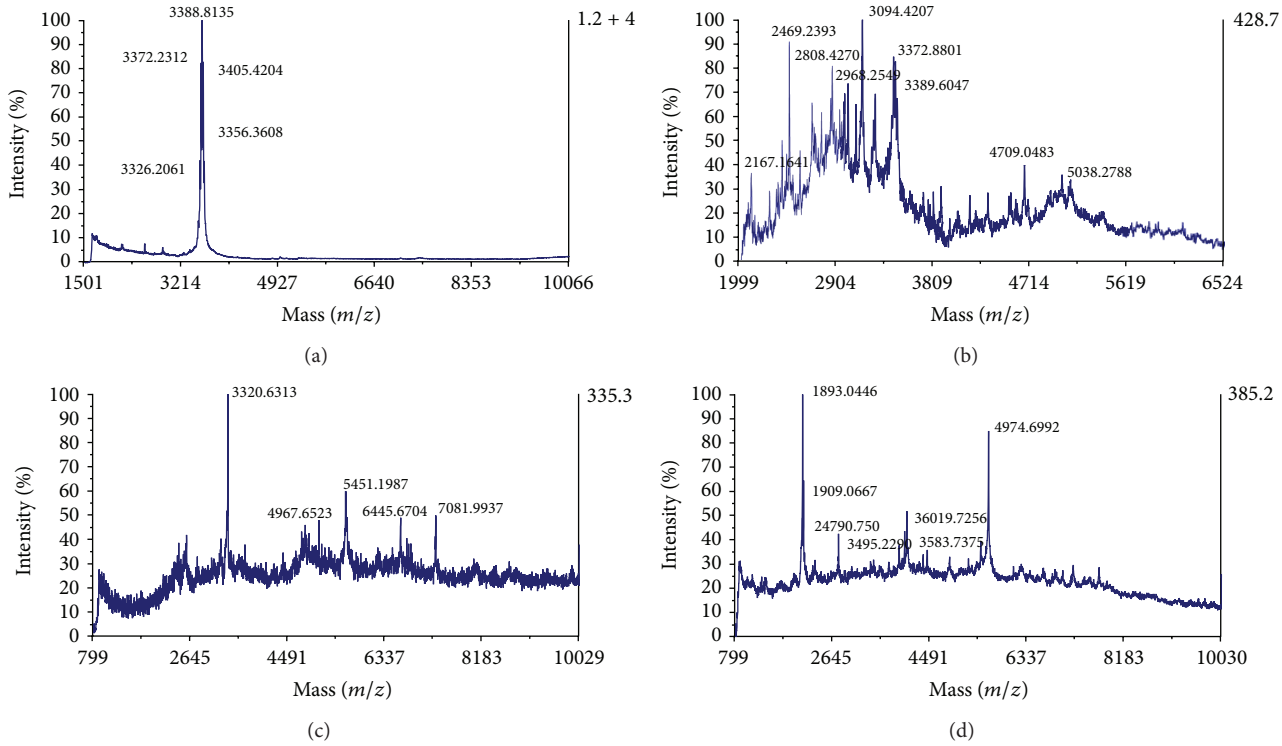


Figure 1: Mass spectrometry analysis of purified supernatants from *P. pastoris* X-33SRCAM602 (a), *P. pastoris* X-33OR-7 (c), *P. pastoris* X-33E-760 (b), and *P. pastoris* X-33L-1077 (d), eluted after RP-FPLC. Numbers indicate the molecular mass in daltons of most of the observed peptide fragments.

pastoris X-33OR-7, *P. pastoris* X-33E-76, and *P. pastoris* X-33L-1077 were purified by a multistep chromatographic procedure, only eluted fractions after the hydrophobic interaction-chromatography step showed full antimicrobial activity against *Pediococcus damnosus* CECT4797 as the indicator microorganism (Table 4). MALDI-TOF MS analysis of eluted fractions after the RP-FPLC step showed that supernatants from *P. pastoris* X-33SRCAM602 showed a major peptide fragment of 3388.8 Da (Figure 1(a)). However, the purified supernatant from *P. pastoris* X-33OR-7 showed a major peptide fragment of 3094.4 Da and peptide fragments of major and minor molecular mass (Figure 1(b)). Similarly, a large display of peptide fragments of different molecular masses was observed in the purified supernatants from *P. pastoris* X-33E-760 (Figure 1(c)) and *P. pastoris* X-33L-1077 (Figure 1(d)). MALDI-TOF MS/MS spectrometry analysis of trypsin-digested peptides from purified supernatants from all recombinant *P. pastoris*, determined that none of the most probable or *de novo* amino acid sequence of the evaluated peptide fragments matched the expected amino acid sequence of the bacteriocins SRCAM 602, OR-7, E-760, and L-1077 (Table 5). However, some of the determined peptide fragments were homologous to those observed in proteins from ABC-transporter systems, histidine kinase protein family, peptidase C1 superfamily, and nucleosome binding proteins.

3.3. Antimicrobial Activity of Purified Supernatants from the Recombinant Yeasts. The purified supernatants from recombinant *P. pastoris*, constructed for expression of the bacteriocins SRCAM 602, OR-7, E-760, and L-1077 showed antimicrobial activity against *Pediococcus damnosus* CECT4797 but did not display a measurable antimicrobial activity against *L. monocytogenes* CECT4032, *E. coli* O157:H7, *Y. ruckeri* LMG3279, *C. jejuni* ATCC33560, and *C. jejuni* NCTC11168, when evaluated by a microtiter plate assay (MPA). The purified supernatants from *P. pastoris* X-33 and *P. pastoris* X-33 (pPICZαA) did not show antagonistic activity neither against *Pediococcus damnosus* CECT4797 nor against any of the indicator bacteria cited above.

4. Discussion

To circumvent the proliferation of emerging pathogenic and antibiotic-resistant bacteria, bacteriocins produced by LAB emerge as natural antimicrobial peptides with potential applications in food preservation, livestock protection, and medical applications [1, 24]. However, the high cost of synthetic bacteriocin synthesis, their low yields, and the production of potential virulence factors from some natural bacterial producers drive the exploration of microbial systems for the biotechnological production of bacteriocins by heterologous LAB and yeasts [6, 8]. Furthermore, the use of synthetic genes may become a useful tool for production and functional expression of bacteriocins by heterologous microbial hosts [12].

The development of heterologous expression systems for bacteriocins may offer a number of advantages over

Table 3: Amino acid sequence of cloned bacteriocins and other class IIa bacteriocins.

Bacteriocin	Amino acid sequence	Number of amino acids
SRCAM 602	ATYYGNGLYCNKQKHYTWVDWNKASREIGKITVNGWVQH	39
OR-7	KTYYGTNGVHCTKNSLWGKVRLKNMKYDQNTTYMGRLQDILLGW ATGAFGKTFH	54
E-760	NRWYCNSAAGGVGGAAVCGLAGYVGEAKENIAGEVRKGWGMAGG FTHNKACKSFPGSGWASG	62
E 50-52	TTKNYGNGVCNSVNWCQCGNVWASCNLATGCAAWLCKLA	39
L-1077	TNYGNGVGVPDAIMAGIIKLIFIFNIRQGYNFGKKAT	37
EntP	ATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH	44
EntA	TTHSGKYYGNGVYCYKNKCTVDWAKATTCIAGMSIGGFLGGAI PGKC	47
HirJM79	ATYYGNGLYCNKEKCWVDWNQAKGEIGKIIVNGWVNHGPWAP RR	44
SakA	ARSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	41

Underlined, the class IIa N-terminal consensus sequence YGNGV(X)C.

Table 4: Antimicrobial activity of fractions generated during purification of supernatants from *P. pastoris* X-SRCAM602, *P. pastoris* X-33OR-7, *P. pastoris* X-33E-760, and *P. pastoris* X-33L-1077, grown in BMMY with methanol.

Strain	Antimicrobial activity (BU/mL) of the purified fractions[a]					
	SN	AS	GF	SE	OE	RP-FPLC
P. pastoris X-33SRCAM602	NA	NA	NA	NA	12,800	1,106,531
P. pastoris X-33OR-7	NA	NA	127	32	1,391	10,027
P. pastoris X-33E-760	NA	NA	NA	NA	687	14,442
P. pastoris X-33L-1077	NA	NA	35	23	2,069	13,213

Most of the data are mean from two independent determinations in triplicate.
[a]Antimicrobial activity against *Pediococcus damnosus* CECT4797 as determined by microtiter plate assay (MPA). BU: bacteriocin units. NA: no activity. Purification fractions: SN: supernatant; AS: ammonium sulfate precipitation; GF: gel filtration; SE: Sepharose fast flow eluate; OE: Octyl Sepharose eluate; RP-FPLC: reversed-phase eluate.

native systems, facilitating the control of bacteriocin gene expression or achieving higher production levels. Although a number of yeast platforms have been used for the production of peptides and proteins, including bacteriocins [10, 11, 25], the use of synthetic genes has been only barely explored for their expression by recombinant yeasts [12]. In this study, the protein expression vector pPICZαA containing an strong and inducible promoter and the Kex2 signal cleavage site for processing of fusion proteins [26] has been used to drive the expression of synthetic genes encoding the mature bacteriocins SRCAM 602, OR-7, E-760, and L-1077, by recombinant *P. pastoris* X-33 derivatives.

Initial results with *P. pastoris* X-33SRCAM602, *P. pastoris* X-33OR-7, *P. pastoris* X-33E-760, and *P. pastoris* X-33L-1077 determined that none of the recombinant yeasts, encoding synthetic genes for expression of the cloned bacteriocins, showed antimicrobial activity when individual colonies were screened by a streak-on-agar test (SOAT). Highly variable yields of secreted proteins have been achieved using the *P. pastoris* expression system and cases of low secretory yields or complete failure in protein production have also been reported [3, 27, 28]. A number of factors may affect the production of foreign peptides by heterologous yeasts including copy number integration of the expression vectors in the yeast DNA, mRNA stability, errors in mRNA translation, uncoordinated rates of protein synthesis, folding and translocation, and undesired proteolysis of heterologous proteins by resident proteases or by proteases in the extracellular space being secreted, cell-wall associated, or released into the culture medium as a result of cell disruption [29–31]. The use of the *P. pastoris* expression system for overproduction of peptides and proteins is known to be somewhat hampered by its unpredictable yields of production of heterologous proteins, which is now believed to be caused in part by their varied efficiencies to traffic through the host secretion machinery [3, 28].

The amino acid sequence following the Kex2 secretion signal may also interfere with the secretion of fused peptides or proteins by recombinant *P. pastoris*. Furthermore, the yields of many recombinant proteins seem to be influenced by the Kex2 P1′ site residue [3]. In this study, the Kex2 P1′ site residues for mature SRCAM 602, OR-7, E-760, and L-1077 were the amino acids A, K, N, and T, respectively (Table 3). However, the cloning in *P. pastoris* of the bacteriocins enterocin A (EntA) and enterocin P (EntP) with the Kex2 P1′ site residues A and T, respectively, showed an overproduction of

Table 5: Results obtained by MALDI-TOF/TOF MS analysis of the eluted RP-FPLC fractions from purified supernatants of the recombinant *P. pastoris* X-33 derivatives.

Bacteriocin	Predicted molecular mass (Da)	Trypsin-digested precursor (Da)	Amino acid sequence (MS/MS analysis)
SRCAM 602	4,630.1	926.65	SANALRPPT
		1,500.95	GDKENAAKASSVPAR
		1,614.97	KTGGNRAVSGAGEIAAR
OR-7	6,215.1	Deficient signal	—
E-760	6,179.8	1,166.63	VGNPLHGIFGR
		1,579.68	SLSAYMFFANEQR
L-1077	4,002.6	1,638.76	IVGSQAGIGEYLFER
		1,718.73	LVELSEQELVDCER

both bacteriocins over their natural producers and an intense antimicrobial activity when colonies of the recombinant yeasts were evaluated by the SOAT [11, 22].

The use of a multistep chromatographic procedure for purification of the expected antimicrobial activity in supernatants of the recombinant *P. pastoris* determined that only eluted fractions after the hydrophobic interaction-chromatography step showed full antimicrobial activity (Table 4), probably due to removal of antimicrobial inhibitors, disaggregation of the bacteriocins, or changes in conformation of the bacteriocins in the hydrophobic solvent. The antimicrobial activity of the supernatant from *P. pastoris* X-33SRCAM602 was much higher than that of the rest of the recombinant *P. pastoris*. While being interesting, this was not an unexpected observation since purification of the circular bacteriocin garvicin ML, produced by *Lactococcus garvieae* DCC43, showed a higher antibacterial activity and a broader antimicrobial spectrum as it was increasingly purified [32]. However, MALDI-TOF MS analysis of the purified supernatants from *P. pastoris* X-33SRCAM602, *P. pastoris* X-33OR-7, *P. pastoris* X-33E-760, and *P. pastoris* X-33L-1077 mostly showed a large display of peptide fragments of different molecular mass than deduced from the calculated molecular mass of the cloned bacteriocins (Figure 1). The different molecular mass of the resulting peptide fragments may suggest the existence of truncated bacteriocins, the interaction of the bacteriocins with unknown biological compounds or the bacteriocins being subjected to posttranslational modifications (PTM) such as phosphorylation, acetylation, methylation, oxidation, formylation, disulfide bond formation, and N-linked and O-linked glycosylation [8, 33]. The presence of cysteine residues in the bacteriocins SRCAM 602, OR-7, and E-760 would permit the formation of disulfide bridges but also permits its oxidation, glutathionylation, and cysteinylation. The absence in all cloned bacteriocins of attachment sites for N-linkages precludes its N-glycosylation, but the presence of threonines and serines makes the bacteriocins sensitive to O-glycosylation [8, 12, 33].

However, MALDI-TOF MS/MS analysis of trypsin-digested peptides from purified supernatants from all recombinant *P. pastoris* determined that none of the evaluated peptide fragments matched the expected amino acid sequence of the bacteriocins SRCAM 602, OR-7, E-760, and L-1077 (Table 5). From the results obtained it may be suggested that very low yields of secreted and/or purified bacteriocins are obtained after cloning of synthetic genes encoding the bacteriocins SRCAM 602, OR-7, E-760, and L-1077 in *P. pastoris*. This observation was also not unexpected because bacteriocins cloned into *Saccharomyces cerevisiae*, *P. pastoris*, *Kluyveromyces lactis*, *Hansenula polymorpha,* and *Arxula adeninivorans* have been produced with variable success regarding their production, secretion, and functional expression [11]. Furthermore, since one of the main bottlenecks in recombinant protein production is the inability of foreign peptides to reach their native conformation in heterologous yeast hosts, it could also happen that incorrectly folded SRCAM 602, OR-7, E-760, and L-1077 are accumulated in the endoplasmic reticulum (ER) of recombinant *P. pastoris*, activating the unfolded protein response (UPR) and the ER-associated degradation (ERDA) of the misfolded bacteriocins, leading to persistent ER stress conditions causing much lower efficiencies to traffic through the host secretion machinery, apoptosis, and cell death [8, 28, 34, 35]. In any case, the inconsistent secretory productivity among recombinant proteins has always been a major obstacle for routine application of *P. pastoris* as an eukaryotic protein expression system in both research and industry [3].

The purified supernatants from the recombinant *P. pastoris* constructed for expression of the bacteriocins SRCAM 602, OR-7, E-760, and L-1077 showed a measurable antimicrobial activity against *Pediococcus damnosus* CECT4797 (Table 4), but not against *L. monocytogenes* CECT4032, *E. coli* O157:H7, *Y. ruckeri* LMG3279, *C. jejuni* ATCC33560, and *C. jejuni* NCTC11168. One of the remarkable features of bacteriocins is that they are very potent, being active in nanomolar concentrations, thereby surpassing by about 1,000-fold the activity of AMPs produced by eukaryotic cells [36]. One of the major reasons for this extreme potency is that bacteriocins apparently recognize specific receptors on target cells, while the interactions between AMPs and microorganisms are mostly nonspecific. Furthermore, the target receptor for class IIa bacteriocins has been identified as proteins of the sugar transporter mannose phosphotransferase system (Man-PTS), with the most potent receptors being those found in *Listeria* spp. [37, 38]. However, the very low yields of secreted or

purified bacteriocins in supernatants of the recombinant *P. pastoris* may be responsible for their nondetected antilisterial activity.

It could be also hypothesized that peptide fragments aggregated to or coeluting with the purified bacteriocins may be responsible for the antimicrobial activity of the eluates against the sensitive indicator *Pediococcus damnosus* CECT4797, but not against any other of the indicator bacteria tested. Many proteins contain encrypted within their primary structure bioactive peptides with antimicrobial activity following hydrolytic release from the native molecule [39, 40]. Incorrect disulfide bond formation, misfolding of the secreted bacteriocin and extensive PTMs were suggested to be responsible for the lower antilisterial activity and the nonmeasurable antimicrobial activity against Gram-negative bacteria of the bacteriocin E 50-52 (BacE50-52), produced by recombinant *P. pastoris* X-33BE50-52S and *K. lactis* GG799BE50-52S [12]. This bacteriocin, originally produced by *Enterococcus faecium* B-30746, was also reported to display a high and broad antimicrobial activity against Gram-positive and Gram-negative bacteria, including *Campylobacter* spp. [20, 41].

The correct processing, secretion and functional expression of the bacteriocins EntP [22], hiracin JM79 (HirJM79) [42] and EntA [11], produced by recombinant yeasts, contrast with the low biological activity of the sakacin A (SakA) and the chimera EntP/SakA, produced by recombinant *P. pastoris* and *K. lactis* producers [8]. Misfolding of SakA and EntP/SakA and induction of the yeasts' UPR may be responsible for apoptosis in recombinant *P. pastoris* producers of SakA and for extensive PTMs in recombinant *P. pastoris* and *K. lactis*, producers of SakA and EntP/SakA [8]. These results, obtained by our research group, also contrast with the low antimicrobial activity against *Pediococcus damnosus* CECT4797 and the absence of antimicrobial activity against Gram-positive and Gram-negative bacteria of the purified supernatants from recombinant *P. pastoris* encoding the mature bacteriocins SRCAM 602, OR-7, E-760, and L-1077.

Nevertheless, it should be also worth to notice that bacteriocins SRCAM 602 [16], OR-7 [17], E-760 [19], and L-1077 [18], although being reported as bacteriocins with a broad antimicrobial activity against Gram-positive and Gram-negative microorganisms including *Campylobacter* spp., have not been fully characterized at their biochemical and genetic level, the genetic identification of their structural and adjacent genes has not been yet reported, and their molecular masses, deduced from their reported amino acid sequences, were not identical to the experimentally determined molecular mass from the purified bacteriocins [16–19]. Furthermore, recent reports suggest that bacteriocin SRCAM 602, previously reported to be produced by *Paenibacillus polymyxa* NRRL B-30509 and claimed to be responsible for inhibition of *C. jejuni*, could not be detected in the purified supernatants of the producer strain while the *srcam602* structural gene was not found via a PCR-based approach using degenerate nucleotide primers or by genomic sequencing of the bacteriocin producer [43]. Similarly, *Bacillus circulans* (now *Paenibacillus terrae*) NRRL B-30644 previously reported to produce SRCAM 1580, a bacteriocin active against *C. jejuni* [16], has been recently suggested not to produce this bacteriocin and no genetic determinants for its production were shown. Instead the anti-*Campylobacter* activity of this strain is due to the production of the lipopeptide tridecaptin A1 whereas this strain also produces the novel lantibiotic paenicidin B, active against Gram-positive bacteria [44].

Although the cloning in recombinant yeasts of synthetic genes encoding bacteriocins drives the production, antimicrobial activity, and specific antimicrobial activity of the cloned bacteriocins in the absence of dedicated immunity and secretion proteins [12], these results contrast with the negligible antimicrobial activity against Gram-positive and Gram-negative bacteria of purified supernatants from recombinant *P. pastoris* encoding the bacteriocins SRCAM 602, OR-7, E-760, and L-1077. Accordingly, since production of bacteriocins from synthetic bacteriocin genes is difficult to predict, further efforts should be performed for a more efficient genetically engineered production and functional expression of other bacteriocin synthetic genes or their chimeras by heterologous producer yeasts. The design of further and novel successful genetic approaches for production and functional expression of bacteriocins by yeasts would facilitate their biotechnological applications as natural antimicrobial agents in food, animal husbandry, and medicine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was partially supported by Project AGL2012-34829 from Ministerio de Economía y Competitividad (MINECO) and Project AGL2009-08348 from the Ministerio de Ciencia e Innovación (MICINN), by Grant GR35-10A from the BSCH-UCM and by Grants S2009/AGR-1489 and S2013/ABI-2747 from the Comunidad de Madrid (CAM). S. Arbulu holds a fellowship (FPI) from the MINECO. J. J. Jiménez and L. Gútiez held a fellowship from the MICINN and the Ministerio de Educación y Ciencia (MEC), Spain, respectively.

References

[1] C. T. Lohans and J. C. Vederas, "Development of class IIa bacteriocins as therapeutic agents," *International Journal of Microbiology*, vol. 2012, Article ID 386410, 13 pages, 2012.

[2] P. D. Cotter, R. P. Ross, and C. Hill, "Bacteriocins-a viable alternative to antibiotics?" *Nature Reviews Microbiology*, vol. 11, no. 2, pp. 95–105, 2013.

[3] S. Yang, Y. Kuang, H. Li et al., "Enhanced production of recombinant secretory proteins in *Pichia pastoris* by optimizing Kex2 P1' site," *PLoS ONE*, vol. 8, no. 9, Article ID e75347, 2013.

[4] P. D. Cotter, C. Hill, and R. P. Ross, "Food microbiology: bacteriocins: developing innate immunity for food," *Nature Reviews Microbiology*, vol. 3, no. 10, pp. 777–788, 2005.

[5] D. Drider, G. Fimland, Y. Héchard, L. M. McMullen, and H. Prévost, "The continuing story of class IIa bacteriocins,"

Microbiology and Molecular Biology Reviews, vol. 70, no. 2, pp. 564–582, 2006.

[6] J. Borrero, J. J. Jiménez, L. Gútiez, C. Herranz, L. M. Cintas, and P. E. Hernández, "Protein expression vector and secretion signal peptide optimization to drive the production, secretion, and functional expression of the bacteriocin enterocin A in lactic acid bacteria," *Journal of Biotechnology*, vol. 156, no. 1, pp. 76–86, 2011.

[7] N. S. Parachin, K. C. Mulder, A. A. B. Viana, S. C. Dias, and O. L. Franco, "Expression systems for heterologous production of antimicrobial peptides," *Peptides*, vol. 38, no. 2, pp. 446–456, 2012.

[8] J. J. Jiménez, J. Borrero, D. B. Diep et al., "Cloning, production, and functional expression of the bacteriocin sakacin A (SakA) and two SakA-derived chimeras in lactic acid bacteria (LAB) and the yeasts *Pichia pastoris* and *Kluyveromyces lactis*," *Journal of Industrial Microbiology & Biotechnology*, vol. 40, no. 9, pp. 977–993, 2013.

[9] M. Gao and Z. Shi, "Process control and optimization for heterologous protein production by methylotrophic pichia pastoris," *Chinese Journal of Chemical Engineering*, vol. 21, no. 2, pp. 216–226, 2013.

[10] M. Ahmad, M. Hirz, H. Pichler, and H. Schwab, "Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production," *Applied Microbiology and Biotechnology*, vol. 98, pp. 5301–5317, 2014.

[11] J. Borrero, G. Kunze, J. Jiménez et al., "Cloning, production, and functional expression of the bacteriocin enterocin A, produced by *Enterococcus faecium* T136, by the *Yeasts Pichia pastoris*, *Kluyveromyces lactis*, *Hansenula polymorpha*, and *Arxula adeninivorans*," *Applied and Environmental Microbiology*, vol. 78, no. 16, pp. 5956–5961, 2012.

[12] J. J. Jiménez, J. Borrero, L. Gútiez et al., "Use of synthetic genes for cloning, production and functional expression of the bacteriocins enterocin A and bacteriocin E 50-52 by *Pichia pastoris* and *Kluyveromyces lactis*," *Molecular Biotechnology*, vol. 56, no. 6, pp. 571–583, 2014.

[13] R. A. J. Darby, S. P. Cartwright, M. V. Dilworth, and R. M. Bill, "Which yeast species shall i choose? *Saccharomyces cerevisiae* versus *Pichia pastoris*," *Methods in Molecular Biology*, vol. 866, pp. 11–23, 2012.

[14] F. Öberg, J. Sjöhamn, M. T. Conner, R. M. Bill, and K. Hedfalk, "Improving recombinant eukaryotic membrane protein yields in *Pichia pastoris*: the importance of codon optimization and clone selection," *Molecular Membrane Biology*, vol. 28, no. 6, pp. 398–411, 2011.

[15] N. J. Stern, E. A. Svetoch, B. V. Eruslanov et al., "*Paenibacillus polymyxa* purified bacteriocin to control *Campylobacter jejuni* in chickens," *Journal of Food Protection*, vol. 68, no. 7, pp. 1450–1453, 2005.

[16] E. A. Svetoch, N. J. Stern, B. V. Eruslanov et al., "Isolation of *Bacillus circulans* and *Paenibacillus polymyxa* strains inhibitory to *Campylobacter jejuni* and characterization of associated bacteriocins," *Journal of Food Protection*, vol. 68, no. 1, pp. 11–17, 2005.

[17] N. J. Stern, E. A. Svetoch, B. V. Eruslanov et al., "Isolation of a *Lactobacillus salivarius* strain and purification of its bacteriocin, which is inhibitory to *Campylobacter jejuni* in the chicken gastrointestinal system," *Antimicrobial Agents and Chemotherapy*, vol. 50, no. 9, pp. 3111–3116, 2006.

[18] E. A. Svetoch, B. V. Eruslanov, V. P. Levchuk et al., "Isolation of *Lactobacillus salivarius* 1077 (NRRL B-50053) and characterization of its bacteriocin, including the antimicrobial activity spectrum," *Applied and Environmental Microbiology*, vol. 77, no. 8, pp. 2749–2754, 2011.

[19] J. E. Line, E. A. Svetoch, B. V. Eruslanov et al., "Isolation and purification of enterocin E-760 with broad antimicrobial activity against Gram-positive and Gram-negative bacteria," *Antimicrobial Agents and Chemotherapy*, vol. 52, no. 3, pp. 1094–1100, 2008.

[20] E. A. Svetoch, B. V. Eruslanov, V. V. Perelygin et al., "Diverse antimicrobial killing by *Enterococcus faecium* E 50-52 bacteriocin," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 6, pp. 1942–1948, 2008.

[21] E. A. Svetoch and N. J. Stern, "Bacteriocins to control *Campylobacter* spp. in poultry-a review," *Poultry Science*, vol. 89, no. 8, pp. 1763–1768, 2010.

[22] J. Gutiérrez, R. Criado, M. Martín, C. Herranz, L. M. Cintas, and P. E. Hernández, "Production of enterocin P, an antilisterial pediocin-like bacteriocin from *Enterococcus faecium* P13, in *Pichia pastons*," *Antimicrobial Agents and Chemotherapy*, vol. 49, no. 7, pp. 3004–3008, 2005.

[23] A. Shevchenko, A. Loboda, W. Ens, and K. G. Standing, "MALDI quadrupole time-of-flight mass spectrometry: a powerful tool for proteomic research," *Analytical Chemistry*, vol. 72, no. 9, pp. 2132–2141, 2000.

[24] World Health Organization (WHO), "Antimicrobial resistance: global report on surveillance 2014," http://www.searo.who.int/thailand/publications/2013/9789241564748/en/.

[25] E. Böer, G. Steinborn, G. Kunze, and G. Gellissen, "Yeast expression platforms," *Applied Microbiology and Biotechnology*, vol. 77, no. 3, pp. 513–523, 2007.

[26] J. M. Cregg, J. L. Cereghino, J. Shi, and D. R. Higgins, "Recombinant protein expression in *Pichia pastoris*," *Applied Biochemistry and Biotechnology—Part B Molecular Biotechnology*, vol. 16, no. 1, pp. 23–52, 2000.

[27] O. J. Burrowes, G. Diamond, and T. C. Lee, "Recombinant expression of pleurocidin cDNA using the *Pichia pastoris* expression system," *Journal of Biomedicine and Biotechnology*, vol. 2005, no. 4, pp. 374–384, 2005.

[28] K. R. Love, T. J. Politano, V. Panagiotou, B. Jiang, T. A. Stadheim, and J. C. Love, "Systematic single-cell analysis of pichia pastoris reveals secretory capacity limits productivity," *PLoS ONE*, vol. 7, no. 6, Article ID e37915, 2012.

[29] H. A. Kang, E.-S. Choi, W.-K. Hong et al., "Proteolytic stability of recombinant human serum albumin secreted in the yeast *Saccharomyces cerevisiae*," *Applied Microbiology and Biotechnology*, vol. 53, no. 5, pp. 575–582, 2000.

[30] M. W. T. Werten and F. A. De Wolf, "Reduced proteolysis of secreted gelatin and Yps1-mediated α-factor leader processing in a *Pichia pastoris* kex2 disruptant," *Applied and Environmental Microbiology*, vol. 71, no. 5, pp. 2310–2317, 2005.

[31] Z. Ni, X. Zhou, X. Sun, Y. Wang, and Y. Zhang, "Decrease of hirudin degradation by deleting the KEX1 gene in recombinant *Pichia pastoris*," *Yeast*, vol. 25, no. 1, pp. 1–8, 2008.

[32] J. Borrero, D. A. Brede, M. Skaugen et al., "Characterization of garvicin ML, a novel circular bacteriocin produced by *Lactococcus garvieae* DCC43, isolated from mallard ducks (*Anas platyrhynchos*)," *Applied and Environmental Microbiology*, vol. 77, no. 1, pp. 369–373, 2011.

[33] Y. Zhao and O. N. Jensen, "Modification-specific proteomics: strategies for characterization of post-translational modifications using enrichment techniques," *Proteomics*, vol. 9, no. 20, pp. 4632–4641, 2009.

[34] B. Gasser, M. Saloheimo, U. Rinas et al., "Protein folding and conformational stress in microbial cells producing recombinant proteins: a host comparative overview," *Microbial Cell Factories*, vol. 7, article 11, 2008.

[35] A. Stolz and D. H. Wolf, "Endoplasmic reticulum associated protein degradation: a chaperone assisted journey to hell," *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1803, no. 6, pp. 694–705, 2010.

[36] E. Breukink, I. Wiedemann, C. van Kraaij, O. P. Kuipers, H.-G. Sahl, and B. de Kruijff, "Use of the cell wail precursor lipid II by a pore-forming peptide antibiotic," *Science*, vol. 286, no. 5448, pp. 2361–2364, 1999.

[37] D. B. Diep, M. Skaugen, Z. Salehian, H. Holo, and I. F. Nes, "Common mechanisms of target cell recognition and immunity for class II bacteriocins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 7, pp. 2384–2389, 2007.

[38] M. Kjos, J. Borrero, M. Opsata et al., "Target recognition, resistance, immunity and genome mining of class II bacteriocins from Gram-positive bacteria," *Microbiology*, vol. 157, no. 12, pp. 3256–3267, 2011.

[39] M. Phelan, A. Aherne, R. J. FitzGerald, and N. M. O'Brien, "Casein-derived bioactive peptides: biological effects, industrial uses, safety aspects and regulatory status," *International Dairy Journal*, vol. 19, no. 11, pp. 643–654, 2009.

[40] G. D. Brand, M. T. Q. Magalhães, M. L. P. Tinoco et al., "Probing protein sequences as sources for encrypted antimicrobial peptides," *PLoS ONE*, vol. 7, no. 9, Article ID e45848, 2012.

[41] E. A. Svetoch, V. P. Levchuk, V. D. Pokhilenko et al., "Inactivating methicillin-resistant *Staphylococcus aureus* and other pathogens by use of bacteriocins OR-7 and E 50-52," *Journal of Clinical Microbiology*, vol. 46, no. 11, pp. 3863–3865, 2008.

[42] J. Sánchez, J. Borrero, B. Gómez-Sala et al., "Cloning and heterologous production of hiracin JM79, a Sec-dependent bacteriocin produced by *Enterococcus hirae* DCH5, in lactic acid bacteria and *Pichia pastoris*," *Applied and Environmental Microbiology*, vol. 74, no. 8, pp. 2471–2479, 2008.

[43] C. T. Lohans, Z. Huang, M. J. Van Belkum et al., "Structural characterization of the highly cyclized lantibiotic paenicidin A via a partial desulfurization/reduction strategy," *Journal of the American Chemical Society*, vol. 134, no. 48, pp. 19540–19543, 2012.

[44] C. T. Lohans, M. J. Van Belkum, S. A. Cochrane et al., "Biochemical, structural, and genetic characterization of tridecaptin A_1, an antagonist of *Campylobacter jejuni*," *ChemBioChem*, vol. 15, no. 2, pp. 243–249, 2014.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 189016, 9 pages
http://dx.doi.org/10.1155/2015/189016

Review Article

Current and Potential Applications of Host-Defense Peptides and Proteins in Urology

Joey Chor Yee Lo and Dirk Lange

The Stone Centre at Vancouver General Hospital, Department of Urologic Sciences, University of British Columbia, 2660 Oak Street, Vancouver, BC, Canada V6H 3Z6

Correspondence should be addressed to Dirk Lange; dirk.lange@ubc.ca

Received 10 August 2014; Revised 21 October 2014; Accepted 24 October 2014

Academic Editor: Joel E. López-Meza

The use of antibiotics has become increasingly disfavored as more multidrug resistant pathogens are on the rise. A promising alternative to the use of these conventional drugs includes antimicrobial peptides or host-defense peptides. These peptides typically consist of short amino acid chains with a net cationic charge and a substantial portion of hydrophobic residues. They mainly target the bacterial cell membrane but are also capable of translocating through the membrane and target intracellular components, making it difficult for bacteria to gain resistance as multiple essential cellular processes are being targeted. The use of these peptides in the field of biomedical therapies has been examined, and the different approaches to using them under various settings are constantly being discovered. In this review, we discuss the current and potential applications of these host-defense peptides in the field of urology. Besides the use of these peptides as antimicrobial agents, the value of these biological molecules has recently been expanded to their use as antitumor and anti-kidney-stone agents.

1. Introduction

The use of antibiotics can be dated back to the 1930s, when the sulfonamide Prontosil was introduced as the first commercially available antibiotic [1]. Several other classes of antibiotics emerged soon after, and by the 1940s, the "golden age of antibiotics" began with the introduction of penicillin [2]. In 1967, the use of antibiotics seemed so promising that the United States surgeon, William H. Stewart, even stated "...we had essentially defeated infectious diseases and could close the book on them..." [3]. However, although this statement looked true at that time, it was soon refuted when pathogenic bacteria with resistance against conventional antibiotics become increasingly prominent by the end of the 20th century; bacteria had gained resistance to multiple drugs [4]. The rise in multidrug resistant (MDR) bacteria became alarming, with a prevalence rate increase of 57% from the 1950s to the 2000s, with more resistance observed towards drugs that had been used for humans and animals for the longest time [4–6]. With MDR pathogens becoming a leading cause of nosocomial infections, and with the lack of novel, effective antibiotics, there is an urgent need to discover alternative drugs to control bacterial infections [4].

Antimicrobial peptides (AMPs) are ancient defense molecules of the innate immune system that has gained substantial attention over recent years [4, 7]. These peptides are found within a wide variety of species, including bacteria, insects, fungi, amphibians, birds, crustaceans, fishes, mammals, and humans, and can be obtained from many different sources, such as neutrophils, macrophages, and epithelial cells [7–9]. Similar to many conventional antibiotics, they have broad spectrum activity against a wide range of microorganisms, including both Gram-positive and Gram-negative bacteria, fungi, viruses, yeast, and protozoa [7, 10, 11]. However, unlike current antibiotics, these AMPs have multiple sites as targets rather than single genes or proteins [4]. Although these peptides are mainly known for their ability to disrupt the cell membrane of target organisms, they are also known for their ability to translocate through the cell membrane and alter other essential cellular activities and promote immune responses, including but not limiting to upregulating or downregulating DNA, RNA, and protein

Table 1: Summary of host-defense peptides discussed.

Peptide name/inducers	Peptide sequence	Current/potential application in urology	References
Lactoferrin-derived peptide HLD1	EATKCFQWQRNMRKVRGPPVSCIKR-NH2	Oral administration for UTI	[17]
Lactoferrin-derived peptide HLD2	TK©FQWQRNMRKVRGPPVS©IKR-NH2		
Tachyplesin III	KWCFRVCYRGICYRKCR-NH2	Antimicrobial coating for urologic devices	[7]
Tet-20	KRWRIRVRVIRKC-NH2		[18]
RK1 (salt-tolerant)	RWKRWWRRKK		[19]
RK2 (salt-tolerant)	RKKRWWRRKK		[19]
Magainin II	GIGKFLHSAKKFGKAFVGEIMNS-NH2		[20–22]
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH2	Target bladder cancer cells	
Cecropin B	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL-NH2		[23]
Peptoids	Most potent one analyzed: H-(Nlys-Nspe-Nspe)4-NH2		[24]
Human β-defensin-1	DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK-NH2		[25–27]
OPN-derived peptides	Many OPN-derived peptides were analyzed; one of the more promising ones being OPN-derived peptide D9: ADAAADDAAADAAADDAA-NH2	Target kidney stones	[28]

synthesis, altering gene expressions, enhancing neutrophil chemotaxis and function, promoting histamine release of mast cells, inhibiting tissue proteases, and stimulating wound healing [7, 8, 12–14]. The ability of these peptides to target multiple systems makes it difficult for bacteria to gain MDR against them, putting them at a great advantage compared to conventional antibiotics [4]. Because AMPs also stimulate the immune system in addition to being "antimicrobial," in 2006, it was suggested that these peptides should be named "host-defense peptides" rather than "antimicrobial peptides," where the latter name was given simply based on their initially discovered characteristic [12]. For this reason, these peptides will be referred to as "host-defense peptides" for the remaining of this review.

Host-defense peptides (HDPs) are typically 12 to 50 amino acids in length, are amphipathic with a net positive charge of +2 to +9, and consist of a substantial portion of hydrophobic residues (≥30%) [8, 12, 15]. These properties allow the peptides to interact with bacterial membranes and insert and form pores; cationic portions of the peptide interact with the negatively charged surface of the bacterial outer membrane via electrostatic bonding [16]. Hydrophobic residues of the HDPs then allow them to be inserted into the lipid bilayer and permeabilize the membrane [16]. The exact mechanisms of how HDPs go beyond the bacterial membrane and affect other essential cellular activities, however, have yet to be discovered [13].

With the promising antimicrobial effects and host immune enhancements offered by HDPs, it is not surprising that they are now of high interest in the biomedical area. Here, we discuss the current and potential applications of these peptides in the field of urology, including urinary tract infections, urological devices, urologic cancers, and kidney stone disease. A summarizing table has been included to help the reader thoroughly understand the HDPs which will be discussed throughout this review (Table 1).

2. Current and Potential Applications of Host-Defense Peptides in the Field of Urology

2.1. Urinary Tract Infections. The urinary tract functions in close proximity with fecal microflora and the outside environment [29, 30]. Yet, it must remain sterile to avoid disease [29, 30]. Our body possesses several mechanisms to help clear the urinary tract of bacteria, including urine flow, changes in urine pH, regular bladder emptying, chemical-defense components of the uroepithelium, and, when stimulated with bacteria, epithelial shedding and influx of effector immune cells [29–31]. More recently discovered is the natural prevalence of HDPs released into our urine upon stimulation with bacteria [29, 30, 32]. When our body fails to keep the urinary tract sterile, bacterial infections may take place. Urinary tract infections (UTI) are one of the most common infections in humans, affecting predominantly females of any age [17].

Several HDPs of the urinary tract have been studied to determine their expression and function, including human beta-defensin-1 (hBD-1), cathelicidin, and ribonuclease 7 [29, 30, 32]. All three peptides are amongst a group of HDPs that are highly expressed by epithelial cells of the urinary tract upon stimulation with bacteria [29, 30, 32]. Although they are present in the urine of both healthy individuals and those with UTI, their level of expression significantly increases with acute pyelonephritis or cystitis, effectively revealing antibacterial activity at micromolar concentrations [29, 30, 32].

However, despite the regular release of HDPs into our urinary tract system by uroepithelial cells, it is apparent that the level of naturally produced HDPs may sometimes not be enough, hence giving rise to the frequent occurrence of UTI. Current therapies for UTI consist of antibiotics [17]. However, as previously mentioned, the high prevalence of MDR pathogens renders the treatment ineffective. Moreover, antibiotics have been associated with adverse effects, and they are often not recommended during pregnancy or in young children [17]. To overcome this predicament, Haversen et al. have examined the effectiveness of human HDP lactoferrin and lactoferrin-derived peptides, HLD1 (EATKCFQWQRNMRKVRGPPVSCIKR-NH2) and HLD2 (TK(C)FQWQRNMRKVRGPPVS(C)IKR-NH2) in clearing UTI when administered orally [17]. Lactoferrin possesses both antimicrobial and anti-inflammatory properties and is associated with host-defense at mucosal surfaces [17]. When orally administered to female mice 30 min after instillation of 10^7 *Escherichia coli* colony forming units (CFU) into the urinary bladder, bacterial numbers in both the kidneys and the bladder were decreased to at least 1000-fold lower than that in control groups which received either phosphate-buffered saline or water when examined 24 h after inoculation [17]. Hence, oral administration of HDPs was shown to be sufficient in treating infection and inflammation at the urinary tract, possibly via renal secretion of the peptides to the site of infection [17]; it has been previously reported that lactoferrin often leaves the body of UTI patients via the urinary tract [33]. Other studies suggest that the molecule may remain intact throughout the gastrointestinal tract, allowing it to be absorbed into the blood under certain medical conditions [34]. Although the exact mechanism of action used by lactoferrin is far from being elucidated, this finding is extremely valuable as it suggests the potential use of orally administered peptides in place of conventional antibiotics. Using this protocol, other HDPs may also be tested to access their effectiveness towards targeting other remote sites of the body when taken orally.

Alternatively, instead of introducing external sources of HDPs into our system, the expression of peptides may be upregulated as a treatment for UTI; past findings have suggested the deficiency in natural HDP production to be one of the main factors leading to the development of certain infectious diseases as well as UTI [35]. This was confirmed by a recent study, where LL-37 levels were significantly lower in UTI patients after infection compared to uninfected individuals [36]. Hence, it has been suggested that induction of certain HDPs may be an effective treatment for UTI [35]. This was confirmed by Hertting et al.; when bladder biopsies were infected with uropathogenic *E. coli*, a significant increase in cathelicidin expression was induced using vitamin D [37]. Similarly, using a mouse model, Rivas-Santiago et al. were able to upregulate the expression of β-defensins 3 and 4 using L-isoleucine [38]. Other approaches include the use of butyrate and vitamin D to upregulate the expression of HDPs LL-37 and cathelicidin, respectively [39, 40].

Estrogen may also indirectly induce HDPs; postmenopausal women suffer from recurrent UTI frequently as a result of low levels of estrogen, leading to structural and chemical changes in the urogenital tract which increases the likelihood of contracting UTI [41]. When estrogen is locally supplemented, the improved integrity of the urinary tract is accompanied by an increased production of HDPs, including β-defensins 1–3, cathelicidin, ribonuclease 7, and psoriasin [41].

Indeed certain inducers are capable of upregulating the expression of various HDPs. This is important as many studies suggest the expression of particular peptides, such as β-defensins 3 and 14, to serve key roles in mucosal defense of the urinary tract, combating infections associated with the system [42].

2.2. Medical Devices. Catheter-associated urinary tract infections (CAUTI) are one of the most common sources of healthcare-associated infections, accounting for 80% of hospital-acquired infections worldwide [43]. In the United States alone, there are approximately 450,000 cases a year, and direct treatment amounts to over $350 million annually [43, 44]. Upon insertion of the urinary catheter into the human body, bacteria adhere onto the surfaces of the implant [18, 19, 43, 45]. Once adhered, they can grow and form colonies, eventually leading to a biofilm and causing infection and encrustation [7]. Biofilms are complex, multilayered communities of microorganisms adhered onto a surface and embedded in self-produced extracellular polymeric substances, which generally consist of extracellular DNA, proteins, and polysaccharides [45–47]. The extracellular matrix reduces the diffusion of antimicrobial compounds and the close proximity of the cells facilitates horizontal gene transfer between antibiotic-resistant and nonresistant bacterial strains, making them extremely resistant to antibiotic treatment [45, 48].

Since fully developed biofilms are difficult to treat, coating urinary catheters with antimicrobial compounds prior to implantation has been of high interest to prevent the formation of biofilms [43]. To date, several different types of coatings have been tested, including antibiotics, silver, triclosan, gendine, and heparin [43]. However, these compounds are often found to be cytotoxic, are associated with the development of antibiotic resistance, or are only effective *in vitro* and not *in vivo* [19, 43].

Recently, HDPs have been examined as a potential coating for urinary catheters and ureteral stents [18, 19, 43]. Tachyplesin III (KWCFRVCYRGICYRKCR-NH2) is a HDP isolated from horseshoe crabs and has been shown to have broad spectrum activity [7]. Minardi et al. investigated the effect of coating Tachyplesin III on ureteral stents in preventing biofilm formation *in vivo* using a rat subcutaneous pouch model and found coated samples to inhibit bacterial growth up to 1000 times [7]. No drug related adverse effects were physically observed in any of the treated animals [7].

HDP implant coatings were further advanced when the use of polymer brushes was introduced [18, 19]. By covalently grafting hydrophilic copolymer (poly(N,N-dimethylacrylamide) (PDMA) and poly(N-(3-aminopropyl) methacrylamide) (PAPMA)) chains onto a surface, and conjugating them to an optimized series of HDPs, Gao et al. were able to demonstrate the effective antimicrobial activity of a peptide-brush coating [18]. Polymer brush

structures served as a flexible linker between HDPs and the surface while maximizing the density of peptides per coating [18]. *In vitro*, when 1–5 × 10^5 CFU/mL of Gram-positive or Gram-negative bacteria was introduced to titanium wires (Ti-wires) coated with peptide Tet-20 (KRWRIRVRVIRKC-NH2), there was a 100,000-fold decrease in CFU for treated Ti-wires 4 hours after incubation in comparison to uncoated controls [18]. The activity was also demonstrated *in vivo* using a rat infection model; when coated and uncoated Ti-wires were implanted into subcutaneous pockets of the rat and were challenged with 10^8 CFU of *Staphylococcus aureus* under a 7-day implantation period, CFU was decreased by 85% for treated rats compared to controls [18]. Moreover, using scanning electron microscopy (SEM), modified 50% haemolytic complement (CH50) analysis, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, the authors were able to demonstrate that peptides gave insignificant platelet activation and adhesion, no complement activation in human blood, and were nontoxic to osteoblast-like cells, respectively [18]. All these results suggest HDPs to be a promising alternative to catheter coatings.

More recently, Li et al. demonstrated the effectiveness of another brush-peptide coating; they utilized allyl glycidyl ether (AGE) polymer brushes in place of PDMA/PAPMA brushes and novel peptides with salt-tolerant properties (engineered from C-terminus of salt-resistant human beta defensin 28) instead of Tet-20 [19]. These novel peptides were referred to as RK1 (RWKRWWRRKK-NH2) and RK2 (RKKRWWRRKK-NH2) [19]. The authors stated that many HDPs succumb to salt inactivation at physiological salt concentrations, and thus the HDPs must be tolerant to salt [19]. The particular brush-peptide coating was immobilized onto polydimethylsiloxane and urinary catheter surfaces and was introduced to *E. coli*, *S. aureus*, and *Candida albicans* [19]. *In vitro* assays showed coated slides exhibited >70% killing activity towards all pathogens tested, with almost 100% inhibition of microbial colonization to surfaces [19]. Additionally, no toxicity towards smooth muscle cells was observed, as demonstrated using the MTT assay [19].

Indeed, brush-peptide coatings may be the next golden coating for urinary catheters to help prevent biofilm formation and infection. However, more clinically relevant *in vivo* models must be used to further test these coatings before they can be made available to the public.

2.3. Cancer. Besides taking a role in UTI and urologic devices, HDPs also serve a prominent role in a disease which affects 14.1 million adults per year worldwide and results in 8.2 million deaths annually: cancer [49]. In this section, we discuss the use of HDPs in bladder cancer, prostate cancer, and kidney cancer.

2.3.1. Bladder Cancer. Each year, approximately 75,000 new cases of bladder cancer are diagnosed, with 20% of them leading to death [50]. If treated by transurethral resection alone, recurrence and progressiveness of nonmuscle invasive bladder cancers can occur in 80% of the cases [20]. Various chemotherapeutic drugs have been established for treatment, including postoperative adjuvant intravesicle instillations of mitomycin, epirubicin, doxorubicin, and immunotherapy with Bacillus Calmette-Guérin (BCG) [23, 51, 52]. However, current treatments have been disappointing with respect to long-term outcomes and, due to their lack of specificity, are often associated with many side effects; 38.8% of patients treated with BCG and 46.4% of patients treated with mitomycin C developed tumor recurrences within 2 years after treatment [24, 53]. BCG is also associated with moderate to severe side effects, including arthritis, febrile episodes, and risk of sepsis [23, 54, 55]. It is also common for patients to develop multidrug resistance, rendering multiple chemotherapeutics ineffective [56]. Thus, it is important to search for alternative treatments.

Interestingly, although the use of HDPs has mainly been used to target pathogens, the peptides, particularly with magainin II (GIGKFLHSAKKFGKAFVGEIMNS-NH2), have recently been reported to have significant cytotoxic effect against a wide range of cancer cell lines, including breast and lung cancers, melanoma, lymphomas, and leukemias [20–22, 57, 58]. Lehmann et al. were particularly interested in evaluating the activity of magainin II, a peptide originally isolated from the skin of African frog *Xenopus laevis*, against bladder cancer cells [20]. Using water soluble 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetra-zolium (WST-1), bromodeoxyuridine (BrdU), and lactate dehydrogenase (LDH) assays, the authors tested antitumor activity of the HDP against 3 different bladder cell lines as well as 2 benign fibroblast cell lines as noncancerous controls [20]. Magainin II significantly inhibited both cell proliferation and DNA synthesis in all bladder cancer cells tested while having no effect on the fibroblast cell lines, demonstrating the specificity of the peptide towards cancer cell lines [20]. Potent concentrations of magainin II for tumor cells were significantly lower than that required to damage normal fibroblasts, erythrocytes, and peripheral blood lymphocytes [59]. The peptide was also shown to be highly resistant against serum proteolysis [60]. In another study using an *in vivo* severe combined immunodeficiency mouse model, introduction of magainins and their analogues to melanoma cells leads to a complete tumor regression [22]. When administered intraperitoneally to mice with ascites tumors, magainin analogues were also able to increase the rodents' survival time [21].

With magainin II looking promising, other studies looked into other HDPs that may also give similar antitumor effects [23]. One HDP family with structural and functional similarity to magainin II was the cecropin family, first isolated from the giant silk moth, *Hyalophora cecropia* [23]. Cecropins have been previously demonstrated to possess specific anticancer activity against small cell lung cancer, mammalian leukemia, gastric cancer cells, and lymphoma and colon carcinoma cell lines [61]. Using the same tests performed to evaluate the tumoricidal activity of magainin II, Lehmann and his colleagues evaluated the potency of cecropin A (KWKLFKKI-EKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH2) and cecropin B (KWKVFKKIEKMGRNIRNGIVKAGPAIAVL-GEAKAL-NH2) against bladder cancer cells [23]. Similar to magainin II, cecropins were selective for cancer cell lines, sparing all benign cells [23]. Inhibition of cell viability and

proliferation was observed at a dose-dependent manner [23]. Scanning electron microscopy allowed visualization of lethal membrane disruption in all bladder cancer cells tested, which was not present in control cells [23]. Moreover, transfection of human bladder tumor cells with cecropin genes has been shown to reduce tumor sizes in nude mouse models [62]. Cecropins have also been shown to be largely resistant against serum and urine proteolysis, giving them an advantage over classic chemotherapeutic agents such as mitomycin, which is highly unstable in urine [63].

Although both magainin and cecropins seem to be promising, some reports have suggested making synthetic modifications to further optimize their bioactivity and rate of biodegradation [24, 64]; Huang et al. worked to bypass potential proteolytic sensitivity by using nonnatural peptidomimetics [24]. They developed poly-N-substituted glycines (peptoids), which mimic the cationic, amphipathic structural feature of magainin II but consist of slight molecular changes, ensuring them to be protease-resistant and more stable [24]. Based on 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assays, the peptoids exhibited fast, potent cytotoxicity at low micromolar concentrations against a wide range of human cancer cell lines, with increased cytotoxicity when treatment duration was longer [24]. When subjected to primary dermal fibroblasts and red blood cells, the peptides showed minimal influence, validating their selectivity for cancer cells [24]. Looking into structure-activity correlations, hydrophobicity and amphipathicity seem to be important for the tumoricidal activity, with peptoid chains of approximately 13 residues having highest potency. The efficacy of peptoids *in vivo* has been validated using a clinically relevant orthotopic xenotransplantation model, where human breast cancer cells were implanted into immunocompromised mice [24]. When peptoid was injected 2 weeks after implantation, tumor growth was significantly inhibited [24].

Although some HDPs appear to have significant specific tumoricidal activity against cancer cell lines, the cytotoxic mechanisms remain to be discovered [23]. The mechanism behind the ability of these peptides to selectively target cancer cells while leaving benign cells spared also remains to be mapped [23]. A possibility may be due to physiochemical differences in the target cell membranes, such as differences in lipoprotein content or fluidity [23]. However, their high selectivity and tumoricidal capabilities may allow for optimal therapy *in vivo* at low therapeutic concentrations, potentially limiting any side effects associated with them [23]. It is also important to point out that the antitumor effect appears to be unaffected by the multidrug resistant cells, which is a common phenotype observed in cancer cells [23, 24]. Such advantages may allow these HDPs to be used as treatments for bladder cancer patients in the near future.

2.3.2. Prostate/Kidney Cancer. Prostate cancer is the second most common cause of death in the United States, and approximately 1 of every 6 men will get diagnosed with the deadly disease during their lifetime [50]. Kidney cancer is not fatal, but with approximately 65,000 new cases a year and 20% of those resulting in death, it still presents a major concern to public health [50]. Although the two types of cancers affect different parts of the urinary tract, one of the similar characteristics between them involves the loss of human β-defensin-1 (DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK-NH2) [25].

Human β-defensin-1 (hBD-1) has been known as a HDP of the urogenital tissues for approximately two decades [32]. However, it was only within the past 10 years when the peptide started gaining extensive attention in its role as an anticancer agent [25–27, 65]. By performing immunohistochemical analysis for hBD-1 in clinical specimens of both prostate cancer and renal cell carcinoma, Donald et al. found significant cancer-specific downregulation of the peptide in 82% and 90% of prostate cancer and renal cell carcinomas, respectively, while adjacent benign regions were unaffected [25]. Based on the authors' analysis on promoter polymorphisms, it was suggested that hBD-1 acts as a tumor suppressor, promoting caspase-3-mediated apoptosis of prostate and renal cancer cells when overexpressed [26, 27]. hBD-1 is located in chromosome 8 at segment region 8p23.2, a region where multiple tumor suppressor genes reside and genetic alternations are common in prostate and renal carcinoma [26, 27].

To gain insight into how hBD-1 may affect the behavior of cancer cells, Bullard et al. cloned the peptide and expressed it ectopically in different prostate cancer cell lines, including DU145, PC3, and LNCap [27]. Introduction of the peptide showed cytotoxic effects against DU145 and PC3, but not LNCap, which suggests hBD-1 targets mainly late-stage prostate carcinoma cells [27]. As such, with the specificity against prostate and renal cancer cells and its tumor-suppressive activity, hBD-1 may be used as an effective anticancer agent [27]. It may be interesting to see what may happen when hBD-1 inducers, such as the ones mentioned in Section 2.1 of this review article, are introduced to the cancer cell lines.

2.4. Kidney Stone Disease. Kidney stone disease is a common pathological disorder in industrialized countries and affects 10–15% of men and 3–5% of women in the United States, with prevalence on the rise [66, 67]. The disease causes significant morbidities, and adverse effects are often experienced when the stones reach an appreciable size, which can become dislodged from the epithelial membrane [28]. Current treatments are limited to increased water intake, supervised dieting, and alkalinization agents [28, 68]. Although such treatments can provide temporary relief, they do not lower the incidence of stone formation [69].

Kidney stones can consist of different types of components, but the main component is calcium oxalate, which comprises 70% of kidney stones [70]. Two polymorphs of calcium oxalate can form, one being the monohydrate (COM) and the other being dihydrate (COD) [71]. COM is the most abundant phase in stone formers and typically constitutes the core of kidney stones [72]. They are rarely excreted via the urine by healthy individuals [70]. COD on the other hand is excreted regularly by both healthy individuals and stone formers [70]. They are less adherent and less stable and cause less damage to cell membrane compared to COM [28, 70, 73].

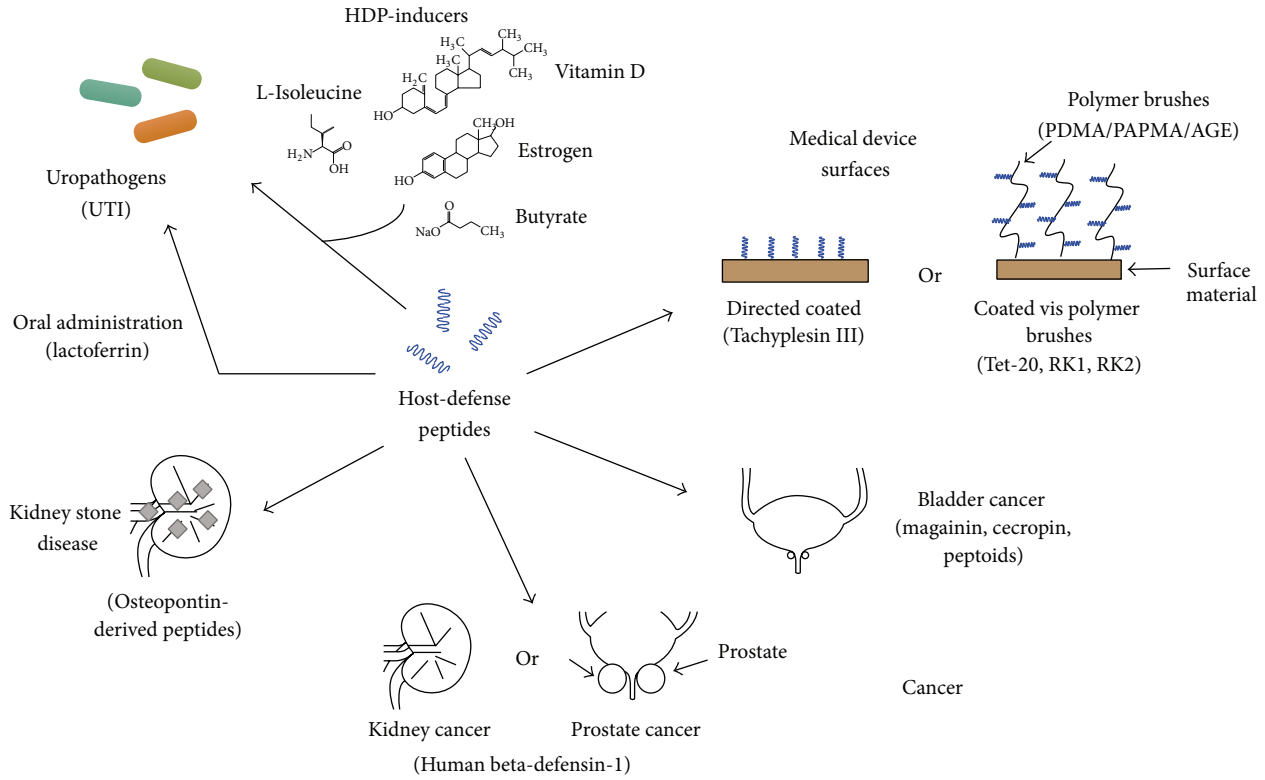


Figure 1: Applications of host-defense peptides in the field of urology.

By preventing the dissolution of COD to COM, crystal deposition and kidney stone formation may be suppressed [73].

The inhibition of COM formation and initiation of COD polymorph has been demonstrated by osteopontin (OPN), which are highly acidic or hydrophilic peptides [70]. Although it is not consistent with the typical cationic and hydrophobic properties that define HDPs, OPN has been reported to play crucial roles as an immune modulator, being involved with chemotactic properties to recruit cells to inflammatory sites, with mediating cell activation and cytokine production and with wound healing [12, 74, 75]. As such, for this review, we consider it as a member of HDP.

OPN-derived peptides have been shown to be effective in inhibiting COM formation while promoting COD precipitation, with the main active properties being the considerable portion of aspartic acid-rich regions, motifs, phosphorylated peptides, hydrophilic residues, net negative charges, and peptide length [28, 70, 76]. Some of the most potent OPN-derived peptides have been shown to be capable of reducing COM growth by more than 90% [77]. Farmanesh et al. have recently focused on designing and screening short peptides with functional moieties made to mimic COM inhibitors [28]. Different peptide sequences were tested to determine the difference in anti-COM activity when alanine and aspartic acid amino acids were arranged differently [28]. The authors found that subtle alterations in the sequence of the acidic residues had profound effects on the anti-COM potential [28]. By using high-throughput *in situ* calcium ion-selective electrode (ISE) screening to rapidly and reproducibly screen large peptide libraries, peptide sequences were discovered which inhibited COM formation more effectively than well-known COM inhibitors, such as citrate [28]. With bulk crystallization which involved optical and scanning electron microscopy, effective inhibitors were validated and were found to have a high tendency to shift for morphology of COM crystals from hexagonal morphology to diamond-shaped platelets, possibly due to the preferential binding of peptides to particular faces of the COM [28]. Effective COM-inhibiting peptides were also found to reduce the growth rates of COM [28]. These particular results suggest these anti-COM peptides may be valuable candidates as future therapies for kidney stone formers.

Table 1 and Figure 1 have been included to aid in understanding the characteristic of HDPs discussed in this review, as well as their current and potential applications in the field of urology (Table 1, Figure 1).

3. Conclusions

As shown in this review, the wide-ranging functionality of HDPs against infection and disease of the urinary tract expands the peptides' activity list to well beyond the "antimicrobial peptide" originally assigned to them. From targeting bacteria and cancer cells to preventing kidney stone formation, no single peptide carried all the different traits necessary to fully treat each condition. Within urology, many potential applications of HDPs have been studied, with very promising results observed. Given that these HDPs do not appear to be susceptible to MDR bacteria and cancer cells, they may

potentially form the compounds of the next "golden age" of new antimicrobials in the near future. However, before they reach that stage, further studies are required to thoroughly understand their advantages, limitations, and mechanisms of action.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Each author has contributed to the research of this review article and drafting and finalizing this paper.

References

[1] G. F. Gibberd, " Prontosil etc. in puerperal Streptococcus infections," *British Medical Journal*, vol. 2, no. 4005, pp. 695–698, 1937.

[2] J. Davies and D. Davies, "Origins and evolution of antibiotic resistance," *Microbiology and Molecular Biology Reviews*, vol. 74, no. 3, pp. 417–433, 2010.

[3] B. Spellberg, R. Guidos, D. Gilbert et al., "The epidemic of antibiotic-resistant infections: a call to action for the medical community from the infectious diseases society of America," *Clinical Infectious Diseases*, vol. 46, no. 2, pp. 155–164, 2008.

[4] S. R. Dennison, F. Harris, M. Mura, L. H. G. Morton, A. Zvelindovsky, and D. A. Phoenix, "A novel form of bacterial resistance to the action of eukaryotic host defense peptides, the use of a lipid receptor," *Biochemistry*, vol. 52, no. 35, pp. 6021–6029, 2013.

[5] D. A. Tadesse, S. Zhao, E. Tong et al., "Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950–2002," *Emerging Infectious Diseases*, vol. 18, no. 5, pp. 741–749, 2012.

[6] I. M. Cullen, R. P. Manecksha, E. McCullagh et al., "The changing pattern of antimicrobial resistance within 42 033 *Escherichia coli* isolates from nosocomial, community and urology patient-specific urinary tract infections, Dublin, 1999–2009," *BJU International*, vol. 109, no. 8, pp. 1198–1206, 2012.

[7] D. Minardi, R. Ghiselli, O. Cirioni et al., "The antimicrobial peptide Tachyplesin III coated alone and in combination with intraperitoneal piperacillin-tazobactam prevents ureteral stent *Pseudomonas* infection in a rat subcutaneous pouch model," *Peptides*, vol. 28, no. 12, pp. 2293–2298, 2007.

[8] R. E. Hancock, "Cationic peptides: effectors in innate immunity and novel antimicrobials," *The Lancet Infectious Diseases*, vol. 1, no. 3, pp. 156–164, 2001.

[9] C. D. Fjell, J. A. Hiss, R. E. W. Hancock, and G. Schneider, "Designing antimicrobial peptides: form follows function," *Nature Reviews Drug Discovery*, vol. 11, no. 1, pp. 37–51, 2012.

[10] M. L. Mangoni, "Host-defense peptides: from biology to therapeutic strategies," *Cellular and Molecular Life Sciences*, vol. 68, no. 13, pp. 2157–2159, 2011.

[11] S. A. Baltzer and M. H. Brown, "Antimicrobial peptides—promising alternatives to conventional antibiotics," *Journal of Molecular Microbiology and Biotechnology*, vol. 20, no. 4, pp. 228–235, 2011.

[12] R. E. Hancock and H.-G. Sahl, "Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies," *Nature Biotechnology*, vol. 24, no. 12, pp. 1551–1557, 2006.

[13] R. L. Williams, H. Y. Sroussi, K. Leung, and P. T. Marucha, "Antimicrobial decapeptide KSL-W enhances neutrophil chemotaxis and function," *Peptides*, vol. 33, no. 1, pp. 1–8, 2012.

[14] A. S. Ali, C. L. Townes, J. Hall, and R. S. Pickard, "Maintaining a sterile urinary tract: the role of antimicrobial peptides," *The Journal of Urology*, vol. 182, no. 1, pp. 21–28, 2009.

[15] A. K. Marr, W. J. Gooderham, and R. E. Hancock, "Antibacterial peptides for therapeutic use: obstacles and realistic outlook," *Current Opinion in Pharmacology*, vol. 6, no. 5, pp. 468–472, 2006.

[16] K. A. Brogden, "Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?" *Nature Reviews Microbiology*, vol. 3, no. 3, pp. 238–250, 2005.

[17] L. A. Haversen, I. Engberg, L. Baltzer, G. Dolphin, L. A. Hanson, and I. Mattsby-Baltzer, "Human lactoferrin and peptides derived from a surface-exposed helical region reduce experimental *Escherichia coli* urinary tract infection in mice," *Infection and Immunity*, vol. 68, no. 10, pp. 5816–5823, 2000.

[18] G. Gao, D. Lange, K. Hilpert et al., "The biocompatibility and biofilm resistance of implant coatings based on hydrophilic polymer brushes conjugated with antimicrobial peptides," *Biomaterials*, vol. 32, no. 16, pp. 3899–3909, 2011.

[19] X. Li, P. Li, R. Saravanan et al., "Antimicrobial functionalization of silicone surfaces with engineered short peptides having broad spectrum antimicrobial and salt-resistant properties," *Acta Biomaterialia*, vol. 10, no. 1, pp. 258–266, 2014.

[20] J. Lehmann, M. Retz, S. S. Sidhu et al., "Antitumor activity of the antimicrobial peptide magainin II against bladder cancer cell lines," *European Urology*, vol. 50, no. 1, pp. 141–147, 2006.

[21] M. A. Baker, W. L. Maloy, M. Zasloff, and L. S. Jacob, "Anticancer efficacy of Magainin2 and analogue peptides," *Cancer Research*, vol. 53, no. 13, pp. 3052–3057, 1993.

[22] P. W. Soballe, W. L. Maloy, M. L. Myrga, L. S. Jacob, and M. Herlyn, "Experimental local therapy of human-melanoma with lytic magainin peptides," *International Journal of Cancer*, vol. 60, no. 2, pp. 280–284, 1995.

[23] H. Suttmann, M. Retz, F. Paulsen et al., "Antimicrobial peptides of the Cecropin-family show potent antitumor activity against bladder cancer cells," *BMC Urology*, vol. 8, article 5, 2008.

[24] W. Huang, J. Seo, S. B. Willingham et al., "Learning from host-defense peptides: cationic, amphipathic peptoids with potent anticancer activity," *PLoS ONE*, vol. 9, no. 2, Article ID e90397, 2014.

[25] C. D. Donald, C. Q. Sun, S. D. Lim et al., "Cancer-specific loss of beta-defensin 1 in renal and prostatic carcinomas," *Laboratory Investigation*, vol. 83, no. 4, pp. 501–505, 2003.

[26] C. Q. Sun, R. Arnold, C. Fernandez-Golarz et al., "Human β-defensin-1, a potential chromosome 8p tumor suppressor: control of transcription and induction of apoptosis in renal cell carcinoma," *Cancer Research*, vol. 66, no. 17, pp. 8542–8549, 2006.

[27] R. S. Bullard, W. Gibson, S. K. Bose et al., "Functional analysis of the host defense peptide Human Beta Defensin-1: new insight into its potential role in cancer," *Molecular Immunology*, vol. 45, no. 3, pp. 839–848, 2008.

[28] S. Farmanesh, J. Chung, D. Chandra, R. D. Sosa, P. Karande, and J. D. Rimer, "High-throughput platform for design and screening of peptides as inhibitors of calcium oxalate monohydrate

crystallization," *Journal of Crystal Growth*, vol. 373, pp. 13–19, 2013.

[29] M. Chromek, Z. Slamová, P. Bergman et al., "The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection," *Nature Medicine*, vol. 12, no. 6, pp. 636–641, 2006.

[30] J. D. Spencer, A. L. Schwaderer, H. Wang et al., "Ribonuclease 7, an antimicrobial peptide upregulated during infection, contributes to microbial defense of the human urinary tract," *Kidney International*, vol. 83, no. 4, pp. 615–625, 2013.

[31] T. Weichhart, M. Haidinger, W. H. Hörl, and M. D. Säemann, "Current concepts of molecular defence mechanisms operative during urinary tract infection," *European Journal of Clinical Investigation*, vol. 38, supplement 2, pp. 29–38, 2008.

[32] E. V. Valore, C. H. Park, A. J. Quayle, K. R. Wiles, P. B. McCray Jr., and T. Ganz, "Human β-defensin-1: an antimicrobial peptide of urogenital tissues," *The Journal of Clinical Investigation*, vol. 101, no. 8, pp. 1633–1642, 1998.

[33] Y. Pan, G. A. Sonn, M. L. Y. Sin et al., "Electrochemical immunosensor detection of urinary lactoferrin in clinical samples for urinary tract infection diagnosis," *Biosensors and Bioelectronics*, vol. 26, no. 2, pp. 649–654, 2010.

[34] D. Legrand, E. Elass, A. Pierce, and J. Mazurier, "Lactoferrin and host defence: an overview of its immuno-modulating and anti-inflammatory properties," *BioMetals*, vol. 17, no. 3, pp. 225–229, 2004.

[35] B. Rivas-Santiago, C. J. Serrano, and J. A. Enciso-Moreno, "Susceptibility to infectious diseases based on antimicrobial peptide production," *Infection and Immunity*, vol. 77, no. 11, pp. 4690–4695, 2009.

[36] K. L. Nielsen, P. Dynesen, P. Larsen, L. Jakobsen, P. S. Andersen, and N. Frimodt-Møller, "Role of urinary cathelicidin LL-37 and human β-defensin 1 in uncomplicated escherichia coli urinary tract infections," *Infection and Immunity*, vol. 82, no. 4, pp. 1572–1578, 2014.

[37] O. Hertting, Å. Holm, P. Lüthje et al., "Vitamin D induction of the human antimicrobial peptide cathelicidin in the urinary bladder," *PLoS ONE*, vol. 5, no. 12, Article ID e15580, 2010.

[38] C. E. Rivas-Santiago, B. Rivas-Santiago, D. A. León, J. Castañeda-Delgado, and R. Hernández Pando, "Induction of β-defensins by l-isoleucine as novel immunotherapy in experimental murine tuberculosis," *Clinical and Experimental Immunology*, vol. 164, no. 1, pp. 80–89, 2011.

[39] M. Schwab, V. Reynders, Y. Shastri, S. Loitsch, J. Stein, and O. Schröder, "Role of nuclear hormone receptors in butyrate-mediated up-regulation of the antimicrobial peptide cathelicidin in epithelial colorectal cells," *Molecular immunology*, vol. 44, no. 8, pp. 2107–2114, 2007.

[40] J. Schauber, R. A. Dorschner, A. B. Coda et al., "Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism," *The Journal of Clinical Investigation*, vol. 117, no. 3, pp. 803–811, 2007.

[41] P. Lüthje, A. Lindén Hirschberg, and A. Brauner, "Estrogenic action on innate defense mechanisms in the urinary tract," *Maturitas*, vol. 77, no. 1, pp. 32–36, 2014.

[42] B. Becknell, J. D. Spencer, A. R. Carpenter et al., "Expression and antimicrobial function of beta-defensin 1 in the lower urinary tract," *PLoS ONE*, vol. 8, no. 10, Article ID e77714, 2013.

[43] J. Lo, D. Lange, and B. Chew, "Ureteral stents and foley catheters-associated urinary tract infections: the role of coatings and materials in infection prevention," *Antibiotics*, vol. 3, no. 1, pp. 87–97, 2014.

[44] P. J. Nowatzki, R. R. Koepsel, P. Stoodley et al., "Salicylic acid-releasing polyurethane acrylate polymers as anti-biofilm urological catheter coatings," *Acta Biomaterialia*, vol. 8, no. 5, pp. 1869–1880, 2012.

[45] F. L. Brancatisano, G. Maisetta, M. Di Luca et al., "Inhibitory effect of the human liver-derived antimicrobial peptide hepcidin 20 on biofilms of polysaccharide intercellular adhesin (PIA)-positive and PIA-negative strains of *Staphylococcus epidermidis*," *Biofouling*, vol. 30, no. 4, pp. 435–446, 2014.

[46] J. W. Costerton, P. S. Stewart, and E. P. Greenberg, "Bacterial biofilms: a common cause of persistent infections," *Science*, vol. 284, no. 5418, pp. 1318–1322, 1999.

[47] N. Høiby, T. Bjarnsholt, M. Givskov, S. Molin, and O. Ciofu, "Antibiotic resistance of bacterial biofilms," *International Journal of Antimicrobial Agents*, vol. 35, no. 4, pp. 322–332, 2010.

[48] N. Hoiby, O. Ciofu, H. K. Johansen et al., "The clinical impact of bacterial biofilms," *International Journal of Oral Science*, vol. 3, no. 2, pp. 55–65, 2011.

[49] "Cancer research UK," *Nature Cell Biology*, vol. 4, no. 3, article E45, 2002.

[50] R. Siegel, J. Ma, Z. Zou, and A. Jemal, "Cancer statistics, 2014," *CA: A Cancer Journal for Clinicians*, vol. 64, no. 1, pp. 9–29, 2014.

[51] W. Oosterlinck, B. Lobel, G. Jakse, P.-U. Malmström, M. Stöckle, and C. Sternberg, "Guidelines on bladder cancer," *European Urology*, vol. 41, no. 2, pp. 105–112, 2002.

[52] A. Stenzl, N. C. Cowan, M. de Santis et al., "Treatment of muscle-invasive and metastatic bladder cancer: update of the EAU guidelines," *European Urology*, vol. 59, no. 6, pp. 1009–1018, 2011.

[53] A. Böhle, D. Jocham, and P. R. Bock, "Intravesical bacillus Calmette-Guerin versus mitomycin C for superficial bladder cancer: a formal meta-analysis of comparative studies on recurrence and toxicity," *The Journal of Urology*, vol. 169, no. 1, pp. 90–95, 2003.

[54] M. Brausi, J. Oddens, R. Sylvester et al., "Side effects of bacillus calmette-guérin (BCG) in the treatment of intermediate- and high-risk Ta, T1 papillary carcinoma of the bladder: results of the EORTC genito-urinary cancers group randomised phase 3 study comparing one-third dose with full dose and 1 year with 3 years of maintenance BCG," *European Urology*, vol. 65, no. 1, pp. 69–76, 2014.

[55] A. P. M. van der Meijden, M. Brausi, V. Zambon et al., "Intravesical instillation of epirubicin, bacillus Calmette-Guerin and bacillus Calmette-Guerin plus isoniazid for intermediate and high risk TA, T1 papillary carcinoma of the bladder: a European Organization for Research and Treatment of Cancer Genito-Urinary Group randomized phase III trial," *The Journal of Urology*, vol. 166, no. 2, pp. 476–481, 2001.

[56] J. I. Fletcher, M. Haber, M. J. Henderson, and M. D. Norris, "ABC transporters in cancer: more than just drug efflux pumps," *Nature Reviews Cancer*, vol. 10, no. 2, pp. 147–156, 2010.

[57] R. A. Cruciani, J. L. Barker, M. Zasloff, H.-C. Chen, and O. Colamonici, "Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 9, pp. 3792–3796, 1991.

[58] Y. Ohsaki, A. F. Gazdar, H.-C. Chen, and B. E. Johnson, "Antitumor activity of magainin analogues against human lung cancer cell lines," *Cancer Research*, vol. 52, no. 13, pp. 3534–3538, 1992.

[59] W. L. Maloy and U. P. Kari, "Structure-activity studies on magainins and other host defense peptides," *Biopolymers*, vol. 37, no. 2, pp. 105–122, 1995.

[60] R. Bessalle, A. Kapitkovsky, A. Gorea, I. Shalit, and M. Fridkin, "All-D-magainin: chirality, antimicrobial activity and proteolytic resistance," *FEBS Letters*, vol. 274, no. 1-2, pp. 151–155, 1990.

[61] R. J. Boohaker, M. W. Lee, P. Vishnubhotla, J. M. Perez, and A. R. Khaled, "The use of therapeutic peptides to target and to kill cancer cells," *Current Medicinal Chemistry*, vol. 19, no. 22, pp. 3794–3804, 2012.

[62] D. Winder, W. H. Günzburg, V. Erfle, and B. Salmons, "Expression of antimicrobial peptides has an antitumour effect in human cells," *Biochemical and Biophysical Research Communications*, vol. 242, no. 3, pp. 608–612, 1998.

[63] H. G. Boman, "Antibacterial peptides: basic facts and emerging concepts," *Journal of Internal Medicine*, vol. 254, no. 3, pp. 197–215, 2003.

[64] N. P. Chongsiriwatana, J. A. Patch, A. M. Czyzewski et al., "Peptoids that mimic the structure, function, and mechanism of helical antimicrobial peptides," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 8, pp. 2794–2799, 2008.

[65] N. Droin, J.-B. Hendra, P. Ducoroy, and E. Solary, "Human defensins as cancer biomarkers and antitumour molecules," *Journal of Proteomics*, vol. 72, no. 6, pp. 918–927, 2009.

[66] J. Tang, P. Mettler, K. Mcfann, and M. Chonchol, "The association of prevalent kidney stone disease with mortality in US adults: the national health and nutrition examination survey III, 1988–1994," *American Journal of Nephrology*, vol. 37, no. 5, pp. 501–506, 2013.

[67] K. K. Stamatelou, M. E. Francis, C. A. Jones, L. M. Nyberg Jr., and G. C. Curhan, "Time trends in reported prevalence of kidney stones in the United States: 1976–1994," *Kidney International*, vol. 63, no. 5, pp. 1817–1823, 2003.

[68] A. D. Rule, A. E. Krambeck, and J. C. Lieske, "Chronic kidney disease in kidney stone formers," *Clinical Journal of the American Society of Nephrology*, vol. 6, no. 8, pp. 2069–2075, 2011.

[69] F. L. Coe and J. R. Asplin, "Stopping the stones," *Science*, vol. 330, no. 6002, pp. 325–326, 2010.

[70] B. P. H. Chan, K. Vincent, G. A. Lajoie, H. A. Goldberg, B. Grohe, and G. K. Hunter, "On the catalysis of calcium oxalate dihydrate formation by osteopontin peptides," *Colloids and Surfaces B: Biointerfaces*, vol. 96, pp. 22–28, 2012.

[71] J. C. Lieske and F. G. Toback, "Renal cell-urinary crystal interactions," *Current Opinion in Nephrology and Hypertension*, vol. 9, no. 4, pp. 349–355, 2000.

[72] N. S. Mandel and G. S. Mandel, "Urinary tract stone disease in the United States veteran population. II. Geographical analysis of variations in composition," *Journal of Urology*, vol. 142, no. 6, pp. 1516–1521, 1989.

[73] B. B. Tomazic and G. H. Nancollas, "The kinetics of dissolution of calcium oxalate hydrates. II. The dihydrate," *Investigative Urology*, vol. 18, no. 2, pp. 97–101, 1980.

[74] D. T. Denhardt, M. Noda, A. W. O'Regan, D. Pavlin, and J. S. Berman, "Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival," *The Journal of Clinical Investigation*, vol. 107, no. 9, pp. 1055–1061, 2001.

[75] T. Uede, Y. Katagiri, J. Iizuka, and M. Murakami, "Osteopontin, a coordinator of host defense system: a cytokine or an extracellular adhesive protein?" *Microbiology and Immunology*, vol. 41, no. 9, pp. 641–648, 1997.

[76] K. Singh, D. Deonarine, V. Shanmugam et al., "Calcium-binding properties of osteopontin derived from non-osteogenic sources," *Journal of Biochemistry*, vol. 114, no. 5, pp. 702–707, 1993.

[77] T. Jung, X. Sheng, C. K. Choi, W.-S. Kim, J. A. Wesson, and M. D. Ward, "Probing crystallization of calcium oxalate monohydrate and the role of macromolecule additives with in situ atomic force microscopy," *Langmuir*, vol. 20, no. 20, pp. 8587–8596, 2004.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 453826, 8 pages
http://dx.doi.org/10.1155/2015/453826

Research Article

Antibacterial Activity of Synthetic Peptides Derived from Lactoferricin against *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212

María A. León-Calvijo,[1] Aura L. Leal-Castro,[2] Giovanni A. Almanzar-Reina,[3] Jaiver E. Rosas-Pérez,[1] Javier E. García-Castañeda,[1] and Zuly J. Rivera-Monroy[1]

[1]*Sciences Faculty, Universidad Nacional de Colombia, Carrera 45, No. 26-85, Bogotá, Colombia*
[2]*Medicine Faculty, Universidad Nacional de Colombia, Carrera 45, No. 26-85, Bogotá, Colombia*
[3]*University Children's Hospital, University of Würzburg, 97080 Würzburg, Germany*

Correspondence should be addressed to Zuly J. Rivera-Monroy; zjriveram@unal.edu.co

Received 30 July 2014; Accepted 24 September 2014

Academic Editor: Alejandra Ochoa-Zarzosa

Peptides derived from human and bovine lactoferricin were designed, synthesized, purified, and characterized using RP-HPLC and MALDI-TOF-MS. Specific changes in the sequences were designed as (i) the incorporation of unnatural amino acids in the sequence, the (ii) reduction or (iii) elongation of the peptide chain length, and (iv) synthesis of molecules with different number of branches containing the same sequence. For each peptide, the antibacterial activity against *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 was evaluated. Our results showed that Peptides I.2 (RWQWRWQWR) and I.4 ($(RRWQWR)_4K_2Ahx_2C_2$) exhibit bigger or similar activity against *E. coli* (MIC 4–33 μM) and *E. faecalis* (MIC 10–33 μM) when they were compared with lactoferricin protein (LF) and some of its derivate peptides as II.1 (FKCRRWQWRMKKLGA) and IV.1 (FKCRRWQWRMKKLGAPSITCVRRAE). It should be pointed out that Peptides I.2 and I.4, containing the RWQWR motif, are short and easy to synthesize; our results demonstrate that it is possible to design and obtain synthetic peptides that exhibit enhanced antibacterial activity using a methodology that is fast and low-cost and that allows obtaining products with a high degree of purity and high yield.

1. Introduction

The World Health Organization has stated that control and/or treatment of infections caused by bacteria resistant to conventional drugs is considered a public health goal [1]. Indiscriminate use and inadequate dosage of conventional antibiotics have contributed to the development of resistant bacterial strains, decreasing the therapeutic options [1]. Over the last few decades, several investigations have addressed the development of drugs that do not induce resistance in pathogens and can thus be considered an alternative for the treatment of bacterial infections. Antimicrobial peptides (AMPs) have received special attention as a possible alternative way to combat infections caused by antibiotic-resistant bacterial strains. AMPs are considered to be an important part of the innate immune response, and they have been isolated from tissues and organisms from every kingdom and phylum and have been characterized [2–4]. AMPs have the following characteristics: they are (i) positively charged, (ii) amphipathic, (iii) structurally diverse, and (iv) of short length. AMPs have exhibited antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, viruses, and parasites [4]. Additionally, AMPs exhibit antibacterial activity over a broad range of pH and temperatures. Interestingly, AMPs have been identified in body fluid proteins in mammals [5], specifically lactoferrin (LF), an 80 kDa non-heme iron-binding protein that is located in mucosal secretions such as breast milk, saliva, seminal plasma, and vaginal mucus [2, 3, 6]. This protein has been associated with

biological activities such as antihypertensive, immunomodulator, antitumor, anti-inflammatory, transcription factor, procoagulant, and protease inhibitor activities, among others [7]. Additionally, it has been reported that LF exhibits antimicrobial activity against pathogenic bacteria, fungi, proto zoa, parasites, and viruses [8–11]. It has been suggested that LF activity is due to the N-terminal domain [10–13]. When the LF protein reaches the digestive tract, it is digested by gastric pepsin, and the protein hydrolyzate contains a peptide called lactoferricin (Lfcin), which belongs to the N-terminal region [12, 13]. Lfcin has shown greater antibacterial activity against Gram-negative and Gram-positive bacteria than that shown by the protein itself. Some authors have stated that the LF antibacterial activity is mainly due to the Lfcin peptide [2, 12–16]. Lfcin has been identified in several mammals, such as humans (LfcinH), bovine (LfcinB), goats, horses, and pigs [2]. The LfcinB (^{17}FKCRRWQWRMKKLGAPSITCVRRA^{41}F) has exhibited greater antibacterial activity than what was exhibited by the LfcinH (^{20}GRRRRSVQWCAVSQPEATKCFQ-WQRNMRKVRGPPVSC-IKRDSPIQC^{67}I). LfcinB inhibits the growth of a wide range of bacteria, viruses, fungi, and parasites [3, 5, 13, 17–21]. Additionally, LfcinB exhibited cytotoxic activity against cancer cell lines, suggesting that LfcinB could be used as an anticancer agent [22–24].

LfcinB contains aromatic amino acids, such as tryptophan (W) and phenylalanine (F), as well as basic residues (e.g., arginine, R and lysine, K) whose side chains provide a net charge of +8 to the peptide. LfcinB contains two cysteine residues that form an intrachain disulfide bridge so that charged and hydrophobic residues are located at opposite sides, providing amphipathic properties to the peptide. Positively charged residues interact electrostatically with the negative charges of bacterial cell wall lipopolysaccharide (LPS), allowing the peptide to approach the bacterial membrane. Then, LfcinB hydrophobic residues interact with the membrane lipid bilayer, causing its disruption and cell lysis [3, 7]. It has been reported that the RRWQWR sequence is the antimicrobial LfcinB center and is considered the smallest motif that exhibits antibacterial and anticancer activity [24, 25]. AMPs can be obtained through solid phase peptide synthesis (SPPS) quickly and inexpensively, with a high degree of purity and good yields [26]. SPPS is a powerful and versatile tool in the design and development of antibacterial agents, which allows the fast and easy production of peptides carrying non-natural amino acid residues and polyvalent molecules, that is, dimeric, tetrameric, and polymeric peptides of a specified amino acid sequence.

In the present paper, the antibacterial activity of synthetic peptides derived from LfcinB containing specific changes in the amino acid sequence was evaluated. These changes were as follows: (i) non-natural amino acid inclusion at specific positions, (ii) sequence length variation, and (iii) multivalent motif presentation, that is, the dimer and tetramer of the RRWQWR sequence. For the experimental strains, *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 were selected. Our results show that antibacterial activity is enhanced for peptides containing multiple presentations of the RWQWR motif and for peptides derived from LfcinB and LfcinH that contain specific changes in the amino acid sequence.

2. Materials and Methods

2.1. Reagents and Materials. Mueller-Hinton, Agar SPC, *E. coli* ATCC 25922, and *E. faecalis* ATCC 29212 were obtained from ATCC, USA. Rink amide resin, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Gln(Trt)-OH, Fmoc-β-Ala-OH, Fmoc-Phe-OH, Fmoc-Met-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Ile-OH, Fmoc-Glu(OtBu)OH, Fmoc-Thr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-Cys(Trt)-OH, Fmoc-Lys(Fmoc)-OH, 6-(Fmoc-amino)hexanoic acid (Fmoc-*Ahx*-OH), 1-hydroxybenzotriazole (HOBt), and *N,N*-dicyclohexylcarbodiimide (DCC) were purchased from AAPPTec (Louisville, KY, USA). *N,N*-Diisopropylethylamine (DIPEA), triisopropylsilane (TIPS), 1,2-Ethanedithiol (EDT), 4-methyl-piperidine, pyridine, ninhydrin, phenol, and KCN were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, diethyl ether, *N,N*-dimethylformamide (DMF), absolute ethanol, dichloromethane (DCM), acetonitrile (ACN), isopropyl alcohol (IPA), and trifluoroacetic acid (TFA) were obtained from Honeywell-Burdick & Jackson (Muskegon, Michigan, USA). All reagents were used without further purification.

2.2. Peptide Synthesis. Peptides were synthesized using the SPPS-Fmoc/tBu methodology [27]. Briefly, Rink amide resin (100 mg) was used as solid support. (i) The resin conditioning and Fmoc group removal were carried out through treatment with 20% 4-methyl-piperidine in DMF at room temperature (RT) for 10 minutes twice. Then, the resin was exhaustively washed with DMF, IPA, and DCM. (ii) For the coupling reaction, 0.21 mmol of Fmoc-amino acids was preactivated with DCC/HOBt (0.20/0.21 mmol) in DMF at RT. The activated Fmoc-amino acid was added to a reactor containing deprotected resin; the coupling reaction was shaken for two hours at RT, and then the resin was washed. (iii) Fmoc group elimination and the incorporation of each amino acid were confirmed through the ninhydrin test [28]. Side chain deprotection reactions and peptide separation from the resin were carried out with a cleavage cocktail containing TFA/water/TIPS/EDT (93/2/2.5/2.5% v/v). The cleavage mixture was filtered and the solution was collected. Crude peptides were precipitated via treatment of the solution with cool ethyl ether, and finally the products were washed with ether 5 times and dried.

2.3. Analytical Methods. Reverse phase HPLC (RP-HPLC) analysis was performed on an Agilent Eclipse XDB-C18 (4.6 × 150 mm, 3.5 μm) column using an Agilent 1200 liquid chromatograph (Omaha, Nebraska, USA). For the analysis of crude peptides (20 μL, 1 mg/mL), a linear gradient was applied from 5% to 70% Solvent B (0.05% TFA in ACN) in Solvent A (0.05% TFA in water) for 45 min at a flow rate of 1.0 mL/min at RT and 210 nm detection. The crude products were purified through solid-phase extraction (SPE), using Supelclean LC-18 SPE columns that were activated

and equilibrated prior to use. Crude peptides were passed through the column, and a gradient was used for their elution [29]. Collected fractions were analyzed using RP-HPLC (as describe above) and MS. MALDI-TOF MS analysis was performed on an Ultraflex III TOF-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflectron mode, using an MTP384 polished steel target (Bruker Daltonics), 2,5-dihydroxybenzoic acid, or sinapinic acid as a matrix, 500 shots with 25–30% power laser.

2.4. Susceptibility Testing. The bacterial strain *E. coli* ATCC 25922 was grown in Mueller Hinton broth (MH) from 18 to 24 hours at 37°C in an aerobic atmosphere. CFU/mL was calculated, and the inoculum was diluted to a 1×10^6 CFU/mL concentration. An aliquot was placed on MH agar plates, mixed, and allowed to solidify. Five wells were drilled using a punch of 8 mm, and then each hollow was filled with 100 μL of peptide (2000 μg/mL). Incubation for 24 hours at 37°C was then performed.

2.5. Antibacterial Activity. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the microdilution assay [30]. Briefly, bacterial strains were incubated for 18 to 24 hours at 37°C in MH broth until an optical density of 0.15 to 0.30 (620 nm) was obtained. Using a 96-well microtiter plate, peptide serial dilution (200, 100, 50, 25, 12.5. 6.2 μg/mL) was performed, and then they were incubated for 24 h at 37°C, with an inoculum of 2×10^6 CFU/mL in MH broth. The final volume in each well was 100 μL. After incubation for 18 h, the absorbance at 620 nm was measured using an Asys Expert Plus ELISA reader. For determining the MBC, using an inoculation loop, a small sample was taken from each well and then was spread on MH agar plates and incubated overnight at 37°C ($n = 2$).

3. Results

Peptides derived from LfcinB and LfcinH proteins were designed (Table 1) and synthesized through SPPS using the Fmoc/tBu strategy. The crude products were characterized using RP-HPLC and then purified via SPE chromatography. In all cases, chromatographic profile of the purified products exhibited a mainly specie. MALDI-TOF-MS analysis showed that synthesized peptides had the expected molecular weight. Table 1 presents a summary of the RP-HPLC and MALDI-TOF-MS analysis.

Designed peptides were organized in four groups as follows: Group I and Group II, peptides containing the sequence RWQWR. The peptides in these groups were designed to establish if the antimicrobial activity could be affected by the introduction of non-natural amino acids, amino acid substitutions, truncated sequences, and/or multiple motif presentation, that is, palindromic or tetrameric sequence. Group III corresponds to sequences derived from N-terminal region of LfcinH. Finally, controls (Group IV) comprised the LFB protein, LfcinB synthetic peptide (Peptide IV.1), and a non-relevant sequence PrM protein belonging to Dengue virus (Peptide IV.3).

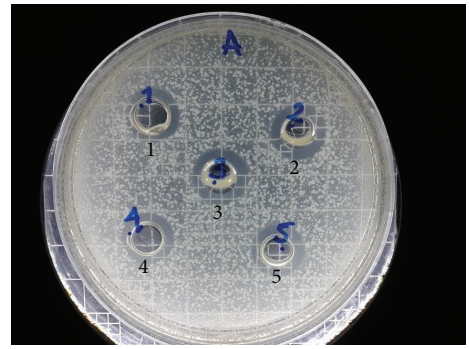


Figure 1: Susceptibility assays against *E. coli* ATCC 25922. Peptide II.8 (1), Peptide II.4 (2), Peptide II.3 (3), Peptide I.3 (4), and Peptide II.5 (5).

Susceptibility assays were performed to determine if the designed peptides exhibited antibacterial activity against the selected strains. All peptides showed an inhibition zone ranging from 12 to 14 mm, indicating that these peptides can inhibit bacterial growth (Figure 1). Significant differences in the size of the inhibition zone caused by the tested peptides were not found. This could be due to the high concentration (2000 μg/mL) used. Then optimal conditions were established to determine the MIC and MBC for each peptide against *E. coli* and *E. faecalis* (Table 1).

4. Discussion

4.1. Antibacterial Activity of Lactoferricin-Derivated Peptides against E. coli ATCC 25922. MIC and MBC values obtained against *E. coli* ATCC 25922 showed that Peptides I.2 and I.4 (Table 1) have the highest antibacterial activity against this strain, MIC 4 and 27 μM, respectively. Peptide I.4 corresponds to a branched peptide that contains 4 copies of the RRWQWR motif; it showed greater antibacterial activity than the sequence RRWQWR itself (Peptide I) and the controls, synthetic LfcinB (Peptide IV.1), and native protein (IV.2). This result indicates that multiple copies of the RRWQWR sequence could enhance the antibacterial activity. Peptide I has been considered as the minimum motif with antibacterial action, and its activity has been attributed to the presence of Trp and Arg residues in an alternating way. These amino acids have been considered important in the mechanism of antibacterial activity of LfcinB [31–33]. For *E. coli*, the palindromic sequence RWQWRWQWR (Peptide I.2) exhibited greater antibacterial activity than that showed by Peptide I and was similar to the controls (IV.1 and IV.2). This palindromic sequence contains the motif WQW, flanked by Arg residues, conferring amphipathic characteristics to peptides that have been considered as relevant in the action mechanism proposed for Lfcin. When a beta-alanine residue was introduced at the N-terminal end (Peptide I.3), the antibacterial activity was reduced. This result suggests that positive charge density over the Arg residue at the N-terminal is relevant to the activity of this peptide, probably because of electrostatic interaction with the bacterial membrane. Our

Table 1: Synthetic peptides derived from lactoferricin protein. Summary of characterization (RP-HPLC and MALDI-TOF MS) and antibacterial activity of purified products.

Group	Peptide code	Sequence	RP-HPLC t_R (min)	MALDI-TOF MS		*E. coli* ATCC 25922		*E. faecalis* ATCC 29212	
				Theoretical Mol wt	*Experimental* *m/z*. $[M + H]^+$	MIC (μM)	MCB (μM)	MIC (μM)	MCB (μM)
I	I	RRWQWR	16,73	985,54	986,92	101,5	101,5	202,9	202,9
	I.1	RRWQWR βA	16,28	1057,56	1057,27	94,6	189,1	189,1	189,1
	I.2	RWQWRWQWR	22,84	1485,75	1487,12	26,9	33,7	26,9	33,7
	I.3	βA RWQWRWQWR	22,47	1556,79	1558,16	64,2	64,2	32,1	32,1
	I.4	$((RRWQWR)_2K$ *Ahx* $C)_2$	20,00	4596,64	2300,57[a]	4,4	4,4	10,9	10,9
II	II.1	^{17}FK^{19}**C**RRWQWRMKKLG^{31}A	19,30	1993,49	1995,02	25,1	25,1	25,1	25,1
	II.2	FK βA RRWQWRMKK	17,42	1718,97	1721,12	29,1	29,1	116,3	116,3
	II.3	FK A RRWQWRMKK	17,81	1718,97	1720,81	116,3	116,3	58,2	116,3
	II.4	FK A RRWQWRM	19,51	1462,78	1464,73	34,2	34,2	68,4	136,7
	II.5	FK βA RRWQWRM	19,17	1462,78	1464,51	68,4	136,7	136,7	136,7
	II.6	FK A R L WQWRM	20,13	1420,75	1420,93	70,4	70,4	140,8	140,8
	II.7	FK ALL WQWRM	23,14	1377,72	1377,9	145,2	145,2	145,2	145,2
	II.8	RRWQWRMKKLG	18,31	1542,87	1545,12	32,4	32,4	64,8	129,6
	II.9	βA RRWQWRMKKLG	18,19	1613,91	1615,3	123,9	123,9	62,0	123,9
	II.10	RRWQWRM RR LG βA	18,52	1669,92	1672,64	59,9	59,9	15,0	29,9
	II.11	RRWQWRMKK βA	17,17	1443,80	1445,08	69,3	69,3	69,3	69,3
III	III.1	^{20}GRRRRSVQWC^{30}A	16,00	1372,73	1373,6	18,2	36,4	72,8	145,7
	III.2	βA GRRRRSVQWCA βA	14,81	1516,76	1515,98	65,9	65,9	65,9	131,9
	III.3	GRRRRSVQWCA βA	15,15	1445,68	1144,93	69,2	138,3	69,2	138,3
	III.4	βA GRRRRSVQWCA	15,20	1443,76	1444,82	17,3	34,6	69,3	138,5
IV	IV.1	FKCRRWQWRMKKLGAPSITCVRRAE	19,12	3104,66	3106,15	32,2	32,2	32,2	32,2
	IV.2	LF protein	—	80000	—	25,0	25,0	25,0	25,0
	IV.3	ITEVEPEDIDT	15,02	1258,58	1259,99	1589,1	1589,1	1589,1	1589,1

[a]This *m/z* signal corresponds to the dimer before oxidation (see Figure 2). The reported antimicrobial LfcinB center [24, 25] is underlined and changes in amino acid sequences are in box.

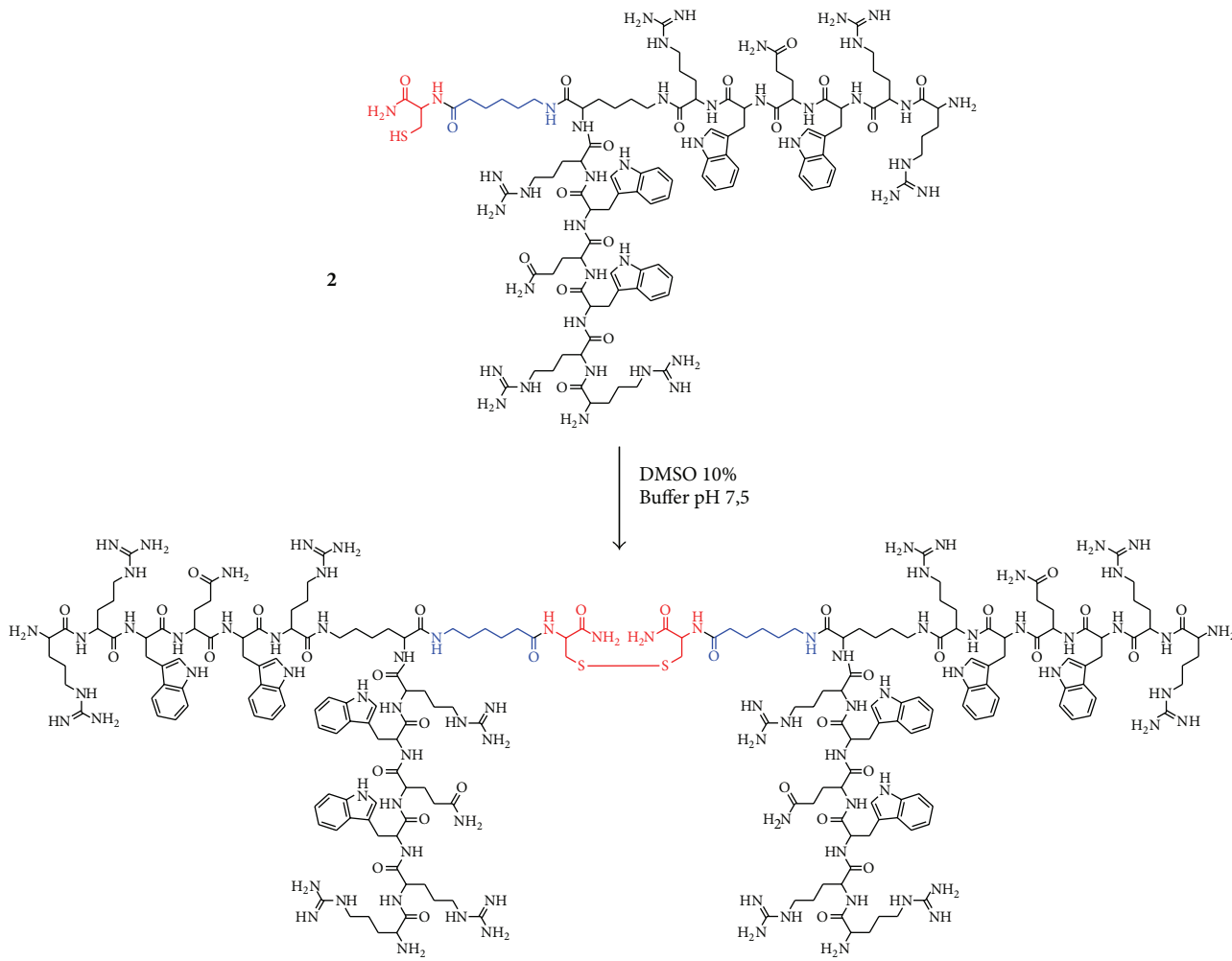


Figure 2: Synthesis of Peptide I.4. A dimer (top) was first synthesized and purified; this molecule contains two copies of the sequence RRWQWR, a spacer (*Ahx*, in blue), and a cysteine residue (in red). The tetra branched peptide (bottom) was obtained by oxidation of dimer molecule.

results indicate that antibacterial activity was increased with the multiplicity of motif RRWQWR and are in agreement with a previous report, where it was demonstrated that MAPs (multiple antigen peptides) of a sequence derived from LfcinH have significant antibacterial activity [34]. However, the synthesis of a sixteen-branched peptide is a high-cost process that gives low yields and is time consuming, due principally to steric hindrance. Our synthetic strategy is simpler because a two-branched peptide was first synthetized using SPPS-Fmoc/tBu. This reduced the problems related to steric hindrance. This method allowed us to obtain a dimeric peptide carrying a cysteine residue with no major difficulties in a process that is rapid and reproducible, gives high yields, and is of high purity. Purified dimer was oxidized using DMSO to generate the tetra-branched peptide (I.4) through disulfide bond formation (Figure 2). Comparing our results with previous reports of other authors, it was reported that RRWQWR presented a MIC of 15 μM against *E. coli* ML35 [33], whereas in our study this sequence (Peptide I) showed a MIC of 100 μM against *E. coli* ATCC 25922, showing that antibacterial activity of this sequence is dependent on the strain.

For Group II, the highest antibacterial activity against *E. coli* was exhibited by Peptide II.1, followed by Peptides II.2, II.8, and II.4. When the results obtained with Peptides II.4 to II.7 are compared, it was possible to establish that (i) cysteine residue at the 17th position is not relevant to the antibacterial activity; previously, for LfcinB, it was reported that reduction of disulfide bridge does not affect the antibacterial activity [35]; (ii) the replacement of Arg by Leu residues at positions 20 and 21 dramatically reduced the activity (Peptides II.6 and II.7); (iii) a beta-alanine residue at the N-terminal end (Peptides II.8 and II.9) considerably reduced the antibacterial activity, similar to the result discussed above (Peptide I.3). Our results suggest that RRWQWRM corresponds to the minimum sequence that exhibits activity against *E. coli*. When this motif was flanked, the antibacterial activity was affected. Peptide II.1 has been tested by other authors and has received several names (LFB, LFB (17-31), LfcinB 17-31, and LfcinB15). Our results for Peptide II.1 (MIC and MCB

25 μM) are in agreement with those reported by others, that is, MIC 24 μM [36], MIC 24 μM [36, 37], MIC 20 μM [38], MIC 30 μg/mL and MBC 40 μg/mL [39], and MIC 32 μg/mL [32]. In the same way, our Peptide II.8 exhibits an activity (MIC and MBC 32 μM (50 μg/mL)) similar to that reported by others, that is, MIC/MBC 32/128 μg/mL [32], 50/50 μg/mL [40] and MIC 50 μM [41].

The antibacterial activity obtained for Peptide III.1 that corresponds to LfcinH (20-30) was MIC 18 μM. Modification of this sequence by the incorporation of a beta-alanine (βA) residue at the N-terminal end does not change the activity (Peptide III.4, MIC 17 μM). However, when the βA was introduced at the C-terminal end, the activity was reduced significantly (Table 1. III.2, III.3). Peptide III.1 is known as hLF (1-11), and it has exhibited antibacterial activity against *E. coli* O54 and has reduced the number of viable bacteria in mice infected with resistant strains of *S. aureus* and *K. pneumonia*. The authors stated that Arg residues at the N-terminal end (^{21}R and ^{22}R) are relevant to the antibacterial activity of this sequence [42].

The antibacterial activity of Peptide IV.1 was similar to that of the native protein LF (control IV.2). The results for synthetic Peptide IV.1 (MIC and MBC 32 μM, corresponding to 100 μg/mL) against *E. coli* ATCC 25922 are in agreement with the results reported by other authors for the same synthetic peptide (MIC/MBC 30/80 μg/mL [39] and MIC 30 μg/mL [38]). Interestingly, it has been reported that LficnB, obtained by protein hydrolysis, presents higher antibacterial activity: MIC 6 μg/mL (*E. coli* O111), MIC 6 μg/mL (*E. coli* IID861) [43], MIC 50 μg/mL (*E. coli* IID861) [44], MIC 32 μg/mL (*E. coli* ATCC 25922), and MIC 64 μg/mL (*E. coli* K88) [32].

4.2. Antibacterial Activity of Lactoferricin-Derivated Peptides against E. faecalis ATCC 29212. The antibacterial activity results for Peptides I.4, I.2, and II.1 against *E. faecalis* were similar to those established for *E. coli*; that is, in the same way as for *E. coli*, Peptide I.4 (tetramer peptide) exhibits the best antibacterial activity against this strain, showing a smaller MIC than Peptide II.1 and the native protein itself. Interestingly, and in contrast to *E. coli*, the inclusion of beta-alanine residue at the N-terminal end does not affect the antibacterial activity against *E. faecalis* (Peptides I.2 and I.3). Additionally, Peptide II.10 shows good activity against this strain (Table 1). Please note that, for this peptide, two lysine residues (K) were replaced by arginine residues (R), suggesting that not only the charge but also its nature is significant and relevant to the activity. For Group II, it is important to note that most of the specific changes performed in the Peptide II.1 sequence reduced the antibacterial activity against *E. faecalis* ATCC 29212. The synthetic LfcinB and the LF native protein exhibit antibacterial activity against *E. faecalis* ATCC 29212 (Table 1). These results contrast with those obtained by Bellamy et al. [43], who reported that the *E. faecalis* ATCC E19433 strain was resistant to all evaluated concentrations of LfcinB. That group had obtained LfcinB by hydrolysis of lactoferrin using pepsin. On the other hand, our results are in agreement with the analysis presented by Chen et al. [45], which demonstrated that peptides containing Arg- and Trp-rich sequences exhibited a high degree of antibacterial activity against *E. faecalis* ATCC 29212. It is also interesting to note that peptides from Group III did not show a high degree of antibacterial activity against *E. faecalis*.

The results obtained for both strains can be summarized as follows: (i) three sequences (Peptides I.2, I.4, and II.1) exhibited a high degree of antibacterial activity against *E. coli* and *E. faecalis*, suggesting that these peptides may present a broad spectrum of antibacterial activity. Some peptides exhibited antibacterial activity against a specific strain; thus, (ii) Peptides II.2, II.4, II.8, III.1, and III.4 only exhibited activity against *E. coli*, and (iii) Peptide II.10 exhibited activity specifically against *E. faecalis*. We studied the influence, for antibacterial activity, of introducing specific changes to peptide sequences from bovine lactoferricin, such as (i) incorporation of non-natural amino acids, (ii) reduction or elongation of the motif, (iii) replacement of basic residues by noncharged residues, and (iv) multiple presentations of the RWQWR motif, such as a tetra-branched or palindromic sequence. We found that these changes directly influenced the antimicrobial activity. The types of microorganisms and their characteristics also affected the action of each peptide. The behavior of the antibacterial activity with the changes in the sequences did not follow a characteristic pattern; its behavior was specific to each microorganism. Our results suggested that peptide antibacterial activity is probably dependent on bacteria and/or the bacterial strain evaluated. This is in agreement with the results found by other authors [2, 32, 40, 46] who had reported that antibacterial activity of peptides derived from LfcinB was dependent on both the kind of bacteria and its strain.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This research was conducted with the financial support of COLCIENCIAS, code 110151928802, Contract no. RC 301-2010. The authors are also thankful to "Jóvenes Investigadores e Innovadores, Virginia Gutiérrez de Pineda," which financed M. A. León. They are grateful to Alejandro Lara, Nataly Huertas, and Carolina Vargas for their excellent technical assistance.

References

[1] World Health Organization, *Technical Consultation: Strategies for Global Surveillance of Antimicrobial Resistance*, WHO, Geneva, Switzerland, 2013.

[2] L. H. Vorland, H. Ulvatne, J. Andersen et al., "Lactoferricin of bovine origin is more active than lactoferricins of human, murine and caprine origin," *Scandinavian Journal of Infectious Diseases*, vol. 30, no. 5, pp. 513–517, 1998.

[3] J. Andrä, K. Lohner, S. E. Blondelle et al., "Enhancement of endotoxin neutralization by coupling of a C12-alkyl chain to a

lactoferricin-derived peptide," *Biochemical Journal*, vol. 385, no. 1, pp. 135–143, 2005.

[4] R. Romero, *Microbiología y parasitología humana bases etiológicas de las enfermedades infecciosas y parasitarias*, Editorial Médi-ca Panamericana, Buenos Aires, Argentina, 2007.

[5] A. Shestakov, H. Jenssen, I. Nordström, and K. Eriksson, "Lactoferricin but not lactoferrin inhibit herpes simplex virus type 2 infection in mice," *Antiviral Research*, vol. 93, no. 3, pp. 340–345, 2012.

[6] E. N. Baker and H. M. Baker, "A structural framework for understanding the multifunctional character of lactoferrin," *Biochimie*, vol. 91, no. 1, pp. 3–10, 2009.

[7] S. Farnaud and R. W. Evans, "Lactoferrin: a multifunctional protein with antimicrobial properties," *Molecular Immunology*, vol. 40, no. 7, pp. 395–405, 2003.

[8] B. Masschalck, R. van Houdt, and C. W. Michiels, "High pressure increases bactericidal activity and spectrum of lactoferrin, lactoferricin and nisin," *International Journal of Food Microbiology*, vol. 64, no. 3, pp. 325–332, 2001.

[9] I. A. García-Montoya, T. S. Cendón, S. Arévalo-Gallegos, and Q. Rascón-Cruz, "Lactoferrin a multiple bioactive protein: an overview," *Biochimica et Biophysica Acta—General Subjects*, vol. 1820, no. 3, pp. 226–236, 2012.

[10] P. F. Levay and M. Viljoen, "Lactoferrin: a general review," *Haematologica*, vol. 80, no. 3, pp. 252–267, 1995.

[11] B. Lönnerdal and S. Iyer, "Lactoferrin: molecular structure and biological function," *Annual Review of Nutrition*, vol. 15, pp. 93–110, 1995.

[12] H. Kuwata, T.-T. Yip, C. L. Yip, M. Tomita, and T. W. Hutchens, "Bactericidal domain of lactoferrin: detection, quantitation, and characterization of lactoferricin in serum by SELDI affinity mass spectrometry," *Biochemical and Biophysical Research Communications*, vol. 245, no. 3, pp. 764–773, 1998.

[13] M. Tomita, H. Wakabayashi, K. Shin, K. Yamauchi, T. Yaeshima, and K. Iwatsuki, "Twenty-five years of research on bovine lactoferrin applications," *Biochimie*, vol. 91, no. 1, pp. 52–57, 2009.

[14] E. W. Odell, R. Sarra, M. Foxworthy, D. S. Chapple, and R. W. Evans, "Antibacterial activity of peptides homologous to a loop region in human lactoferrin," *FEBS Letters*, vol. 382, no. 1-2, pp. 175–178, 1996.

[15] K. Yamauchi, M. Tomita, T. J. Giehl, and R. T. Ellison III, "Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment," *Infection and Immunity*, vol. 61, no. 2, pp. 719–728, 1993.

[16] S. Farnaud, A. Patel, E. W. Odell, and R. W. Evans, "Variation in antimicrobial activity of lactoferricin-derived peptides explained by structure modelling," *FEMS Microbiology Letters*, vol. 238, no. 1, pp. 221–226, 2004.

[17] A. M. Di Biase, A. Tinari, A. Pietrantoni et al., "Effect of bovine lactoferricin on enteropathogenic Yersinia adhesion and invasion in HEp-2 cells," *Journal of Medical Microbiology*, vol. 53, no. 5, pp. 407–412, 2004.

[18] J. H. Andersen, S. A. Osbakk, L. H. Vorland, T. Traavik, and T. J. Gutteberg, "Lactoferrin and cyclic lactoferricin inhibit the entry of human cytomegalovirus into human fibroblasts," *Antiviral Research*, vol. 51, no. 2, pp. 141–149, 2001.

[19] T. Isamida, T. Tanaka, Y. Omata, K. Yamauchi, K. I. Shimazaki, and A. Saito, "Protective Effect of Lactoferricin against *Toxoplasma gondii* Infection in Mice," *Journal of Veterinary Medical Science*, vol. 60, no. 2, pp. 241–244, 1998.

[20] H. Jenssen, K. Sandvik, J. H. Andersen, R. E. W. Hancock, and T. J. Gutteberg, "Inhibition of HSV cell-to-cell spread by lactoferrin and lactoferricin," *Antiviral Research*, vol. 79, no. 3, pp. 192–198, 2008.

[21] N. Mistry, P. Drobni, J. Näslund, V. G. Sunkari, H. Jenssen, and M. Evander, "The anti-papillomavirus activity of human and bovine lactoferricin," *Antiviral Research*, vol. 75, no. 3, pp. 258–265, 2007.

[22] J. S. Mader, D. Smyth, J. Marshall, and D. W. Hoskin, "Bovine lactoferricin inhibits basic fibroblast growth factor- and vascular endothelial growth factor165-induced angiogenesis by competing for heparin-like binding sites on endothelial cells," *The American Journal of Pathology*, vol. 169, no. 5, pp. 1753–1766, 2006.

[23] J. S. Mader, J. Salsman, D. M. Conrad, and D. W. Hoskin, "Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines," *Molecular Cancer Therapeutics*, vol. 4, no. 4, pp. 612–624, 2005.

[24] A. Richardson, R. de Antueno, R. Duncan, and D. W. Hoskin, "Intracellular delivery of bovine lactoferricin's antimicrobial core (RRWQWR) kills T-leukemia cells," *Biochemical and Biophysical Research Communications*, vol. 388, no. 4, pp. 736–741, 2009.

[25] D. J. Schibli, P. M. Hwang, and H. J. Vogel, "The structure of the antimicrobial active center of lactoferricin B bound to sodium dodecyl sulfate micelles," *The FEBS Letters*, vol. 446, no. 2-3, pp. 213–217, 1999.

[26] C. P. J. M. Brouwer, M. Rahman, and M. M. Welling, "Discovery and development of a synthetic peptide derived from lactoferrin for clinical use," *Peptides*, vol. 32, no. 9, pp. 1953–1963, 2011.

[27] P. Lloyd-Williams, F. Albericio, and E. Giralt, *Chemical Approaches to the Synthesis of Peptides and Proteins*, CRC Press, University of Barcelona, Barcelona, Spain, 1997.

[28] E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, "Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides," *Analytical Biochemistry*, vol. 34, no. 2, pp. 595–598, 1970.

[29] W. Kamysz, M. Okrój, E. Łempicka, T. Ossowski, and J. Łukasiak, "Fast and efficient purification of synthetic peptides by solid-phase extraction," *Acta Chromatographica*, no. 14, pp. 180–186, 2004.

[30] I. Wiegand, K. Hilpert, and R. E. W. Hancock, "Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances," *Nature Protocols*, vol. 3, no. 2, pp. 163–175, 2008.

[31] B. E. Haug and J. S. Svendsen, "The role of tryptophan in the antibacterial activity of a 15-residue bovine lactoferricin peptide," *Journal of Peptide Science*, vol. 7, no. 4, pp. 190–196, 2001.

[32] Y. Liu, F. Han, Y. Xie, and Y. Wang, "Comparative antimicrobial activity and mechanism of action of bovine lactoferricin-derived synthetic peptides," *BioMetals*, vol. 24, no. 6, pp. 1069–1078, 2011.

[33] L. T. Nguyen, J. K. Chau, N. A. Perry, L. de Boer, S. A. J. Zaat, and H. J. Vogel, "Serum stabilities of short tryptophan- and arginine-rich antimicrobial peptide analogs," *PLoS ONE*, vol. 5, no. 9, Article ID e12684, 2010.

[34] M. Azuma, T. Kojima, I. Yokoyama et al., "Antibacterial activity of multiple antigen peptides homologous to a loop region in human lactoferrin," *Journal of Peptide Research*, vol. 54, no. 3, pp. 237–241, 1999.

[35] K. S. Hoek, J. M. Milne, P. A. Grieve, D. A. Dionysius, and R. Smith, "Antibacterial activity of bovine lactoferrin-derived peptides," *Antimicrobial Agents and Chemotherapy*, vol. 41, no. 1, pp. 54–59, 1997.

[36] M. B. Strøm, Ø. Rekdal, and J. S. Svendsen, "Antibacterial activity of 15-residue lactoferricin derivatives," *Journal of Peptide Research*, vol. 56, no. 5, pp. 265–274, 2000.

[37] M. B. Strøm, B. Erik Haug, Ø. Rekdal, M. L. Skar, W. Stensen, and J. S. Svendsen, "Important structural features of 15-residue lactoferricin derivatives and methods for improvement of antimicrobial activity," *Biochemistry and Cell Biology*, vol. 80, no. 1, pp. 65–74, 2002.

[38] Ø. Rekdal, J. Andersen, L. H. Vorland, and J. S. Svendsen, "Construction and synthesis of lactoferricin derivatives with enhanced antibacterial activity," *Journal of Peptide Science*, vol. 5, pp. 32–45, 1999.

[39] L. H. Vorland, H. Ulvatne, J. Andersen et al., "Antibacterial effects of lactoferricin B," *Scandinavian Journal of Infectious Diseases*, vol. 31, no. 2, pp. 179–184, 1999.

[40] L. T. Nguyen, D. J. Schibli, and H. J. Vogel, "Structural studies and model membrane interactions of two peptides derived from bovine lactoferricin," *Journal of Peptide Science*, vol. 11, no. 7, pp. 379–389, 2005.

[41] J. Svenson, V. Vergote, R. Karstad, C. Burvenich, J. S. Svendsen, and B. de Spiegeleer, "Metabolic fate of lactoferricin-based antimicrobial peptides: effect of truncation and incorporation of amino acid analogs on the in vitro metabolic stability," *Journal of Pharmacology and Experimental Therapeutics*, vol. 332, no. 3, pp. 1032–1039, 2010.

[42] P. H. Nibbering, E. Ravensbergen, M. M. Welling et al., "Human lactoferrin and peptides derived from its N terminus are highly effective against infections with antibiotic-resistant bacteria," *Infection and Immunity*, vol. 69, no. 3, pp. 1469–1476, 2001.

[43] W. Bellamy, M. Takase, H. Wakabayashi, K. Kawase, and M. Tomita, "Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin," *Journal of Applied Bacteriology*, vol. 73, no. 6, pp. 472–479, 1992.

[44] H. Wakabayashi, H. Matsumoto, K. Hashimoto, S. Teraguchi, M. Takase, and H. Hayasawa, "N-acylated and D enantiomer derivatives of a nonamer core peptide of lactoferricin B showing improved antimicrobial activity," *Antimicrobial Agents and Chemotherapy*, vol. 43, no. 5, pp. 1267–1269, 1999.

[45] P.-W. Chen, C.-L. Shyu, and F. C. Mao, "Antibacterial activity of short hydrophobic and basic-rich peptides," *The American Journal of Veterinary Research*, vol. 64, no. 9, pp. 1088–1092, 2003.

[46] T. Z. Oo, N. Cole, L. Garthwaite, M. D. P. Willcox, and H. Zhu, "Evaluation of synergistic activity of bovine lactoferricin with antibiotics in corneal infection," *Journal of Antimicrobial Chemotherapy*, vol. 65, no. 6, pp. 1243–1251, 2010.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 615153, 9 pages
http://dx.doi.org/10.1155/2015/615153

Research Article

Molecular Detection and Sensitivity to Antibiotics and Bacteriocins of Pathogens Isolated from Bovine Mastitis in Family Dairy Herds of Central Mexico

Ma. Fabiola León-Galván,[1,2] José E. Barboza-Corona,[1,2] A. Arianna Lechuga-Arana,[3] Mauricio Valencia-Posadas,[2,3] Daniel D. Aguayo,[4] Carlos Cedillo-Pelaez,[5] Erika A. Martínez-Ortega,[3] and Abner J. Gutierrez-Chavez[2,3]

[1]*Food Department, Life Sciences Division, University of Guanajuato, Campus Irapuato-Salamanca, 36500 Irapuato, GTO, Mexico*
[2]*Graduate Program in Biosciences, Life Sciences Division, University of Guanajuato, Campus Irapuato-Salamanca, 36500 Irapuato, GTO, Mexico*
[3]*Agronomy Department, Life Sciences Division, University of Guanajuato, Campus Irapuato-Salamanca, 36500 Irapuato, GTO, Mexico*
[4]*Department of Physics, University of Antwerp, Campus Groenenborger, Groenenborgerlaan 171, 2020 Antwerp, Belgium*
[5]*Experimental Immunology Laboratory, National Institute of Pediatrics, Ministry of Health, 04530 México, DF, Mexico*

Correspondence should be addressed to Abner J. Gutierrez-Chavez; ajgutierrez@ugto.mx

Received 6 August 2014; Revised 11 November 2014; Accepted 9 December 2014

Academic Editor: Joel E. López-Meza

Thirty-two farms ($n = 535$ cows) located in the state of Guanajuato, Mexico, were sampled. Pathogens from bovine subclinical mastitis (SCM) and clinical mastitis (CLM) were identified by 16S rDNA and the sensitivity to both antibiotics and bacteriocins of *Bacillus thuringiensis* was tested. Forty-six milk samples were selected for their positive California Mastitis Test (CMT) (≥3) and any abnormality in the udder or milk. The frequency of SCM and CLM was 39.1% and 9.3%, respectively. Averages for test day milk yield (MY), lactation number (LN), herd size (HS), and number of days in milk (DM) were 20.6 kg, 2.8 lactations, 16.7 animals, and 164.1 days, respectively. MY was dependent on dairy herd (DH), LN, HS, and DM ($P < 0.01$), and correlations between udder quarters from the CMT were around 0.49 ($P < 0.01$). Coagulase-negative staphylococci were mainly identified, as well as *Staphylococcus aureus*, *Streptococcus uberis*, *Brevibacterium stationis*, *B. conglomeratum*, and *Staphylococcus agnetis*. Bacterial isolates were resistant to penicillin, clindamycin, ampicillin, and cefotaxime. Bacteriocins synthesized by *Bacillus thuringiensis* inhibited the growth of multiantibiotic resistance bacteria such as *S. agnetis*, *S. equorum*, *Streptococcus uberis*, *Brevibacterium stationis*, and *Brachybacterium conglomeratum*, but they were not active against *S. sciuri*, a microorganism that showed an 84% resistance to antibiotics tested in this study.

1. Introduction

In Mexico, the national milk production has an average annual growth rate of ~1.3%, representing an increase of 9,784 to 10,677 million liters per year during the period from 2003 to 2010 [1]. The backyard livestock is one of the oldest production systems in Mexico; however, the governments have not considered it important enough [2]. In the last few years, family dairy herds or small-scale dairy enterprises contribute to the national milk production with values ranging from 35 to 40% [3]. Milk is mainly sold locally in different sale channels directly to consumers, or through intermediaries or the rural or commercial industry. Intermediaries collect milk either to supply fluid milk in urban areas or to manufacture traditional cheese that is in remarkable demand in cities or suburban areas [4, 5].

According to the Food and Agriculture Organization [6], small herds are a majority in the developing world. In these

herds, animal health care is scarce because producers carry out neither preventive medicine nor a hygienic handling of milk during milking [4]. Even though mastitis is the largest cause of antimicrobial use in dairy herds [7, 8], very little is known about the use of antibiotics in small dairy herds. Mastitis is the inflammation of the mammary gland and it is a complex and costly disease in dairy herds [9, 10]. Subclinical mastitis (SCM) has a tendency to persist because it usually remains undetected. About 70 to 80% of the estimated $140 to $300 dollar loss per cow per year from mastitis relates to decreased milk production caused by asymptomatic subclinical mastitis [11]. The bacterial contamination of milk from the affected cows makes it unhealthy for human consumption and has zoonotic importance [12]. The mastitis occurrence in Mexico has been reported [13, 14], but there are few reports about bovine udder health, including the etiology of intramammary infections (IMI), antimicrobial susceptibility patterns, and mastitis frequency [15].

Alternatively, bacteriocins are antimicrobial peptides ribosomally synthesized by prokaryotes that inhibit or kill phylogenetically related and/or unrelated microorganism that share the same microbial niche. These peptides have a potential for diversified use in different areas such as food, pharmaceutical industries, agriculture, and apiculture [16, 17]. In particular, bacteriocins produced by *Bacillus thuringiensis,* the most important microbial insecticide, have showed potential to inhibit *Staphylococcus aureus* isolates associated with bovine mastitis [18]. Unfortunately, no other bacteria associated with this disease in Mexico have been tested using antimicrobial peptides synthesized by *B. thuringiensis*. In this study, our objective was to isolate and to identify molecularly microorganisms from bovine mastitis, determine antimicrobial susceptibility to antibiotic and bacteriocins synthesized by *B. thuringiensis*, and estimate the frequency of mastitis in family dairy herds from the central region of Mexico.

2. Material and Methods

2.1. Study Area and Herds. The study was developed in four municipalities in the state of Guanajuato, Mexico: Abasolo, Cuerámaro, Irapuato, and Silao. This region is located in central Mexico, to the south of the Mexican high plateau. Geographically, there are three climatic zones defined in Guanajuato with a pleasant climate with temperatures ranging from 11.7°C to 24.2°C, an average altitude of 2,015 meters above sea level, and annual average rainfall of 635 mm. Guanajuato is located at west longitude 99°40′–102°6′ and north latitude 21°51′–19°55′. Thirty-two family dairy herds were included in this study, which were selected for convenience based on the readiness to participate in the research and the existence of productive and reproductive data at the sampling time. All farms included in this study were classified as family dairy herds, according to [19], who report that farms, including the management system and facilities, should be directly served by the owner and family members, as is the case in the present study [20]. Most herds were Holstein-Friesian breed type with different herd sizes, cows with a different number of days in milk, number of calving, age, and level of milk yield.

2.2. Milk Sample Collection. Subclinical mastitis (SCM) was detected by reactive application (Masti test, BIVE, Mexico) to California Mastitis Test (CMT) in all lactation cows, including a total of 535 animals, following the method described by Schalm and Noorlander [21]. The results were interpreted in scores (range 0–4): 0 for no reaction, 1 a trace, 2 a weak positive, 3 a distinct positive, and 4 a strong positive, or in the case of clinical mastitis cases considering visual abnormalities such as flakes, clots, or any color changes in the milk, or by detecting slight swelling of the affected quarter udder. Once the udder quarters affected by subclinical (CMT 3) and clinical (any visual abnormality) mastitis were identified, teats were disinfected with swabs soaked in 70% ethyl alcohol. After discarding the first few streams, 10–15 mL milk samples were collected in sterile caped tubes and numbered, according to standard procedures of the National Mastitis Council [22]. Samples were cooled and immediately transported to the Laboratory of Proteomic and Genic Expression of the Life Science Division at the University of Guanajuato, Mexico.

2.3. Microbiological Culture and Isolation. Forty-six milk samples from udder quarters affected by mastitis were sent to microbiological analysis. Each sample was taken in clean conditions and a sown dilution of 1 : 10, 1 : 100, and 1 : 1000. The dilution was made using PBS buffer (130 mM $NaCl_2$, 10 mM $NaPO_4$, and pH 7.2) and then it was added to culture medium with agar as per standard procedures [22]. The different culture media used were Todd-Hewitt, Tryptic Soy Agar, and culture medium containing peptone trypticase, 10 g/L; yeast extract, 1.0 g/L; KH_2PO_4, 3.0 g/L; K_2HPO_4, 4.8 g/L; $(NH_4)_2SO_4$, 30 g/L; $MgSO_4{\cdot}7H_2O$, 0.2 g/L; L-cysteine $HCl{\cdot}H_20$, 0.5 g/L; sodium propionate, 15 g/L; agar, 15 g/L; pH 6.0–7.9, all with the addition of 5% of sheep blood. The plates were incubated under aerobic conditions at 37°C for 72 h. For molecular identification those culture plates with growing of one or two different colonies were included. Culture plates showing the growth of three or more different colonies were discarded and registered as contaminate sample [22]. They also were subcultured in LB liquid at 37°C for 72 h, and after this time 20% glycerol was added. Bacteria stocks were stored at −80°C.

2.4. 16S rDNA Amplification. For confirmation of the identity, isolation of genomic DNA was carried out by picking one colony from fresh culture plate. The 16S rDNA was amplified by colony-PCR using 10 pM of the universal oligonucleotide set that amplifies both bacterial domains: forward UBF 5′-AGAGTTTGATCCTGGCTGAG-3′ and reverse 1492 R5′-GGTTACCTTGTTACGACTT-3′. For the amplification of 16S rDNA a proof fidelity enzyme (BioRad) was used under the following conditions: 5 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 58°C, and 1 : 30 min at 72°C; and finally 5 min at 72°C. An aliquot of 5 μL of the PCR products was subjected to electrophoresis in 1% agarose gels and stained with ethidium bromide to visualize the amplified products. The sequencing

was performed in Molecular Cloning Laboratories (MCLAB; San Francisco, CA, USA). Amplicons were treated for analysis restriction of amplified fragments (ARDRA) with 10 U of enzymes *Mbo*I and *Bam*HI (New England, Bio-Lab UK). The digestion reaction was performed at 37°C for 3 h. The digestion products were analyzed in 2% agarose gels stained with ethidium bromide and digitalized. The amplified fragment from microorganisms that presented different restriction patterns was selected for sequencing. The sequencing was performed in Molecular Cloning Laboratories (MCLAB; San Francisco, CA, USA).

2.5. Bioinformatics Analysis. The ambiguous bases from the 5′ and 3′ terminal sequences were eliminated, and the resulting sequences were confirmed using BioEdit software. Sequences were then compared against the Ribosomal Database Project and GenBank using BLAST against the NCBI nonredundant nucleotide database "nt."

2.6. Antibiotic Susceptibility Testing. For susceptibility testing, isolates were suspended in 5 mL trypticase soy broth (TSB) at 28°C or 37°C to a turbidity of 0.5 on a scale of McFarland and with a sterile swab extension covered by the surface of a Petri dish with Muller-Hinton agar gel (MH) (Difco). The antibiotic susceptibility was identified by routine diagnostic methods using standard disk diffusion for Gram-positive and Gram-negative (MultiBac I.D., México D.F). Zones of inhibition (in mm) were recorder after ~18 h of incubation at 35–37°C. The zones of inhibition (mm) were determined and compared with the standards of performance of the supplier to determine whether the tested strain was sensitive (S), intermediate (I), or resistant (R).

2.7. Susceptibility to Antimicrobial Peptides of B. thuringiensis. Mexican strains of *B. thuringiensis* subsp. *morrisoni*, *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *kenyae*, *B. thuringiensis* subsp. *entomocidus*, and *B. thuringiensis* subsp. *tolworthi* produce the bacteriocins Morricin 269, Kurstacin 287, Kenyacin 404, Entomocin 420, and Tolworthcin 524, respectively. These bacteria were cultured at 28°C, 200 rpm, for 24 h in tryptic soy broth (TSB). Extracellular proteins were precipitated with ammonium sulfate to 80% saturation at 4°C, resuspended in 100 mM phosphate buffer (pH 7.0), and dialyzed overnight using a 1 kDa cut-off membrane (Amersham Biosciences) to obtain partially purified bacteriocins. To carry out the well-diffusion assay, indicator bacteria were cultivated overnight in tryptic soy broth (TSB), and 105 μL (~1×10^9 cell/mL) of each culture was mixed with 15 mL of TSB with warm soft agar 0.7% (w/v) and plated. Five wells of 8 mm in diameter were dug into the agar and 100 μL of partially purified Morricin (~150 U), Kenyacin (~260 U), Entomocin (~260 U), Tolworthcin (~260 U), and Kurstacin (~360 U), whose inhibitory activities were standardized with *Bacillus cereus* 183 as indicator bacterium, was added to each well. Then samples were incubated for 12 h at 4°C to allow diffusion of samples, followed by an additional incubation at 28°C or 37°C for 1 day before diameters of zones of inhibition were measured. The minimum detectable zone measured for analytic purposes was 1 mm beyond the well diameter. One unit (U) of bacteriocin activity was defined as equal to 1 mm^2 of the zone of inhibition of growth of the target indicator bacterium [17, 18, 21]. Additionally, the inhibitory effect of bacteriocins against bacteria was also performed using gel-screening assay. Partial purified bacteriocins in Laemmli's buffer without β-mercaptoethanol were loaded in two continuous sodium dodecyl sulfate-(SDS-) polyacrylamide gels for electrophoresis (SDS-PAGE). One gel was stained with Coomassie blue and the other was fixed in 25% (v/v) isopropanol and 10% (v/v) acetic acid. The gel was washed with double-distilled water and equilibrated in phosphate buffer (pH 6.5). The gel was overlaid with TSB with soft agar 0.7% (w/v) containing ~1×10^9 cell/mL of indicator bacteria and incubated at 28°C. The next day zones of inhibition were examined and molecular mass of the bacteriocins was calculated [17].

2.8. Data and Statistical Analyses. Data registered in the herds were entered into a spreadsheet in electronic format with Excel for Windows and edited to guarantee the quality of analyses. The dependent variables studied were the test day milk yield in kg (MY) and CMT results by udder quarter. Independent variables were lactation number (LN), family dairy herd (DH), herd size (HS), number of days in milk (DM), and municipality (M). Descriptive analysis was used for the variables included in this study. Normality was evaluated for the dependent variables to define the type of statistical analysis. The variables were recorded and grouped into the next categories: HS (0–15, 16–25, and >25 cows), LN (1, 2, 3, and >4), and DM (0–90, 91–180, and >180 days). In order to know the independence between some variables and because most of these were discrete and without normal distribution, the chi-square test was applied between MY, DH, HS, LN, and DM. To estimate the probability of association between results of CMT, udder quarters were estimated using the Spearman rank correlation. For the statistical analyses, the Statgraphics Centurion program version 15.2 was used.

3. Results and Discussion

3.1. Characteristics and Parameters of Dairy Farms. Table 1 shows the variability among farms according to the herd size (from 3 to 47 heads), number of lactations (from 1.7 to 4.1 lactations), number of days in milk (from 52 to 275 days), and average of test day milk yield (from 9.0 to 26.4 Kg). The family dairy herds are one of the dominant and widely distributed production systems in Mexico, in small scale units run by the family. It was estimated that 10% of all milk production in Mexico comes from family dairy herds. According to the livestock census carried out in 2007, it was found that ~73% of units correspond to the small farms [4]. It is interesting that although in Mexico a decrement in the family dairy participation has been reported to domestic supplies it has been observed that it does not have a direct influence on the number of small farms as it remains without important changes. The herd size of the family farms reported in this study is much lower than suggested from family

Table 1: Descriptive statistics for variables studied from family dairy herds ($n = 32$) from the central region of Mexico.

Parameter	Mean	Standard deviation	Minimum	Maximum
Herd size (heads)	16.7	9.4	3	47
Days in milk	161.4	108.2	7	730
Lactation number	2.8	1.6	1	16
Milk yield (Kg)	20.6	7.2	3	45

dairy farms in Los Altos, Jalisco, Mexico, where an average population of 61 lactating cows was described. However, the average of milk yield per cow obtained in this study (20.6 L/d) was higher than 17.8 L/d (Jalisco) [23] and 11.4 L/d (Jalisco and Michoacán) [24]. As indicated above, of all the dairy production systems in Mexico, the familial system is the most heterogeneous. The farms that compose this system range from subsistence operations (milk and cheese are used exclusively to feed the family) to large-scale operations (milk sale is the primary but not the unique source of income for the family). The family production system is centered in the west central region of the country, including the states of Jalisco, Michoacán, Aguascalientes, and Guanajuato [23].

3.2. Frequencies of Subclinical and Clinical Bovine Mastitis. CMT global results in this study showed that 48% of animals ($n = 257$) were negative, and 52% of animals ($n = 278$) showed a positive reaction to SCM. However, SCM per animal among herds ranged from 11 to 75%. Concerning the quarter reaction degree of CMT, milk samples registered a 10.3% (trace), 6.12% (grade 1), 2.9% (grade 2), 5.28% (grade 3), and 1.59% dry-off gland, and the remaining percentage was negative (72.2%). In general, at least one case of clinical mastitis was detected in 66% of the dairy herds studied (21 of 32). The percentage of CLM per animal among herds ranged from 0 to 25 (Table 2). In small-scale dairy herds, hygiene and health management are often poor, a situation that contributes to the development of clinical mastitis cases [13]. This might explain the higher prevalence of CLM registered in this study (0–25%), especially when datum is compared with the results obtained in family dairy herds from State of Mexico (3.4–9.8%) [13] and also with Jalisco (4.0%) [25]. Furthermore, it is necessary to consider that there is a clear variation in the epidemiology of mastitis and mastitis inducers among different regions in Mexico [25]. The frequency of clinical cases based on quarter udder signs was 78% (25/32), moderately acute; 16% (5/32), chronic; and 6.2% (2/32), severe acute. Statistical analysis showed that MY was dependent on the farm, HS, DIM, and LN ($P < 0.01$) (Table 3). The estimated correlation between results of CMT for each udder quarter ranged from 0.46 to 0.52 ($P < 0.01$). Mastitis is an expensive disease, where a high proportion of dairy farms might have avoidable losses [26]. The frequency of SCM obtained in our study (11–75%) was as high as that obtained (23–52%) in dairy cattle located in province of Huaral, Lima, Peru [27]. In addition, our data are comparable with two studies carried out in smallholder and/or family dairy farms located in the Jalisco and State of Mexico, Mexico, where SCM prevalence per animal was of 34.1 and 48.3%, respectively [13, 25]. It is important to indicate that both SCM and CLM were associated with herd size, parity, management practices, and time of lactation [25]. The prevalence of mastitis might change between countries and geographical regions, but frequently the highest prevalence is

Table 2: Frequency of subclinical (SCM) and clinical (CLM) mastitis in family dairy herds from the central region of Mexico.

	Frequency (%)*	
Farm	SCM	CLM
1	4/12 (33)	1/12 (8)
2	0/3 (0)	0/3 (0)
3	4/8 (50)	2/8 (25)
4	8/14 (57)	2/14 (14)
5	4/9 (44)	0/9 (0)
6	5/9 (56)	0/9 (0)
7	3/14 (21)	0/14 (0)
8	5/17 (29)	0/17 (0)
9	10/16 (63)	1/16 (6)
10	5/10 (50)	0/10 (0)
11	9/26 (35)	2/26 (8)
12	6/16 (38)	0/16 (0)
13	3/11 (27)	0/11 (0)
14	9/12 (75)	3/12 (25)
15	13/28 (46)	6/28 (21)
16	7/13 (54)	2/13 (15)
17	19/31 (61)	6/31 (19)
18	2/12 (17)	2/12 (17)
19	3/19 (16)	1/19 (5)
20	8/20 (40)	3/20 (15)
21	8/47 (17)	9/47 (19)
22	17/36 (47)	4/36 (11)
23	2/18 (11)	4/18 (22)
24	18/29 (62)	0/29 (0)
25	3/12 (25)	1/12 (8)
26	7/15 (47)	2/15 (13)
27	5/9 (56)	0/9 (0)
28	10/16 (63)	2/16 (13)
29	3/9 (33)	1/9 (11)
30	4/24 (17)	4/24 (17)
31	3/6 (50)	0/6 (0)
32	2/14 (14)	1/14 (7)

*Denominator represents the total number of animals in the herd.

Table 3: Results of the chi-square independence test between different variables.

Variables	Statistical values	Probability
Milk yield—herd	1684.197	0.0000
Milk yield—herd size	1706.651	0.0064
Milk yield—days in milk	2548.930	0.0000
Milk yield—lactation number	533.235	0.0000

Table 4: Potential microbial pathogens isolated from dairy cattle and their susceptibility to antibiotics[a].

Bacteria	Accession number	Antibiotics[b]											
Gram-positive		E	PE	TE	AM	CFX	CPF	CLM	SXT	VA	CF	DC	GE
Staphylococcus aureus	KP224443	S	R	S	R	R	S	R	S	S	S	S	S
Staphylococcus agnetis	JQ394696	S	R	S	R	R	S	R	S	S	S	S	S
Staphylococcus epidermidis	KP224442	S	R	S	I	R	S	R	S	S	S	S	S
Staphylococcus sciuri	KP224448	I	R	I	R	R	R	R	R	R	R	R	R
Staphylococcus haemolyticus	KP224444	S	R	S	R	R	S	R	S	S	S	S	S
Staphylococcus equorum	KP224447	S	R	S	R	S	S	I	S	S	S	S	S
Streptococcus dysgalactiae	KP224445	S	R	S	R	R	S	R	S	S	S	S	S
Streptococcus uberis	KP224446	S	I	S	R	S	S	I	S	S	R	S	S
Brevibacterium stationis	KP224449	I	R	S	I	I	S	R	S	S	S	S	S
Brachybacterium conglomeratum (1)° cvbnm	KP224450	S	R	I	S	R	S	R	S	S	S	S	S
Gram-negative		CL	AK	CB	NET	NF	NOF	CF	AM	CFX	CPF	SXT	GE
Raoultella sp.	KP224451	S	S	R	S	I	S	R	R	S	S	S	S

[a]R, resistant; S, susceptible; I, intermediate.

[b]Erythromycin (E), penicillin (PE), tetracycline (TE), ampicillin (AM), cefotaxime (CFX), ciprofloxacin (CPF), clindamycin (CLM), sulfamethoxazole-trimethoprim (SXT), vancomycin (VA), cephalothin (CF), dicloxacillin (DC), gentamicin (GE), amikacin (AK), carbenicillin (CB), chloramphenicol (CL), netilmicin (NET), nitrofurantoin (NF), and norfloxacin (NOF).

found in countries with a poorly developed dairy sector and with a lack of udder health control programs.

3.3. Isolation and Bacterial Identification. A total of eleven milk samples plated (24%) were selected for bacterial isolation and identification. The remaining milk samples plated were not considered for showing a lack of growth or a contaminated bacteria growth. It is necessary to highlight that most of the milk samples of this study were from SCM cases, where (i) the colony-forming units of the organism in the milk were below the detection limit of the assay, (ii) special media or growth conditions were required, or (iii) presence of inhibitors in the milk sample, such as antibiotics, had interfered with the growth of the pathogen. If it is common that 20–30% of clinical quarters will result in no microbial growth, this percentage could be increased when milk samples come from SCM as in this study. Also, clinical signs could be present but the pathogen might be eliminated or controlled by the cow's immune system [22]. Bacterial PCR amplification and subsequent ARDRA analyses of 16S rDNA gene were successful for all samples. The 16S rDNA sequences that presented different ARDRA profiles were selected for sequencing (Table 4). Five genera and eleven bacterial species involved in cases of mastitis were identified. Table 4 shows that 42% of the isolated microorganisms were coagulase-negative staphylococci (CNS). Similar results were obtained in smallholder dairy farms from Jalisco state of Mexico, where the most common udder pathogens were CNS (15.6%), followed by *S. aureus* (5.9%), *S. agalactiae* (6.8%), *Corynebacterium* spp. (14%), and coliform bacteria (4.1%) [25]. CNS are considered minor pathogens, especially in comparison with major pathogens such as *S. aureus*, streptococci, and coliforms [28]. However, these bacteria are of great interest because they are regularly isolated from milk samples obtained from cows and are currently considered emerging pathogens of bovine mastitis and the main cause of intramammary infection (IMI) in modern dairy herds [29–31].

Alternatively, a total of 124 milk samples were collected from 124 multiparous lactating dairy (Holstein) cows at the province of Nanning, China. Positive CMT was recorded from 65 (52.4%) glands. Bacteria were isolated from 45 (36.3%) of milk samples. Distributions of microbial isolates responsible for infected milk samples have been reported as follows: *S. aureus* (47%), CNS (27%), *Escherichia coli* (9%), *S. agalactiae* (9%), *S. uberis* (4%), and *Cryptococcus neoformans* (4%) [32]. In another study, Lago et al. [33] found 422 cows affected by clinical mastitis in 449 quarters, where coliform bacteria were the most commonly isolated pathogen (24% of clinical mastitis cases).

According to results of sequence analysis of isolates conducted in the present study (retrieved from the GenBank, http://www.ncbi.nlm.nih.gov/, using the nucleotide-nucleotide BLAST algorithm) *Staphylococcus agnetis* (NCBI/EMBL accession JQ 394696) was isolated from milk samples of mastitis cases. It is important to emphasize that *S. agnetis* is mentioned only once before in the literature as a pathogen causing mastitis in dairy cattle. Recently, it has been reported that *S. agnetis* was associated with bovine mastitis based on the characteristics of 12 isolates originating from milk samples of cows with subclinical or mild clinical IMI and one isolate from the apex of the teat [34]. We also identified the bacteria *Brevibacterium stationis* and *Brachybacterium conglomeratum*. Although these microorganisms have not been reported as etiological agents of cow mastitis, they have occasionally been isolated from goat raw milk samples and also from different areas of the farm (e.g., teat surfaces, milking parlors, hay, air, and dust) [35].

3.4. Antibiotic Susceptibility Patterns. Six isolates of this study (54.54%) showed resistance to two or three antimicrobial agents, mostly to penicillin, clindamycin, and cefotaxime;

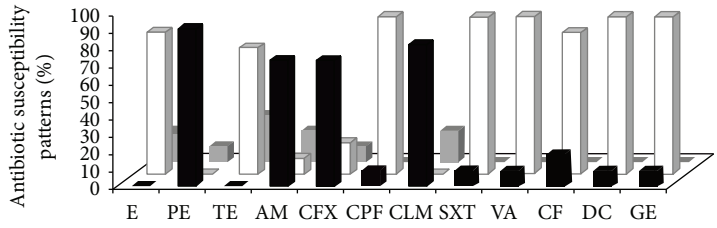


Figure 1: Percentage of sensitivity *in vitro* by standard disk diffusion (MultiBac-ID) of different antibiotics against bacterial isolates from bovine mastitis. Graphic bars represent the percentage of sensitive (white), intermediate (grey), or resistant (black). Erythromycin (E), penicillin (PE), tetracycline (TE), ampicillin (AM), cefotaxime (CFX), ciprofloxacin (CPF), clindamycin (CLM), sulfamethoxazole-trimethoprim (SXT), vancomycin (VA), cephalothin (CF), dicloxacillin (DC), and gentamicin (GE).

meanwhile resistance to four or more antimicrobial agents was found in 5 isolates (45.45%). All isolates showed a variable susceptibility (~60%) to the 12 antimicrobials tested. Special consideration showed *Staphylococcus sciuri* isolated that was resistant to 10 of 12 antimicrobials tested and the rest were detected with intermediate susceptibility (Table 4). The microorganisms were mainly resistant to penicillin (90%), clindamycin and cefotaxime (both 80%), and ampicillin (70%). In this study we found a high frequency of penicillin-resistant bacteria, which is higher than those reported in subclinical milk samples obtained from dairy herds located in state of Michoacán, Mexico (74%) [18]. In addition, percentage of ampicillin-resistant microorganisms (i.e., 70%) was very similar to that reported (67.4%) in a study performed in dairy herds of south of Brazil [36]. In particular, in Mexico it is very common that, at the end of period of lactation in dairy cattle, farmers use a prophylactic dose of antimicrobial (i.e., penicillins and cephalosporins) into the udder. Although the purpose of this treatment is the prevention of future mastitis, it is obvious that this procedure might generate penicillin-resistant microorganisms [37]. In addition, some isolates were highly sensitive (90%) to trimethoprim/sulfamethoxazole, dicloxacillin, ciprofloxacin, and gentamicin (Figure 1). All microorganisms shown in Table 4 were identified as Gram-positive bacteria. Only one Gram-negative microorganism was isolated and identified as *Raoultella* sp., which had resistance to ampicillin, carbenicillin, and cephalothin. *Raoultella* sp. (before *Klebsiella* sp.) is one of the most frequent Gram-negative pathogens isolated from bovine clinical mastitis [38]; and it has been isolated from bedding material [39].

The emergence of antimicrobial resistance among pathogens that affect animal health is a growing concern in veterinary medicine. Furthermore, the use of antimicrobial drugs has also been considered as a potential health risk for humans [40, 41]. *S. agnetis* showed resistance (33.0%) to penicillin, ampicillin, cefotaxime, and clindamycin (Table 4). It should be noted that, in another study, *S. agnetis* was resistant to lysozyme, polymyxins, and deferoxamine, and it was susceptible to novobiocin and lysostaphin [34]. Phylogenetically, *S. agnetis* is a novel species of the genus *Staphylococci* and can be differentiated from the coagulase-positive species, such as *S. hyicus, S. simulans, S. schleiferi, S. chromogenes, S. intermedius,* and *S. epidermidis*. Compared to *S. aureus*, streptococci, and coliforms, coagulase-negative staphylococcus (CNS) has been considered an emerging bovine mastitis pathogen in several countries [30, 42, 43] with a high degree of resistance to some conventional drugs [30, 40, 43, 44]. CNS mastitis responds much better to antimicrobial treatment than *S. aureus* mastitis, but resistance to different antimicrobials is more common in CNS than *S. aureus*. CNS tends to be more resistant to antimicrobials than *S. aureus* and can easily develop multiresistance. The most common resistance mechanism in staphylococci is β-lactamase production, which results in resistance to penicillin G and aminopenicillin [28].

3.5. Inhibitory Activity of Bacteriocins. We recently showed that antimicrobial peptides or bacteriocins (Morricin 269, Kurstacin 287, Kenyacin 404, Entomocin 420, and Tolworthcin 524) synthesized by *B. thuringiensis* are able to inhibit food-borne pathogenic bacteria [17]. In addition it was demonstrated that *Staphylococcus* strains isolated from bovine with mastitis are also susceptible to this kind of bacteriocins [18]. In the present study the five bacteriocins inhibited the growth of *S. agnetis, S. equorum, Streptococcus uberis, B. stationis*, and *B. conglomeratum*, bacteria that showed multiantibiotic resistance (Table 5). Unfortunately, bacteriocins did not show activity on *S. sciuri,* microorganism with an 84% resistance to antibiotics tested in this study. This bacterium has been found to be associated not only with bovine subclinical mastitis [45], but also with serious infections in humans such as endocarditis [46], peritonitis [47], wound infections [48], and urinary infections [49]. In addition, we did not find susceptibility of *S. aureus* to the bacteriocins, which is very interesting as we previously demonstrate that different isolates of this bacterium are susceptible to the five antimicrobial peptides tested in this work [18]. We do not have a clear explanation for this observation, but it has been shown that, within the same genus or strains of the same species, microorganisms can differ in their susceptibilities to a particular bacteriocin. For example, (i) *B. licheniformis* strain P40 produces an antimicrobial peptide with inhibitory action to *S. intermedius* but not to *S. aureus* [50]. (ii) Also, *S. aureus* strains isolated from dairy cow mastitis [18] showed different susceptibilities

Table 5: Inhibitory activity (U[a]) of partial purified bacteriocin determined by the well-diffusion method against potential microbial pathogens associated with mastitis in dairy bovines.

Indicator bacteria	Bacteriocins				
	Morricin 269	Kurstacin 287	Kenyacin 404	Entomocin 420	Tolworthcin 524
Bacillus cereus 183[b]	151	365	264	264	264
Staphylococcus aureus	0	0	0	0	0
Streptococcus dysgalactiae	0	0	365	365	330
Staphylococcus agnetis	53	28	142	148	104
Staphylococcus epidermidis	0	0	0	0	0
Streptococcus uberis	204	296	264	296	233
Staphylococcus sciuri	0	0	0	0	0
Staphylococcus haemolyticus	0	0	0	0	0
Staphylococcus equorum	186	245	231	374	225
Brevibacterium stationis	62	28	329	204	150
Brachybacterium conglomeratum	103	44	296	480	150
Raoultella sp.	264	264	296	296	264

[a]One unit is defined as 1 mm^2 of the zone of inhibition as determined by the well-diffusion method (see text). Data are the average of triplicate assays. A value of "0" indicates no inhibition.
[b]Bacterium used as positive control. It was used to determine the units of bacteriocins contained in the crude extracts used in the assay [17, 18].

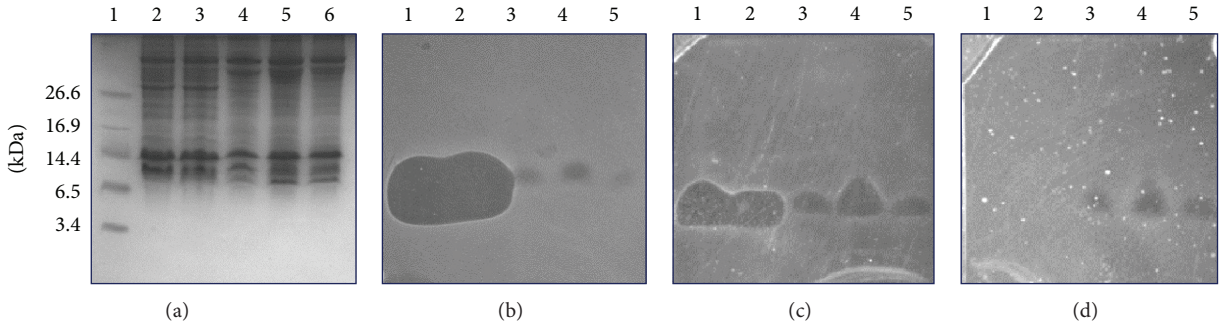


Figure 2: Inhibitory detection of bacteriocins against bacteria using gel-screening assay. (a) SDS-PAGE; gel was overlaid with (b) *Bacillus cereus* 183 (control), (c) *Raoultella* sp., and (d) *Staphylococcus agnetis*. Bacteria (c) and (d) were isolated from bovines with mastitis. Lane 1, Morricin 269; lane 2, Kurstacin 287; lane 3, Kenyacin 404; lane 4, Entomocin 420; lane 5, Tolworthcin 524. Growth inhibition zones show the relative position of bacteriocins with molecular mass of ~10 kDa. Protein marker (BioRad) was used to estimate the molecular masses of bacteriocins.

to the five bacteriocins used in this study. In addition, it seems that pathogenic microorganisms have acquired the ability to sense and to respond to bacteriocins in different way, often resulting in reduced negative charge of their cell envelope due to specific surface modifications, which in consequence induce the generation of bacteriocin-resistant bacteria [51].

Alternatively, in order to detect the molecular mass of the bacteriocins, we carried out gel-screening assays using *Raoultella* sp. and *S. agnetis* as reporter bacteria. Morricin 269, Kurstacin 287, Kenyacin 404, Entomocin 420, and Tolworthcin 524 exhibited molecular mass of ~10 kDa as shown previously (Figure 2) [17]. It is important to indicate that because we used different units of bacteriocins, we did not carry out comparisons in the inhibitory effects of the different bacteriocins against the bacteria assayed in this work, as our purpose was only to detect whether microorganisms were susceptible or not to the antimicrobial peptides.

4. Conclusions

In this work, the most common udder pathogens isolated from mastitis milk samples were coagulase-negative staphylococci (42%), followed by streptococci (17%), and *S. aureus, B. stationis, B. conglomeratum*, and *Raoultella* sp. with an 8% each. We found that 72.7% of isolates had a resistance pattern to three or more antimicrobial agents mainly to penicillin, clindamycin, and cefotaxime. Studies on the prevalence rate of clinical and subclinical mastitis of different mastitis pathogens in a cow population from small-scale dairy herds are scarce. Although it is difficult to compare results obtained in this work with those obtained in other countries, CNS, *S. aureus*, and streptococci have been reported to be the most prevalent pathogens [52, 53]. Alternatively, bacteriocins of *B. thuringiensis* inhibited the growth of different bacteria tested here and they could have a viable potential for use

in integrated management programs to control or prevent mastitis in animals. However, it is obvious that a higher number of bacterial isolates with different genus or different strains of the same genera and species obtained from bovine mastitis must be tested in future studies.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Acknowledgments

This research was supported by Grants from SEP-PROMEP (103.5/10/4684) and University of Guanajuato, Mexico (Project 262/2013), to Abner J. Gutierrez-Chavez and J. E. Barboza-Corona, respectively. The authors also appreciate the support received from the Fundación Guanajuato Produce AC for the development of this research through the FGP 583/12 Project. E. A. Martínez-Ortega is an undergraduate student supported by the Universidad de Guanajuato. They thank Dr. Luz Edith Casados-Vázquez and Jaime J. Badajoz-Martínez from the Universidad de Guanajuato for their technical support during this study. They appreciate the contribution of the producers to this study as well.

References

[1] Secretaria de Economia (SE), *Analisis de Sector Lacteo en Mexico*, Direccion General de Industrias Basicas, Secretaria de Economia, Mexico City, Mexico, 2012.

[2] R. E. Caceido-Rivas, J. L. Garita-Goiz, and N. M. Paz-Calderon, "Salud animal de una cuenca lechera bajo el sistema de traspatio, Puebla, Mexico," *Actas Iberoamericanas de Conservacion Animal*, vol. 1, pp. 323–326, 2011.

[3] O. Val-Arreola, E. Kebreab, and J. France, "Modeling small-scale dairy farms in central Mexico using multi-criteria programming," *Journal of Dairy Science*, vol. 89, no. 5, pp. 1662–1672, 2006.

[4] R. Jiménez-Jiménez, V. Espinosa-Ortiz, F. Alonso-Pesado et al., "Globalization effects in family farms: a case of Mexican dairy production," in *The Economic Geography of Globalization*, P. Pachura, Ed., InTech, Rijeka, Croatia, 2011.

[5] G. Álvarez-Fuentes, J. G. Herrera-Haro, G. Alonso-Bastida, and A. Barreras-Serrano, "Raw milk quality produced in small dairy farms in the South of Mexico City," *Archivos de Medicina Veterinaria*, vol. 44, no. 3, pp. 237–242, 2012.

[6] Food Agriculture Organization (FAO), "Status of and prospects for smallholder milk production—a global perspective," in *Pro-Poor Live-stock Policy Initiative*, T. Hemme and J. Otte, Eds., Food Agriculture Organization, Rome, Italy, 2010.

[7] J. S. Moon, A. R. Lee, H. M. Kang et al., "Antibiogram and coagulase diversity in staphylococcal enterotoxin-producing *Staphylococcus aureus* from bovine mastitis," *Journal of Dairy Science*, vol. 90, no. 4, pp. 1716–1724, 2007.

[8] S. P. Oliver, S. E. Murinda, and B. M. Jayarao, "Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: a comprehensive review," *Foodborne Pathogens and Disease*, vol. 8, no. 3, pp. 337–355, 2011.

[9] R. Hussain, M. T. Javed, and A. Khan, "Changes in some bio - chemical parameters and somatic cell counts in the milk of buffalo and cattle suffering from mastitis," *Pakistan Veterinary Journal*, vol. 32, no. 3, pp. 418–421, 2012.

[10] S. Atasever, "Estimation of correlation between somatic cell count and coagulation score of bovine milk," *International Journal of Agriculture and Biology*, vol. 14, no. 2, pp. 315–317, 2012.

[11] G. Leitner, U. Merin, and N. Silanikove, "Effects of glandular bacterial infection and stage of lactation on milk clotting parameters: Comparison among cows, goats and sheep," *International Dairy Journal*, vol. 21, no. 4, pp. 279–285, 2011.

[12] A. Sharif, M. Umer, and G. Muhammad, "Mastitis control in dairy production," *Journal of Agriculture and Social Science*, vol. 5, pp. 102–105, 2009.

[13] A. M. M. López, S. D. Zarco, F. S. García et al., "*Staphylococcus aureus* biotypes in cows presenting subclinical mastitis from family dairy herds in the central-eastern state of Mexico," *Revista Mexicana De Ciencias Pecuarias*, vol. 3, no. 2, pp. 265–274, 2012.

[14] H. Castañeda Vázquez, S. Jäger, W. Wolter, M. Zschöck, M. A. Castañeda Vazquez, and A. El-Sayed, "Isolation and identification of main mastitis pathogens in mexico," *Arquivo Brasileiro de Medicina Veterinaria e Zootecnia*, vol. 65, no. 2, pp. 377–382, 2013.

[15] R. E. Miranda-Morales, V. Rojas-Trejo, R. Segura-Candelas et al., "Prevalence of pathogens associated with bovine mastitis in bulk tank milk in Mexico," *Annals of the New York Academy of Sciences*, vol. 1149, pp. 300–302, 2008.

[16] N. M. de la Fuente-Salcido, L. E. Casados-Vázquez, and J. E. Barboza-Corona, "Bacteriocins of *Bacillus thuringiensis* can expand the potential of this bacterium to other areas rather than limit its use only as microbial insecticide," *Canadian Journal of Microbiology*, vol. 59, no. 8, pp. 515–522, 2013.

[17] J. E. Barboza-Corona, H. Vázquez-Acosta, D. K. Bideshi, and R. Salcedo-Hernández, "Bacteriocin-like inhibitor substances produced by Mexican strains of *Bacillus thuringiensis*," *Archives of Microbiology*, vol. 187, no. 2, pp. 117–126, 2007.

[18] J. E. Barboza-Corona, N. de la Fuente-Salcido, N. Alva-Murillo, A. Ochoa-Zarzosa, and J. E. López-Meza, "Activity of bacteriocins synthesized by *Bacillus thuringiensis* against *Staphylococcus aureus* isolates associated to bovine mastitis," *Veterinary Microbiology*, vol. 138, no. 1-2, pp. 179–183, 2009.

[19] A. L. Villamar and C. E. Olivera, *Situación Actual y Perspectiva de la Producción de Leche de Bovino en México*, Coordinación General de Ganadería. Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación, Mexico City, Mexico, 2005.

[20] R. G. Sanchez and V. A. Sanchez, *La Ganadería Bovina del Estado de Michoacán*, Fundación PRODUCE Michoacán, 2nd edition, 2005.

[21] O. Schalm and D. Noorlander, "Experiments and observations leading to the development of California mastitis test," *Journal of the American Veterinary Medical Association*, vol. 130, no. 5, pp. 199–204, 1957.

[22] J. S. Hogan, R. N. Gonzales, R. J. Harmon et al., *Laboratory Handbook on Bovine Mastitis*, National Mastitis Council, Madison, Wis, USA, 2009.

[23] M. Wattiaux, J. Blazek, and J. J. Olmos-Colmenero, *Bird's Eye View of the Mexican Dairy Industry and On-the-Ground Assessment of Production Systems in the Highlands (Los Altos)*

of Jalisco, Mexico, The Babcock Institute, Madison, Wis, USA, 2012.

[24] L. C. J. Castro, G. R. Sánchez, L. F. E. Iruegas et al., "Tendencias y oportunidades de desarrollo de la red de leche en Mexico," *Boletin Informativo*, vol. 133, no. 317, pp. 1–135, 2001.

[25] H. C. Vázquez, S. Jäger, W. Wolter, M. Zschöck, M. A. C. Vazquez, and A. El-Sayed, "Isolation and identification of main mastitis pathogens in mexico," *Arquivo Brasileiro de Medicina Veterinaria e Zootecnia*, vol. 65, no. 2, pp. 377–382, 2013.

[26] H. Hogeveen, K. Huijps, and T. J. G. M. Lam, "Economic aspects of mastitis: new developments," *New Zealand Veterinary Journal*, vol. 59, no. 1, pp. 16–23, 2011.

[27] C. Velasquez and J. Vega, "Milk quality and subclinical mastitis in dairy herds in the province of huaura, Lima-Peru," *Revista de Investigaciones Veterinarias del Peru*, vol. 23, no. 1, pp. 65–71, 2012.

[28] S. Taponen and S. Pyörälä, "Coagulase-negative staphylococci as cause of bovine mastitis-Not so different from *Staphylococcus aureus*?" *Veterinary Microbiology*, vol. 134, no. 1-2, pp. 29–36, 2009.

[29] K. L. Smith and J. S. Hogan, "The world of mastitis," in *Proceedings of the 2nd International Symposium on Mastitis and Milk Quality*, pp. 1–12, Vancouver, Canada, 2001.

[30] Y. H. Schukken, R. N. González, L. L. Tikofsky et al., "CNS mastitis: nothing to worry about?" *Veterinary Microbiology*, vol. 134, no. 1-2, pp. 9–14, 2009.

[31] S. Pyörälä and S. Taponen, "Coagulase-negative staphylococci—emerging mastitis pathogens," *Veterinary Microbiology*, vol. 134, no. 1-2, pp. 3–8, 2009.

[32] F. L. Yang, X. S. Li, B. X. He et al., "Malondialdehyde level and some enzymatic activities in subclinical mastitis milk," *African Journal of Biotechnology*, vol. 10, no. 28, pp. 5534–5538, 2011.

[33] A. Lago, S. M. Godden, R. Bey, P. L. Ruegg, and K. Leslie, "The selective treatment of clinical mastitis based on on-farm culture results: I. Effects on antibiotic use, milk withholding time, and short-term clinical and bacteriological outcomes," *Journal of Dairy Science*, vol. 94, no. 9, pp. 4441–4456, 2011.

[34] S. Taponen, K. Supré, V. Piessens, E. van Coillie, S. de Vliegher, and J. M. K. Koort, "*Staphylococcus agnetis* sp. nov., a coagulasevariable species from bovine subclinical and mild clinical mastitis," *International Journal of Systematic and Evolutionary Microbiology*, vol. 62, no. 1, pp. 61–65, 2012.

[35] L. Quigley, O. O'Sullivan, C. Stanton et al., "The complex microbiota of raw milk," *FEMS Microbiology Reviews*, vol. 37, no. 5, pp. 664–698, 2013.

[36] H. Ceotto, J. dos Santos Nascimento, M. A. V. de Paiva Brito, and M. do Carmo de Freire Bastos, "Bacteriocin production by *Staphylococcus aureus* involved in bovine mastitis in Brazil," *Research in Microbiology*, vol. 160, no. 8, pp. 592–599, 2009.

[37] J. Rushton, J. Pinto Ferreira, and K. D. Stärk, "Antimicrobial resistance: the use of antimicrobials," *OECD Food, Agriculture and Fisheries Papers*, vol. 68, 2014.

[38] J. Hogan and K. L. Smith, "Coliform mastitis," *Veterinary Research*, vol. 34, no. 5, pp. 507–519, 2003.

[39] M. A. Muñoz, F. L. Welcome, Y. H. Schukken, and R. N. Zadoks, "Molecular epidemiology of two *Klebsiella pneumoniae* mastitis outbreaks on a dairy farm in New York State," *Journal of Clinical Microbiology*, vol. 45, no. 12, pp. 3964–3971, 2007.

[40] B. B. Kaliwal, S. O. Sadashiv, M. M. Kurjogi, and R. D. Sanakal, "Prevalence and antimicrobial susceptibility of coagulase-negative *Staphylococci* isolated from bovine mastitis," *Veterinary World*, vol. 4, no. 4, pp. 158–161, 2011.

[41] L. E. Redding, F. Cubas-Delgado, M. D. Sammel et al., "The use of antibiotics on small dairy farms in rural Peru," *Preventive Veterinary Medicine*, vol. 113, no. 1, pp. 88–95, 2014.

[42] E. Gentilini, G. Denamiel, A. Betancor, M. Rebuelto, M. Rodriguez Fermepin, and R. A. De Torres, "Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitis in Argentina," *Journal of Dairy Science*, vol. 85, no. 8, pp. 1913–1917, 2002.

[43] S. Piepers, L. de Meulemeester, A. de Kruif, G. Opsomer, H. W. Barkema, and S. de Vliegher, "Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium," *Journal of Dairy Research*, vol. 74, no. 4, pp. 478–483, 2007.

[44] M. Brinda, V. Herman, and I. Fodor, "Phenotypic characterization of coagulase-negative staphylococci isolated from mastitic milk in cows," *Lucrari Stiintifice Medicine Veterinara*, vol. 43, pp. 97–101, 2010.

[45] B.-M. Thorberg, M.-L. Danielsson-Tham, U. Emanuelson, and K. Persson Waller, "Bovine subclinical mastitis caused by different types of coagulase-negative *Staphylococci*," *Journal of Dairy Science*, vol. 92, no. 10, pp. 4962–4970, 2009.

[46] G. Hedin and M. Widerström, "Endocarditis due to *Staphylococcus sciuri*," *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 17, no. 9, pp. 673–675, 1998.

[47] F. Wallet, L. Stuit, E. Boulanger, M. Roussel-Del Vallez, P. Dequiedt, and R. J. Courcol, "Peritonitis due to *Staphylococcus sciuri* in a patient on continuous ambulatory peritoneal dialysis," *Scandinavian Journal of Infectious Diseases*, vol. 32, no. 6, pp. 697–698, 2000.

[48] T. Horri, Y. Suzuki, T. Kimura, T. Kanno, and M. Maekawa, "Intravenous catheter-related septic shock caused by Staphylococcus sciuri and *Escherichia vulneris*," *Scandinavian Journal of Infectious Diseases*, vol. 33, no. 12, pp. 930–932, 2001.

[49] S. Stepanović, P. Ježek, D. Vuković, I. Dakić, and P. Petráš, "Isolation of members of the *Staphylococcus sciuri* group from urine and their relationship to urinary tract Infections," *Journal of Clinical Microbiology*, vol. 41, no. 11, pp. 5262–5264, 2003.

[50] F. Cladera-Olivera, G. R. Caron, and A. Brandelli, "Bacteriocin-like substance production by *Bacillus licheniformis* strain P40," *Letters in Applied Microbiology*, vol. 38, no. 4, pp. 251–256, 2004.

[51] N. Y. Yount and M. R. Yeaman, "Immunocontinuum: perspectives in antimicrobial peptide mechanisms of action and resistance," *Protein and Peptide Letters*, vol. 12, no. 1, pp. 49–67, 2005.

[52] A. Pitkälä, M. Haveri, S. Pyörälä, V. Myllys, and T. Honkanen-Buzalski, "Bovine mastitis in Finland 2001—prevalence, distribution of bacteria, and antimicrobial resistance," *Journal of Dairy Science*, vol. 87, no. 8, pp. 2433–2441, 2004.

[53] K. Östensson, V. Lam, N. Sjögren, and E. Wredle, "Prevalence of subclinical mastitis and isolated udder pathogens in dairy cows in Southern Vietnam," *Tropical Animal Health and Production*, vol. 45, no. 4, pp. 979–986, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 102129, 11 pages
http://dx.doi.org/10.1155/2015/102129

Review Article

Biologically Active and Antimicrobial Peptides from Plants

Carlos E. Salas,[1] Jesus A. Badillo-Corona,[2] Guadalupe Ramírez-Sotelo,[2] and Carmen Oliver-Salvador[2]

[1] *Departamento de Bioquímica, Universidade Federal de Minas Gerais, 31270-901 Belo Horizonte, Brazil*
[2] *Unidad Profesional Interdisciplinaria de Biotecnología, Instituto Politécnico Nacional, Avenida Acueducto S/N, Colonia Barrio La Laguna Ticomán, 07320 Mexico City, Mexico*

Correspondence should be addressed to Carmen Oliver-Salvador; moliver@ipn.mx

Received 15 August 2014; Revised 13 October 2014; Accepted 31 October 2014

Academic Editor: Dennis K. Bideshi

Bioactive peptides are part of an innate response elicited by most living forms. In plants, they are produced ubiquitously in roots, seeds, flowers, stems, and leaves, highlighting their physiological importance. While most of the bioactive peptides produced in plants possess microbicide properties, there is evidence that they are also involved in cellular signaling. Structurally, there is an overall similarity when comparing them with those derived from animal or insect sources. The biological action of bioactive peptides initiates with the binding to the target membrane followed in most cases by membrane permeabilization and rupture. Here we present an overview of what is currently known about bioactive peptides from plants, focusing on their antimicrobial activity and their role in the plant signaling network and offering perspectives on their potential application.

1. Introduction

No doubt proteins were designed to be versatile molecules. The number of functions in which they participate during metabolism supports this affirmation. Proteins act as defense, integrating the immunological system, as part of the enzymatic network required during metabolism, as a nutrient, as storage, contractile, structural, and motile molecules, as transporters, and as signaling and regulatory mediators. These are well-established functions for which proteins have gained undisputed roles. Aside from these functions other roles are associated with these molecules, such as antifreezers, sweeteners, and antioxidants. A relatively new role involves their ability to interact with cellular membranes in a nonreceptor-ligand type of binding.

Antimicrobial peptides (AMPs) are often the first line of defense against invading pathogens and play an important role in innate immunity [1]. The list of identified antimicrobial peptides has been growing steadily over the past twenty years. Initially, the skin of frogs and lymph from insects were shown to contain antimicrobial peptides, but now over 1500 antimicrobial peptides have been described, in living organisms including those from microorganisms, insects, amphibians, plants, and mammals [2].

In 1963, Zeya and Spitznagel described a group of basic proteins in leukocyte lysosomes endowed with antibacterial activity [3]. Later, Hultmark et al. [4] purified three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. The vaccinated insects survived a posterior challenge with high doses of the infecting bacteria, indicating the relevance of the bactericidal proteins. Additional research identified a 35-residue peptide (cecropin) as responsible for the antibacterial effect. Further investigation by Boman and other groups confirmed that antimicrobial peptides (AMPs) are distributed ubiquitously in all invertebrates investigated, generating academic and commercial interest [1, 5–9].

Because the rapid increase in drug-resistant infections poses a challenge to conventional antimicrobial therapies, there is a need for alternative microbicides to control infectious diseases [2, 10–13]. Bioactive peptides can fulfill this role because they display antibacterial, antiviral, antifungal, and/or antiparasitic activities. A comparative analysis of these molecules reveals that there are no unique structural requirements useful to discriminate these activities and to facilitate their classification. Most bioactive peptides have a high content of cysteine or glycine residues; the disulphide bridges that may be formed between cysteinyl residues

increase their stability. Most of them contain charged amino acids, primarily cationic, and also hydrophobic domains. Both, β-sheets or α-helices, looped or extended, structures or combinations of these domains can be found in natural bioactive peptides [3, 6, 7, 14–24]; their length varies between 12 and 55 residues. There is evidence that cationic charged peptides are relevant for antibacterial or antiviral activity but few exemptions of anionic peptides also exist.

This review updates information on plant bioactive peptides. When little or no available information exists on a specific group, we use examples taken from other lifer forms, assuming that upcoming studies may reveal information on peptides whose attributes have not yet been found in plants. The review does not cover in detail the antimicrobial mechanism underlying the effect of bioactive peptides since two recent reviews on the subject were published [4, 5, 11, 14, 15, 25–31].

2. Antimicrobial Peptides Isolated from Plants

As mentioned above, AMPs are part of important immunological barriers to counter microorganism microbial infections and represent another aspect of the resistance phenomenon known as the hypersensitive response (HR). This phenomenon was described by H. Marshall Ward in cultures of leaf rust (*Puccinia dispersar* or *Puccinia triticina*) and by several plant pathologists 100 years ago [1, 5, 7, 8]. The hypersensitive reaction (HR) is considered the maximum expression of plant resistance to pathogen attack and is defined as a fast death of the plant cells associated with growth restriction and pathogen isolation. Cell death that happens during HR is considered a lysosomal-type of programmed cell death (PCD) or autophagy [2, 10, 12], unlike mammalian apoptosis. Also, signaling by resistance gene products (RGP) triggered during the HR response is not associated with death effectors (mammalian caspases), or with the death complex equivalent to the mammalian apoptosome. It is hypothesized that RGP signaling is required to initiate deployment of non-HR defenses, most likely via the production of so-called "dead signals" like ROS (reactive oxygen species), NO (nitric oxide), and SA (salicylic acid), all of them initiators of resistance in the absence of a HR [3, 14, 16]. Therefore, HR is viewed as part of a continuum of effects mediated by defense elicitors [4, 5, 15, 25, 27–29].

Although many AMPs are generically active against various kinds of infectious agents, they are generally classified as antibacterial, fungicides, antiviral, and antiparasitic. The antibacterial activity of peptides results from the amphiphilic character and presence of motifs with high density of positively charged residues within their structure [6–9]. This type of arrangement facilitates peptide attachment and insertion into the bacterial membrane to create transmembrane pores resulting in membrane permeabilization. The amphipathic nature of antimicrobial peptides is required for this process, as hydrophobic motifs directly interact with lipid components of the membrane, while hydrophilic cationic groups interact with phospholipid groups also found in the membrane.

The antifungal activity of AMP was initially attributed to either fungal cell lysis or interference with fungal cell wall synthesis. A comparison of plants antifungal peptides suggests a particular structural-activity arrangement involving polar and neutral amino acids [11–13, 32]. However, like for antibacterial peptides, there are no obvious conserved structural domains clearly associated with antifungal activity. The cell wall component "chitin" has been implied as fungal target for bioactive peptides [6, 7, 15, 17–24]. Peptide binding induces fungal membrane permeabilization and/or pore formation [4, 11, 14, 15, 26, 29–31].

The antiviral effect of some AMPs depends on their interaction with the membrane by electrostatic association with negative charges of glycosaminoglycans facilitating binding of AMP and competing with viruses [11]. Such is the case of the mammalian cationic peptide lactoferrin that prevents binding of herpes simplex virus (HSV) by binding to heparan moieties and blocking virus-cell interactions [3, 32–34]. Alternatively, defensins (described below) bind to viral glycoproteins making HSV unable to bind to the surface of host cells [25, 27]. The antiviral effect of peptides can also be explained by obstruction of viral interaction with specific cellular receptors, as shown during binding of HSV and the putative B5 cell surface membrane protein displaying a heptad repeat alpha-helix fragment. The effect was demonstrated with the synthetic 30-mer peptide that has the same sequence found in the heptad repeat that inhibits HSV infection of B5-expressing porcine cells and human HEp-2 cells [7, 15, 19, 20, 22–24]. Another mechanism involves the interaction between AMP and viral glycoprotein as shown with a retrocyclin-2 analogue that binds with high affinity (Kd = 13.3 nM) to immobilized HSV-2 glycoprotein B (gB2) while it does not bind to enzymatically deglycosylated gB2 [25, 28]. A less specific interaction between AMP and viruses causes disruption or destabilization of viral envelope yielding viruses unable to infect host cells [15, 17, 19, 21–24]. Finally, a peptide mediated activation of intracellular targets induces an antiviral effect as demonstrated with the antiviral peptide NP-1 from rabbit neutrophils that crosses the cell membrane migrating into the cytoplasm and organelles, followed by inhibition of viral gene expression in the infected cell. The proposed mechanism involves downregulation of VP16 viral protein entry into the nucleus that prevents expression of early viral genes required to propagate viral infection [4, 11, 26, 30, 31].

The initial characterization of molecules displaying AMP activity was followed by isolation of purothionin, the first plant-derived AMP. Purothionin is active against *Pseudomonas solanacearum*, *Xanthomonas phaseoli* and *X. campestris*, *Erwinia amylovora*, *Corynebacterium flaccumfaciens*, *C. michiganense*, *C. poinsettiae*, *C. sepedonicum,* and *C. fascians* [25]. Since then, several plant peptides have been discovered. The major groups include thionins (types I–V), defensins, cyclotides, 2S albumin-like proteins, and lipid transfer proteins [15, 19, 22–24]. Other less common AMPs include knottin-peptides, impatiens, puroindolines, vicilin-like, glycine-rich, shepherins, snakins, and heveins (Table 1) [35–44].

Full isolation of plant AMP has been attained in some cases. It is the case of lunatusin a peptide with molecular

Table 1: Selected plant antimicrobial peptides.

Peptide	Biological activity	Peptide size	Reference
Thionins (types I–V)	Antibacterial	45–47 residues	[15, 22–24]
Thionein: alpha-1-purothionin (*Triticum aestivum)*	Antibacterial	5 kDa 45 residues	[15, 25, 81]
Cyclotides: kalata B1 and B2 (*Oldenlandia affinis*)	Antibacterial, Antifungal, insecticide nematicide	28–37 residues	[15, 19, 22–24]
2S albumin-like *Malva parviflora*, *Raphanus sativus*	Antibacterial, allergen	105 residues	[15, 24]
Lipid transfer proteins (LTPs) (*Zea mays*)	Antibacterial	90–95 residues	[15, 22–24]
Knottin-peptides: PAFP-S (*Phytolacca americana*) knottin-type (*Mirabilis jalapa*)	Antibacterial	36-37 residues	[15, 35–43]
Puroindolines: PIN*A* and PIN*B* (*Triticum aestivum*)	Antibacterial	13 kDa	[15, 35–43]
Snakins (*Solanum tuberosum*)	Antibacterial	63 residues, 6.9 kDa	[15, 35–43]
Heveins (*Hevea brasiliensis*)	Antibacterial and antifungal	43 residues, 4.7 kDa	[15, 35–43]
Peptides (*Phaseolus vulgaris*)	Antibacterial and antifungal	2.2 and 6 kDa	[2, 49, 50]
Peptide PvD1 (*Phaseolus vulgaris*)	Antibacterial and antifungal	6 kDa	[60, 75]
Defensin-like (*Phaseolus vulgaris*)	Antibacterial	7.3 kDa	[15, 50]
Defensins (*Triticum aestivum* and *Hurdeum vulgare*)	Antibacterial and antifungal	5 kDa	[25, 53]
Lunatusin (*Phaseolus lunatus*)	Antibacterial[a] and antiviral	7.0 kDa	[45]
Vulgarinin (*Phaseolus vulgaris*)	Antibacterial, antifungal, and antiviral	7.0 kDa	[46]
Hispidulin (*Benincasa hispida)*	Antibacterial and antifungal	5.7 kDa	[48]
Lc-def (*Lens culinaris*)	Antifungal	47 residues	[37, 79]
Cicerin (*Cicer arietinum*)	Antifungal and antiviral	8.2 kDa	[49, 60, 61]
Arietin (*Cicer arietinum*)	Antifungal and antiviral	5.6 kDa	[36, 49, 60, 61]
Peptide So-D1 (*Spinacia oleracea*)	Antifungal and antibacterial	22 residues	[36, 44]
Ay-AMP *Amaranthus hypochondriacus*	Antifungal	3.18 kDa	[47]
PR1, PR2 Chitinases (*Vitis vinifera*)	Antifungal	26 and 43 kDa	[19, 38, 41, 64]
Proteins from latex of *Calotropis procera* (CpLP)	Antifungal	13 kDa	[38, 60, 61]
Proteinases from *Carica candamarcensis*, *Carica papaya* and *Cryptostegia grandiflora* (Cg24-I)	Antifungal	23–25 kDa	[36, 60, 61]
Impatiens (*Impatiens balsamina*) Ib-AMP1, Ib-AMP2, Ib-AMP3, and Ib-AMP4	Antibacterial	20 residues	[36, 52, 53, 57]
Shepherins (*Capsella bursa-pastoris*)	Antibacterial and antifungal	28 residues	[38, 41]
Vicilin-like (*Macadamia integrifolia*)	Antibacterial and antifungal	45 residues	[38]
Peptides[a] (*Brassica napus*)	Antiviral	ND	[82]
Proteinases from *Ananas comosus*, *Carica papaya, Ficus carica*, and *Asclepias sinaica*	Anthelmintic	23-24 kDa	[52, 53, 57]

[a]Mitogenic activity; ND: not determined.

mass of 7 kDa purified from Chinese lima bean (*Phaseolus lunatus* L.) (Table 1). Lunatusin exerted antibacterial action on *Bacillus megaterium*, *Bacillus subtilis*, *Proteus vulgaris,* and *Mycobacterium phlei*. The peptide also displays antifungal activity towards *Fusarium oxysporum*, *Mycosphaerella arachidicola,* and *Botrytis cinerea*. Interestingly, the antifungal activity was retained after incubation with trypsin [45].

Another peptide, named vulgarinin, from seeds of haricot beans (*Phaseolus vulgaris*), with a molecular mass of 7 kDa showed antibacterial action against *Mycobacterium phlei*, *Bacillus megaterium*, *B. subtilis,* and *Proteus vulgaris* and antifungal activity against *Fusarium oxysporum*, *Mycosphaerella arachidicola*, *Physalospora piricola,* and *Botrytis cinerea*. Its antifungal activity was also retained after incubation with trypsin. Another example is a peptide from *Amaranthus hypochondriacus* seeds that displays antifungal activity (Table 1) [46, 47].

Both lunatusin and vulgarinin inhibited HIV-1 reverse transcriptase and inhibited translation in a cell-free rabbit reticulocyte lysate system, suggesting a similarity of action between these two peptides and that antimicrobial activity might be linked to protein synthesis [46]. Lunatusin also elicited a mitogenic response in mouse splenocytes [45] and proliferation of breast cancer MCF-7b cell line while vulgarinin inhibited proliferation of leukemia L1210 and M1 cell lines and breast cancer MCF-7 cell line [46].

A peptide named hispidulin was purified from seeds of the medicinal plant *Benincasa hispida* that belongs to the Cucurbitaceae family (Table 1). Hispidulin exhibits a molecular mass of 5.7 kDa, is composed of 49 amino acid residues, and displays broad and potent inhibitory effects against various human bacterial and fungal pathogens [48]. Two additional antifungal peptides with novel N-terminal sequences, designated *cicerin* and *arietin*, were isolated from seeds of chickpea (*Cicer arietinum*), respectively. These peptides exhibited molecular masses of approximately 8.2 and 5.6 kDa, respectively. Arietin expressed higher translation-inhibitory activity in a rabbit reticulocyte lysate system and higher antifungal potency toward *Mycosphaerella arachidicola*, *Fusarium oxysporum,* and *Botrytis cinerea* than cicerin. Both lack mitogenic and anti-HIV-1 reverse transcriptase activities [2, 49, 50].

There are also some studies on AMP peptides from dry seeds of *Phaseolus vulgaris* cv. brown kidney beans; these AMPs exhibit antifungal and antibacterial activity [2, 50, 51]. Another AMP (So-D1-7) was isolated from a crude cell wall preparation from spinach leaves (*Spinacia oleracea* cv. Matador) and was active against Gram-positive (*Clavibacter michiganensis*) and Gram-negative (*Ralstonia solanacearum*) bacterial pathogens, as well as against fungi, such as, *Fusarium culmorum, F. solani*, *Bipolaris maydis*, and *Colletotrichum lagenarium* [44].

Antiparasitic peptides are another group of bioactive peptides. Following an initial report describing the lethal effect of *magainin* isolated from Xenopus skin on *Paramecium caudatum*, another peptide (cathelicidin) confirmed the antiparasitic activity of AMPs [52–56].

Anthelmintic activity is also a recognized feature attributed to vegetable proteinases (Table 1). For instance, bromelain, the stem enzyme of *Ananas comosus* (Bromeliaceae), shows anthelmintic effect against *Haemonchus contortus* [52, 53], similar to the reference drug pyrantel tartrate. A similar effect was confirmed with proteinases from papaya (*Carica papaya*), pineapple (*A. comosus*), fig (*Ficus carica*), and Egyptian milkweed (*Asclepia sinaica*) *in vitro* against the rodent gastrointestinal nematode *Heligmosomoides polygyrus* [57]. The anthelmintic effect cannot be fully explained by the proteolytic effect of these enzymes, as the inhibited enzymes partially preserve antiparasitic activity. It is suggested that selected domains within the proteinase molecule different from the active site could be responsible for the antiparasitic effect (unpublished observations). The notion that specific regions within a protein are responsible for the biocide effect is supported by the observation that some AMPs become functional upon protein hydrolysis, like in egg [58, 59] and milk proteins hydrolysates [58, 60–63]. At present, there are not many studies on plant protein hydrolysates with antibiotic properties; this situation encourages the search in protein databases for motifs featuring the signature of AMPs.

Plant proteinases also display antifungal activity as demonstrated with latex proteinases from *Calotropis procera*, *Carica candamarcensis,* and *Cryptostegia grandiflora* [27, 60, 61]. Using a collection composed of *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *F. solani*, *Rhizoctonia solani*, *Neurospora* sp., and *Aspergillus niger*, fungal germination, growth, and IC_{50} were determined. The observed IC_{50} for *Rhizoctonia solani* with proteinases from *C. procera* was 20.7± 1.6 μg/mL while with proteinases from *C. candamarcensis* was 25.3±2.4 μg/mL. Chitinases are also chitinolytic enzymes found in different plants that display antifungal activity [64].

Plant Defensins. There is no consensus about the size of defensins. According to some authors defensins are AMPs that range from 18 to 48 amino acids, while other groups define them as having 12–54 residues. Regardless of their size they contain several conserved cysteinyl residues structuring disulphide bridges that contribute to their stability. Two kinds of defensins have been described, α-defensin and β-defensin, the latter probably emerged earlier based on its similarity with insect forms. Defensins are among the best-characterized cysteine-rich AMPs in plants [27, 65]. All known members of this family have four disulphide bridges and are folded into a globular structure that includes three L-strands and a K-helix [65, 66]. Initially, these proteins were described in human neutrophils [66, 67], more specifically in granules of phagocytes and intestinal Paneth cells [67–71]. Later, they were described in human, chimpanzee, rat, mouse, marine arthropods, plants, and fungi [68–71].

Defensins are structurally classified in four categories, which correlate with morphological and/or developmental changes in fungi following treatment with defensins [72–75]. Defensins of group I cause inhibition of Gram-positive bacteria and fungi, and fungal inhibition occurs with marked morphological distortions of hyphae (branching); those of group II are active against fungi, without inducing hyphal branching, and are inactive against bacteria; those of group III are active against Gram-positive and Gram-negative

bacteria but are inactive against fungi; while group IV are active against Gram-positive and Gram-negative bacteria, and against fungi, without causing hyphal branching. The selective action assigned to these four groups of defensins suggests that specific determinants within each group are responsible for targeting different groups of infectious agents.

Several defensins have been purified from plants. The PvD1 defensin from *Phaseolus vulgaris* (cv. Perola) seeds is a 6 kDa peptide (Table 1). Its N-terminal has been sequenced and the comparative analysis in databases shows high similarity with sequences of different defensins isolated from other plants species. PvD1 has been shown to inhibit the growth of yeasts, *Candida albicans*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, *Kluyveromyces marxiannus,* and *Saccharomyces cerevisiae*. PvD1 also inhibits phytopathogenic fungi including *Fusarium oxysporum*, *F. solani*, *F. lateritium,* and *Rhizoctonia solani* [51, 72]. Analysis of cloned PvD1 cDNA yielded a fragment that contains 314 bp, encoding a 47-amino-acid polypeptide displaying strong similarity with plant defensins from *Vigna unguiculata* (93%), *Cicer arietinum* (95%), and *Pachyrhizus erosus* (87%).

An antifungal peptide with a defensin-like sequence and exhibiting a molecular mass of (7.3 kDa) was purified from dried seeds of *Phaseolus vulgaris* "cloud bean" (Table 1). The peptide exerted antifungal activity against *Mycosphaerella arachidicola* with an IC_{50} value of 1.8 μM and it was also active against *Fusarium oxysporum* with an IC_{50} value of 2.2 μM [52]. From lentil (*Lens culinaris*), a 47-amino-acid-residue (Lc-def) defensin was purified from germinated seeds (Table 1). The molecular mass (5.4 kDa) and the complete amino acid sequence were determined. Lc-def has eight cysteines forming four disulphide bonds; it shows high sequence homology with defensins from legumes and exhibits activity against *Aspergillus niger* [50, 76].

A 5.4 kDa antifungal peptide, with an N-terminal sequence highly similar to defensins and with inhibitory activity against *Mycosphaerella arachidicola* (IC_{50} = 3 μM), *Setosphaeria turcica,* and *Bipolaris maydis*, was isolated from the seeds of *Phaseolus vulgaris* cv. brown kidney bean (Table 1). The antifungal activity of the peptide against *M. arachidicola* was stable in a wide pH range (3–12) and progressively decreases at pHs <2 and >12. Similarly, its activity remains stable between 0 and 80°C and partially declines between 90 and 100°C. Deposition of Congo red at the hyphal tips of *M. arachidicola* was induced by this peptide indicating inhibition of hypha growth. The lack of antiproliferative activity of brown kidney bean antifungal peptide toward tumor cells, in contrast to the presence of such activity seen in other antifungal AMPs, suggests that different domains are responsible for the antifungal and antiproliferative activities [50].

The biotechnological potential of defensins became evident following experiments aimed at increasing plant resistance to pathogens by genetic transformation of various recipient plants. In a number of cases increased resistance to specific pathogens was obtained in transgenic plants overexpressing a defensing gene [24].

3. Peptides from Plant Hydrolysates

Plant protein hydrolysates represent an option for production of bioactive peptides. Hydrolysis can be done enzymatically or under acidic conditions; the former is preferred because it is milder and effectively produces bioactive peptides from a variety of sources, like legumes, rice, chia seeds, and so forth. Particularly, studies with enzymatic hydrolysates from leguminous plants, like common bean (*P. vulgaris* L.), are relevant since this is a fundamental ingredient of human diet in several cultures and because it represents up to 10% of total proteins ingested in developing countries [77, 78].

The characterization of bioactive peptides released by hydrolysis demonstrates that they preserve their nutritional value, and at least, some of them behave as biologically active substances. Protein hydrolysates show antioxidant, antitumoral, antithrombotic, antimicrobial, or antihypertensive activities, thus qualifying as functional foods [77, 79]. Particularly, total hydrolysates (TH) or peptide fractions from leguminous such as chickpea, soya bean, pea, lentil, mung bean, and common beans demonstrate important antioxidant and angiotensin-I converting enzyme activities (ACE) [79, 80].

Our studies using concentrates following enzymatic hydrolysates from three common bean varieties of *P. vulgaris* L., plus black (PB), azufrado higuera (AH), and pinto saltillo (PS), show evidence of antimicrobial activity. The bactericidal activity determined by growth inhibition demonstrated that ten out of twelve bacterial strains were inhibited by these THs and also by the 3–10 kDa peptide fraction obtained by subsequent ultrafiltration of TH. The ultrafiltrate fraction from TH with cutoff of 1 kDa (<1 kDa) also demonstrated antimicrobial activity against *Shigella dysenteriae* in each of the bean varieties (PB, AH, and PS) at 0.1, 0.4, and 0.3 mg/mL, respectively [81]. A similar antimicrobial activity was seen in beans *Phaseolus lunatus* digested with pepsin followed by pancreatin [81]. Both TH and the partially purified peptide fraction (<10 kDa) exhibited antimicrobial activity against *Staphylococcus aureus* and *Shigella flexneri*. The largest antimicrobial effect was seen with the <10 kDa fraction and the determined MIC was 0.39 mg/mL against *S. aureus* and 0.99 mg/mL for *S. flexneri* [81].

Antiretroviral activity has also been described in alcalase hydrolysates of rapeseed (*Brassica napus*) protein. The antiviral effect seen in human immunodeficiency virus (HIV) is due to inhibition of the viral protease, possibly by a 6 kDa peptide. When rapeseed hydrolysate was purified by size-exclusion chromatography, two fractions of 6 kDa enriched in this protease inhibitor were isolated [82].

4. Role of Peptides in Plant Signalling

Since plants are stationary attached to earth, they must withstand aggressions from predatory activities by herbivores including man or pathogens and environmental variations like water supply, temperature changes, and manmade aggressions. To successfully meet these challenges, they have developed an efficient signaling network to elicit appropriate cellular responses. As in mammals, their signaling processes rely on efficient and specific interactions between organic

molecules or simple ions (ligand) and their receptors to communicate and respond to these signals.

As result many plant peptides and proteins evolved as signaling molecules and play a key role in homeostasis, defense, growth, differentiation, and senescence. Most of these actions require the coaction of hormones (auxin, ethylene, abscisic acid (ABA), gibberellic acid, and cytokinins), acting as coregulators in these processes. As part of their defense strategies, a group of peptides evolved to inactivate microorganisms menacing plant essential functions. The antimicrobial peptides comprising this category are discussed in the previous section.

In this section, we focus on peptides whose main established functions provide a physiological attribute to the plant, but it should be noted that a peptide might participate in a defense strategy against infectious agents, while being at the same time a component of a metabolic function of the host plant without intervention of an infective agent. Some examples that illustrate this situation include a defensive peptide of 7.45 kDa from white cloud beans (*Phaseolus vulgaris* cv.) that shows reverse transcriptase inhibitory activity when probed *in vitro* [83, 84]. This type of effect does not follow a logical evolutionary explanation, unless a retroviral form yet unidentified is found in plants. In another similar situation, it is being shown that purothionin, the AMP from wheat endosperm, can substitute for thioredoxin/from spinach chloroplasts in the dithiothreitol-linked activation of chloroplast fructose-1,6 bisphosphatase, suggesting a role for the thiol carrier during regulation of redox molecules [83, 85].

Human β-defensins also display diverse immune related functions in addition to their antimicrobial activity. Such is the case of human β-defensin-2 that promotes histamine release and prostaglandin D2 production in mast cells. The immune modulatory role of β-defensin-2 has been further studied following the finding that β-defensin-2 binds to the chemokine receptor CCR-6, the cognate receptor for macrophage inflammatory protein-3α/CCL20 [85, 86]. Secretion of protein-3α along with other cytokines is linked to migration of immature dendritic cells from blood to the skin and from sites of inflammation to local lymph nodes triggering activation of memory specific T cells [86, 87]. In addition, β-defensins are associated with stimulation of toll-like receptor-4, thus serving as an additional mechanism for amplification of the innate host defense response [87, 88]. In summary, it is evident that at least some antimicrobial molecules evolved from host metabolites and share other functions.

In plants, most of these signaling molecules are found in seeds, highlighting the necessity to preserve the genetic material that represents the informational basis to sustain the species. Following *in silico* screening in *A. thaliana* about 15 peptide families were identified plus additional groups described in other species, most of them monocot [88, 89]. Aside from partial repositories available like in the case of secreted peptides in *A. thaliana* obtained by *in silico* analysis of unannotated sequences [89, 90], PhytAMP, a database dedicated to antimicrobial plant peptides http://phytamp.pfba-lab-tun.org/main.php [90, 91], C-PAmP, a database of computationally predicted plant antimicrobial peptides http://bioserver-2.bioacademy.gr/Bioserver/C-PAmP/ [2, 91], the antimicrobial peptide database that includes an algorithm to determine Boman's index http://aps.unmc.edu/AP/FAQ.php[2, 92] or attempts to identify a specific family of signaling peptides [88, 92], no comprehensive database is available that deposits all the signaling peptides described to date. The annotation of these sequences would be valuable to identify and catalogue new peptide sequences that continuously emerge.

Signaling peptides encompass a myriad of highly diversified sequences showing variation within and across species and without a common phylogenetic origin. These circumstances defy the efforts to classify them as a single group [88, 93–95]. A classification attempt involving their suggested functions includes homeostatic, innate immune responses (defensive), expansion and proliferation, organ maintenance and organogenesis, and sexual related functions. Three peptide classes, natriuretic class (PNP), phytosulfokines (PSK), and rapid alkalinization factors (RAF), participate in homeostatic functions. PNP has been purified from several species [93–96]. A number of effects are attributed to PNP, such as H^+, K^+, and Na^+ fluxes in roots probably mediated by cGMP [96–98], transient increase of cGMP levels, water uptake in mesophyll cells, water exit from xylem, and osmotic dependent protoplast swelling [97–99]. Unconfirmed evidence suggests that a leucine-rich brassinosteroid receptor (AtBR1) displaying guanylyl cyclase activity and kinase-like structure could act as natriuretic peptide receptor [99, 100].

PSKs are sulfated pentapeptides containing two sulfated Tyr residues synthesized as precusrsors. The ligand acts on phytosulfokine receptors (PSKR) which are leucine-rich repeat receptors displaying guanylate cyclase activity [100, 101].

The alkalinization RALF factor and homologues (RALF-like) are 5 kDa peptides, expressed in a tissue specific manner. Its role in roots is associated with hair growth control by modulation of intra- and extracellular pH [101, 102]. Indirect effects such as K^+ and Ca^{+2} currents are linked to proton-pump changes [102, 103]. Some of the actions attributed to RALF may involve the participation of abscisic acid too [103–105].

The meristematic region at the top of the shoot responds to many actions related to growth and differentiation of the plant. The apical meristem contains stem cells that generate signaling peptides following a genetic program influenced by the surrounding habitat. The CLE family includes several groups of peptides capable of triggering signaling pathways. CLV3 is a 13-residue peptide of this family that plays a fundamental role by promoting stem cell differentiation during meristematic development [104–106]. A battery of transgenic assays using the recombinant forms of CLE peptides showed that overexpression of 10 CLE genes, like the CLV3 positive control, resulted in growth arrest at the shoot apical meristem [106, 107]. Contrary to the initial observation that fully active CLV3 was 13 residues long, a recent report provides evidence that CLV3 must contain five additional N-terminal residues that are critical for optimal activity *in vitro* [107–110].

The identified receptor for CLV3 is CLV1 plus the isoforms CLV2 and CRN [108–112]. These leucine-rich repeat

receptors are membrane associated and display cytoplasmic kinase domain. Additional genes include POL, KAPP, and WUS that likely act as downregulators of this pathway [111–113]. Senescence-controlling proteins have been also identified; BAX inhibitor-1, the evolutionarily conserved cell death suppressor found in yeast, is also present in plants. It seems that BAXI-1 acts by delaying methyl jasmonate-induced senescence [106, 113]. A similar situation is encountered at the other end (root meristem) where CLE peptides influence root growth, as well. Overexpression of CLE peptides following transformation assays was observed for CLV3, CLV9, CLV10, CLV11, and CLV13 and linked to root growth inhibition, while overexpression of CLE2, CLE4, CLE5, CLE6, CLE7, CLE18, CLE25, and CLE26 was associated with root growth induction [106, 114]. Overall, it seems that these CLE peptides keep a balance between differentiation and stem cell status.

Vascular meristematic development is controlled by a CLE bearing twelve-amino-acid peptide designated by Ito et al. [114, 115] as tracheary differentiation inhibitory factor (TDIF). The cognate receptor (TDR) contains a leucine-rich repeat and kinase domains as described earlier and is located at the membrane of procambial cells. Its putative role involves suppression of xylem vessel differentiation [115, 116].

The self-incompatibility response during fertilization of hermaphrodite plants is another example of signaling mechanism. In Brassicaceae the pollen determinant and ligand are the S-locus pollen peptide (SP11) [116–118]. The interaction between SP11 and the S-locus receptor kinase (SRK) triggers a signaling cascade leading to inhibition of self-pollination. Structural features of the ligand and the receptor play an important role in this interaction, in such way that interaction between noncognate pairs of ligand receptors fails to occur. Aside from SP-11, additional pollen factors might be needed for the appropriate interaction between SP11 and SRK receptor [118–120].

An additional signaling pathway involves the genesis of stomata pores on leaves that regulate gas exchange with the environment. In *A. thaliana*, such family of ligands designated as "epidermal patterning factor like" (EPFL) contain eleven members ranging in sizes between 5 and 9 kDa. While EPF1 and EPF2 inhibit stomata formation, EPFL9 stimulates stomata formation [119–121]. A recent report shows evidence that EPFL5 represses stomata development by inhibiting meristemoid maintenance in *A. thaliana* [121, 122]. The membrane receptors for transducing the EPFL signal are ER, ER1, and ER2 as described by Shpak et al. [122, 123]. Plant pores adjust their opening/closure condition in response to nutritional needs and humidity by changing turgor pressure of guard cells through intervention of CO_2 and ABA leading to increase in Ca^{+2} sensitivity (for a review see [123, 124]). Also, the number of stomata cells varies as a function of CO_2 via a light induced mechanism. A recent review discusses the various pathways involving stomata development in *A. thaliana* [124, 125].

5. Perspectives

Biologically active peptides represent an excellent example of the advantage of the evolutionary process capable of selecting assortments of amino acids with antimicrobial activity. In the likely event of evolutionary changes within the target offender, new forms of peptides naturally emerge to counter the resistant infectious agent. Changing the assortments of amino acids and/or their order in the peptide are simple alternatives that evolved successfully in living systems during millenniums. Research is needed to elucidate the strategies adopted by life forms producing AMPs to counter the defensive plots posed by invading germs.

Several options are available to improve the quality, selectivity, durability, and safety of AMPs. For instance, the functional and immunological properties of proteins can be improved by partial hydrolysis and the resulting hydrolysate can be used in food systems as additives for beverage and infant formulae, as food texture enhancer or as pharmaceutical ingredient [125, 126]. Bioactive peptides can be computationally modeled, genetically manipulated, and expressed in different systems to serve a practical purpose. In addition to their microbicide activities, other intriguing functions (opioid, antithrombotic, immunomodulatory, and antihypertensive) are emerging [58, 126, 127]. These attributes provide natural alternatives with potential to be used as food ingredients in a variety of applications [58, 127].

Another promising application of AMPs relates to their use on bacterial biofilms. Biofilms are thin layers of microorganisms that colonize onto surfaces, such as implants, dental plaques, ear skin, intestine, and occasioning highly challenging infections and diseases. Several studies demonstrate the efficacy of AMPs into blocking biofilm formation. Singh et al. [127, 128] showed that lactoferrin and LL-37, a human cathelicidin AMP or its derivative, blocked formation of *P. aeruginosa* biofilms at concentrations lower than those required to kill the planktonic cells and, also, reduced biofilm thickness of colonized *P. aeruginosa* by 60% and destroyed microcolony structures of treated biofilms. It also was found effective against both Gram-positive and Gram-negative bacteria [128, 129]. In addition, AMPs have potential to be used in treating persister cells, which are latent phenotypic variants highly tolerant to antibiotics [129, 130].

Since membrane integrity is essential for bacterial survival regardless of the metabolic stage of the cell and because AMPs target the membrane, they show good potential to kill persister microbes. In a recent study, a synthetic cationic peptide, (RW) NH2, was found to kill more than 99% of *E. coli* HM22 persister cells in planktonic culture [15, 19, 22–24, 130].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors would like to acknowledge the support by Fapemig and CNPq from Brazil and by Instituto Politecnico Nacional, SIP 20144606 and SIP 20141244 Mexico. COS and JABC are scholars from COFAA-IPN.

References

[1] I. Y. Park, J. H. Cho, K. S. Kim, Y.-B. Kim, M. S. Kim, and S. C. Kim, "Helix stability confers salt resistance upon helical antimicrobial peptides," *The Journal of Biological Chemistry*, vol. 279, no. 14, pp. 13896–13901, 2004.

[2] Z. Wang and G. Wang, "APD: the antimicrobial peptide database," *Nucleic Acids Research*, vol. 32, pp. D590–D592, 2004.

[3] H. I. Zeya and J. K. Spitznagel, "Antibacterial and enzymic basic proteins from leukocyte lysosomes: separation and identification," *Science*, vol. 142, no. 3595, pp. 1085–1087, 1963.

[4] D. Hultmark, H. Steiner, T. Rasmuson, and H. G. Boman, "Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*," *European Journal of Biochemistry*, vol. 106, no. 1, pp. 7–16, 1980.

[5] Y. Yamaguchi and A. Huffaker, "Endogenous peptide elicitors in higher plants," *Current Opinion in Plant Biology*, vol. 14, no. 4, pp. 351–357, 2011.

[6] R. E. W. Hancock, "Peptide antibiotics," *The Lancet*, vol. 349, no. 9049, pp. 418–422, 1997.

[7] H. G. Boman, "Innate immunity and the normal microflora," *Immunological Reviews*, vol. 173, no. 1, pp. 5–16, 2000.

[8] H. M. Ward, *Disease in Plants*, Macmillan, 1901.

[9] J.-P. S. Powers, A. Rozek, and R. E. W. Hancock, "Structure-activity relationships for the β-hairpin cationic antimicrobial peptide polyphemusin I," *Biochimica et Biophysica Acta: Proteins and Proteomics*, vol. 1698, no. 2, pp. 239–250, 2004.

[10] L. A. J. Mur, P. Kenton, A. J. Lloyd, H. Ougham, and E. Prats, "The hypersensitive response; the centenary is upon us but how much do we know?" *Journal of Experimental Botany*, vol. 59, no. 3, pp. 501–520, 2008.

[11] T. C. Mettenleiter, "Brief overview on cellular virus receptors," *Virus Research*, vol. 82, no. 1-2, pp. 3–8, 2002.

[12] A. A. Bahar and D. Ren, "Antimicrobial peptides," *Pharmaceuticals*, vol. 6, no. 12, pp. 1543–1575, 2013.

[13] H. Jenssen, P. Hamill, and R. E. W. Hancock, "Peptide antimicrobial agents," *Clinical Microbiology Reviews*, vol. 19, no. 3, pp. 491–511, 2006.

[14] N. L. van der Weerden, R. E. W. Hancock, and M. A. Anderson, "Permeabilization of fungal hyphae by the plant defensin NaD1 occurs through a cell wall-dependent process," *Journal of Biological Chemistry*, vol. 285, no. 48, pp. 37513–37520, 2010.

[15] R. Nawrot, J. Barylski, G. Nowicki, J. Broniarczyk, W. Buchwald, and A. Goździcka-Józefiak, "Plant antimicrobial peptides," *Folia Microbiologica*, vol. 59, no. 3, pp. 181–196, 2014.

[16] S. Grün, C. Lindermayr, S. Sell, and J. Durner, "Nitric oxide and gene regulation in plants," *Journal of Experimental Botany*, vol. 57, no. 3, pp. 507–516, 2006.

[17] N. Sitaram and R. Nagaraj, "Interaction of antimicrobial peptides with biological and model membranes: structural and charge requirements for activity," *Biochimica et Biophysica Acta: Biomembranes*, vol. 1462, no. 1-2, pp. 29–54, 1999.

[18] S. Yokoyama, Y. Iida, Y. Kawasaki, Y. Minami, K. Watanabe, and F. Yagi, "The chitin-binding capability of Cy-AMP1 from cycad is essential to antifungal activity," *Journal of Peptide Science*, vol. 15, no. 7, pp. 492–497, 2009.

[19] C. P. Selitrennikoff, "Antifungal Proteins," *Applied and Environmental Microbiology*, vol. 67, no. 7, pp. 2883–2894, 2001.

[20] A. Perez, Q.-X. Li, P. Perez-Romero et al., "A new class of receptor for herpes simplex virus has heptad repeat motifs that are common to membrane fusion proteins," *Journal of Virology*, vol. 79, no. 12, pp. 7419–7430, 2005.

[21] W. Edward Robinson Jr., B. McDougall, D. Tran, and M. E. Selsted, "Anti-HIV-1 activity of indolicidin, an antimicrobial peptide from neutrophils," *Journal of Leukocyte Biology*, vol. 63, no. 1, pp. 94–100, 1998.

[22] B. Stec, "Plant thionins: the structural perspective," *Cellular and Molecular Life Sciences*, vol. 63, no. 12, pp. 1370–1385, 2006.

[23] P. B. Pelegrini, B. F. Quirino, and O. L. Franco, "Plant cyclotides: an unusual class of defense compounds," *Peptides*, vol. 28, no. 7, pp. 1475–1481, 2007.

[24] H. U. Stotz, J. G. Thomson, and Y. Wang, "Plant defensins: defense, development and application," *Plant Signaling & Behavior*, vol. 4, no. 11, pp. 1010–1012, 2009.

[25] R. Fernandez-de Caleya, B. Gonzalez-Pascual, F. García-Olmedo, and P. Carbonero, "Susceptibility of phytopathogenic bacteria to wheat purothionins in vitro," *Applied Microbiology*, vol. 23, no. 5, pp. 998–1000, 1972.

[26] Y. Liu, W. Gong, C. C. Huang, W. Herr, and X. Cheng, "Crystal structure of the conserved core of the herpes simplex virus transcriptional regulatory protein VP16," *Genes and Development*, vol. 13, no. 13, pp. 1692–1703, 1999.

[27] M. Bruix, M. A. Jiménez, J. Santoro et al., "Solution structure of γ1-H and γ1-P thionins from barley and wheat endosperm determined by 1H-NMR: a structural motif common to toxic arthropod proteins," *Biochemistry*, vol. 32, no. 2, pp. 715–724, 1993.

[28] B. Yasin, W. Wang, M. Pang et al., "θ defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry," *Journal of Virology*, vol. 78, no. 10, pp. 5147–5156, 2004.

[29] P. B. Pelegrini, R. P. Del Sarto, O. N. Silva, O. L. Franco, and M. F. Grossi-De-Sa, "Antibacterial peptides from plants: what they are and how they probably work," *Biochemistry Research International*, vol. 2011, Article ID 250349, 9 pages, 2011.

[30] S. Sinha, N. Cheshenko, R. I. Lehrer, and B. C. Herold, "NP-1, a rabbit α-defensin, prevents the entry and intercellular spread of herpes simplex virus type 2," *Antimicrobial Agents and Chemotherapy*, vol. 47, no. 2, pp. 494–500, 2003.

[31] M. Wachinger, A. Kleinschmidt, D. Winder et al., "Antimicrobial peptides melittin and cecropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression," *Journal of General Virology*, vol. 79, no. 4, pp. 731–740, 1998.

[32] S. Laquerre, R. Argnani, D. B. Anderson, S. Zucchini, R. Manservigi, and J. C. Glorioso, "Heparan sulfate proteoglycan binding by herpes simplex virus type 1 glycoproteins B and C, which differ in their contributions to virus attachment, penetration, and cell-to-cell spread," *Journal of Virology*, vol. 72, no. 7, pp. 6119–6130, 1998.

[33] J. H. Andersen, H. Jenssen, K. Sandvik, and T. J. Gutteberg, "Anti-HSV activity of lactoferrin and lactoferricin is dependent on the presence of heparan sulphate at the cell surface," *Journal of Medical Virology*, vol. 74, no. 2, pp. 262–271, 2004.

[34] D. WuDunn and P. G. Spear, "Initial interaction of herpes simplex virus with cells is binding to heparan sulfate," *Journal of Virology*, vol. 63, no. 1, pp. 52–58, 1989.

[35] Y. Liu, J. Luo, C. Xu et al., "Purification, characterization, and molecular cloning of the gene of a seed-specific antimicrobial protein from pokeweed," *Plant Physiology*, vol. 122, no. 4, pp. 1015–1024, 2000.

[36] R. H. Tailor, D. P. Acland, S. Attenborough et al., "A novel family of small cysteine-rich antimicrobial peptides from seed of Impatiens balsamina is derived from a single precursor protein," *The Journal of Biological Chemistry*, vol. 272, no. 39, pp. 24480–24487, 1997.

[37] D. Palumbo, M. Iannaccone, A. Porta, and R. Capparelli, "Experimental antibacterial therapy with puroindolines, lactoferrin and lysozyme in Listeria monocytogenes-infected mice," *Microbes and Infection*, vol. 12, no. 7, pp. 538–545, 2010.

[38] J. P. Marcus, J. L. Green, K. C. Goulter, and J. M. Manners, "A family of antimicrobial peptides is produced by processing of a 7S globulin protein in Macadamia integrifolia kernels," *Plant Journal*, vol. 19, no. 6, pp. 699–710, 1999.

[39] F. R. G. Terras, K. Eggermont, V. Kovaleva et al., "Small cysteine-rich antifungal proteins from radish: their role in host defense," *The Plant Cell*, vol. 7, no. 5, pp. 573–588, 1995.

[40] U. Zottich, M. Da Cunha, A. O. Carvalho et al., "Purification, biochemical characterization and antifungal activity of a new lipid transfer protein (LTP) from Coffea canephora seeds with α-amylase inhibitor properties," *Biochimica et Biophysica Acta: General Subjects*, vol. 1810, no. 4, pp. 375–383, 2011.

[41] C. Remuzgo, T. S. Oewel, S. Daffre et al., "Chemical synthesis, structure-activity relationship, and properties of shepherin I: a fungicidal peptide enriched in glycine-glycine-histidine motifs," *Amino Acids*, vol. 46, no. 11, pp. 2573–2586, 2014.

[42] M. Berrocal-Lobo, A. Segura, M. Moreno, G. López, F. García-Olmedo, and A. Molina, "Snakin-2, an antimicrobial peptide from potato whose gene is locally induced by wounding and responds to pathogen infection," *Plant Physiology*, vol. 128, no. 3, pp. 951–961, 2002.

[43] M. Fujimura, Y. Minami, K. Watanabe, and K. Tadera, "Purification, characterization, and sequencing of a novel type of antimicrobial peptides, *Fa*-AMP1 and *Fa*-AMP2, from seeds of buckwheat (*Fagopyrum esculentum* Moench.)," *Bioscience, Biotechnology and Biochemistry*, vol. 67, no. 8, pp. 1636–1642, 2003.

[44] A. Segura, M. Moreno, A. Molina, and F. García-Olmedo, "Novel defensin subfamily from spinach (*Spinacia oleracea*)," *FEBS Letters*, vol. 435, no. 2-3, pp. 159–162, 1998.

[45] J. H. Wong and T. B. Ng, "Lunatusin, a trypsin-stable antimicrobial peptide from lima beans (*Phaseolus lunatus* L.)," *Peptides*, vol. 26, no. 11, pp. 2086–2092, 2005.

[46] H. W. Jack and B. N. Tzi, "Vulgarinin, a broad-spectrum antifungal peptide from haricot beans (*Phaseolus vulgaris*)," *International Journal of Biochemistry and Cell Biology*, vol. 37, no. 8, pp. 1626–1632, 2005.

[47] L. A. Rivillas-Acevedo and M. Soriano-García, "Isolation and biochemical characterization of an antifungal peptide from *Amaranthus hypochondriacus* seeds," *Journal of Agriculture and Food Chemistry*, vol. 55, no. 25, pp. 10156–10161, 2007.

[48] S. Sharma, H. N. Verma, and N. K. Sharma, "Cationic bioactive peptide from the seeds of benincasa hispida," *International Journal of Peptides*, vol. 2014, Article ID 156060, 12 pages, 2014.

[49] X. Y. Ye, T. B. Ng, and P. F. Rao, "Cicerin and arietin, novel chickpea peptides with different antifungal potencies," *Peptides*, vol. 23, no. 5, pp. 817–822, 2002.

[50] Y. S. Chan, J. H. Wong, E. F. Fang, W. L. Pan, and T. B. Ng, "An antifungal peptide from *Phaseolus vulgaris* cv. brown kidney bean," *Acta Biochim Biophys Sinica*, vol. 44, no. 4, pp. 307–315, 2012.

[51] X. Wu, J. Sun, G. Zhang, H. Wang, and T. B. Ng, "An antifungal defensin from *Phaseolus vulgaris* cv. "Cloud Bean"," *Phytomedicine*, vol. 18, no. 2-3, pp. 104–109, 2011.

[52] A. B. R. Thomson, M. Keelan, A. Thiesen, M. T. Clandinin, M. Ropeleski, and G. E. Wild, "Small bowel review: normal physiology part 1," *Digestive Diseases and Sciences*, vol. 46, no. 12, pp. 2567–2587, 2001.

[53] P. Hördegen, J. Cabaret, H. Hertzberg, W. Langhans, and V. Maurer, "In vitro screening of six anthelmintic plant products against larval Haemonchus contortus with a modified methylthiazolyl-tetrazolium reduction assay," *Journal of Ethnopharmacology*, vol. 108, no. 1, pp. 85–89, 2006.

[54] M. Zasloff, "Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 15, pp. 5449–5453, 1987.

[55] Y. Park, S.-H. Jang, D. G. Lee, and K.-S. Hahm, "Antinematodal effect of antimicrobial peptide, PMAP-23, isolated from porcine myeloid against *Caenorhabditis elegans*," *Journal of Peptide Science*, vol. 10, no. 5, pp. 304–311, 2004.

[56] S. Tagboto and S. Townson, "Antiparasitic properties of medicinal plants and other naturally occurring products," *Advances in Parasitology*, vol. 50, pp. 199–295, 2001.

[57] G. Stepek, D. J. Buttle, I. R. Duce, A. Lowe, and J. M. Behnke, "Assessment of the anthelmintic effect of natural plant cysteine proteinases against the gastrointestinal nematode, *Heligmosomoides polygyrus*, *in vitro*," *Parasitology*, vol. 130, no. 2, pp. 203–211, 2005.

[58] N. Benkerroum, "Antimicrobial peptides generated from milk proteins: a survey and prospects for application in the food industry. A review," *International Journal of Dairy Technology*, vol. 63, no. 3, pp. 320–338, 2010.

[59] E. Abeyrathne, H. Y. Lee, C. Jo, K. C. Nam, and D. U. Ahn, "Enzymatic hydrolysis of ovalbumin and the functional properties of the hydrolysates," *Poultry science*, vol. 93, no. 10, pp. 2678–2686, 2014.

[60] M. V. Ramos, D. P. Souza, M. T. R. Gomes et al., "A phytopathogenic cysteine peptidase from latex of wild rubber vine Cryptostegia grandiflora," *The Protein Journal*, vol. 33, no. 2, pp. 199–209, 2014.

[61] D. P. Souza, C. D. T. Freitas, D. A. Pereira et al., "Laticifer proteins play a defensive role against hemibiotrophic and necrotrophic phytopathogens," *Planta*, vol. 234, no. 1, pp. 183–193, 2011.

[62] I. López-Expósito, A. Quirós, L. Amigo, and I. Recio, "Casein hydrolysates as a source of antimicrobial, antioxidant and antihypertensive peptides," *Le Lait*, vol. 87, no. 4-5, pp. 241–249, 2007.

[63] M. Paul and G. A. Somkuti, "Hydrolytic breakdown of lactoferricin by lactic acid bacteria," *Journal of Industrial Microbiology and Biotechnology*, vol. 37, no. 2, pp. 173–178, 2010.

[64] G. Le Henanff, T. Heitz, P. Mestre, J. Mutterer, B. Walter, and J. Chong, "Characterization of *Vitis vinifera* NPR1 homologs involved in the regulation of pathogenesis-related gene expression," *BMC Plant Biology*, vol. 9, article 54, 2009.

[65] F. T. Lay and M. A. Anderson, "Defensins—components of the innate immune system in plants," *Current Protein & Peptide Science*, vol. 6, no. 1, pp. 85–101, 2005.

[66] T. Ganz, M. E. Selsted, D. Szklarek et al., "Defensins. Natural peptide antibiotics of human neutrophils," *The Journal of Clinical Investigation*, vol. 76, no. 4, pp. 1427–1435, 1985.

[67] A. Patil, A. L. Hughes, and G. Zhang, "Rapid evolution and diversification of mammalian α-defensins as revealed by comparative analysis of rodent and primate genes," *Physiological Genomics*, vol. 20, pp. 1–11, 2005.

[68] C. Tian, B. Gao, Q. Fang, G. Ye, and S. Zhu, "Antimicrobial peptide-like genes in Nasonia vitripennis: a genomic perspective," *BMC Genomics*, vol. 11, no. 1, article 187, 2010.

[69] T. Saito, S. I. Kawabata, T. Shigenaga et al., "A novel big defensin identified in horseshoe crab hemocytes: isolation, amino acid sequence, and antibacterial activity," *Journal of Biochemistry*, vol. 117, no. 5, pp. 1131–1137, 1995.

[70] B. P. H. J. Thomma, B. P. A. Cammue, and K. Thevissen, "Plant defensins," *Planta*, vol. 216, no. 2, pp. 193–202, 2002.

[71] L. Galgóczy, L. Kovács, and C. Vágvölgyi, "Defensin-like antifungal proteins secreted by filamentous fungi," in *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Technology*, pp. 550–559, 2010.

[72] P. D. Games, I. S. dos Santos, É. O. Mello et al., "Isolation, characterization and cloning of a cDNA encoding a new antifungal defensin from *Phaseolus vulgaris* L. seeds," *Peptides*, vol. 29, no. 12, pp. 2090–2100, 2008.

[73] W. F. Broekaert, B. P. A. Cammue, M. F. C. de Bolle, K. Thevissen, G. W. de Samblanx, and R. W. Osborn, "Antimicrobial peptides from plants," *Critical Reviews in Plant Sciences*, vol. 16, no. 3, pp. 297–323, 1997.

[74] R. W. Osborn, G. W. De Samblanx, K. Thevissen et al., "Isolation and characterisation of plant defensins from seeds of Asteraceae, Fabaceae, Hippocastanaceae and Saxifragaceae," *FEBS Letters*, vol. 368, no. 2, pp. 257–262, 1995.

[75] F. García-Olmedo, A. Molina Fernández, J. M. Alamillo, and P. Rodriguez Palenzuela, "Plant defence peptides," *Peptide Science*, vol. 47, no. 6, pp. 479–491, 1998.

[76] E. I. Finkina, E. I. Shramova, A. A. Tagaev, and T. V. Ovchinnikova, "A novel defensin from the lentil Lens culinaris seeds," *Biochemical and Biophysical Research Communications*, vol. 371, no. 4, pp. 860–865, 2008.

[77] N. P. Möller, K. E. Scholz-Ahrens, N. Roos, and J. Schrezenmeir, "Bioactive peptides and proteins from foods: indication for health effects," *European Journal of Nutrition*, vol. 47, no. 4, pp. 171–182, 2008.

[78] J. Boye, F. Zare, and A. Pletch, "Pulse proteins: processing, characterization, functional properties and applications in food and feed," *Food Research International*, vol. 43, no. 2, pp. 414–431, 2010.

[79] J. Ruiz-Ruiz, G. Dávila-Ortíz, L. Chel-Guerrero, and D. Betancur-Ancona, "Angiotensin I-converting enzyme inhibitory and antioxidant peptide fractions from hard-to-cook bean enzymatic hydrolysates," *Journal of Food Biochemistry*, vol. 37, no. 1, pp. 26–35, 2013.

[80] T. de Jesús Ariza-Ortega, E. Y. Zenón-Briones, J. L. Castrejón-Flores, J. Yáñez-Fernández, Y. de las Mercedes Gómez-Gómez, and M. Del Carmen Oliver-Salvador, "Angiotensin-I-converting enzyme inhibitory, antimicrobial, and antioxidant effect of bioactive peptides obtained from different varieties of common beans (*Phaseolus vulgaris* L.) with in vivo antihypertensive activity in spontaneously hypertensive rats," *European Food Research and Technology*, vol. 239, no. 5, pp. 785–794, 2014.

[81] E. Borjórquez-Balam, J. C. Ruiz Ruiz, M. Segura-Campos, D. Betancur Ancona, and L. Chel Guerrero, "Evaluación de la capacidad antimicrobiana de fracciones peptídicas de hidrolizados proteínicos de frijol lima (Phaseolus lunatus)," in *Bioactividad de péptidos derivados de proteínas alimentarias*, M. Segura-Campos, L. Chel Guerrero, and D. Betancur Ancona, Eds., pp. 139–154, OmniaScience Monographs, 2013.

[82] M. D. M. Yust, J. Pedroche, C. Megías et al., "Rapeseed protein hydrolysates: a source of HIV protease peptide inhibitors," *Food Chemistry*, vol. 87, no. 3, pp. 387–392, 2004.

[83] T. C. Johnson, K. Wada, B. B. Buchanan, and A. Holmgren, "Reduction of purothionin by the wheat seed thioredoxin system," *Plant Physiology*, vol. 85, no. 2, pp. 446–451, 1987.

[84] J. H. Wong, X. Q. Zhang, H. X. Wang, and T. B. Ng, "A mitogenic defensin from white cloud beans (*Phaseolus vulgaris*)," *Peptides*, vol. 27, no. 9, pp. 2075–2081, 2006.

[85] A. D. Befus, C. Mowat, M. Gilchrist, J. Hu, S. Solomon, and A. Bateman, "Neutrophil defensins induce histamine secretion from mast cells: mechanisms of action," *Journal of Immunology*, vol. 163, no. 2, pp. 947–953, 1999.

[86] D. Yang, O. Chertov, S. N. Bykovskaia et al., "β-Defensins: Linking innate and adaptive immunity through dendritic and T cell CCR6," *Science*, vol. 286, no. 5439, pp. 525–528, 1999.

[87] M.-C. Dieu-Nosjean, A. Vicari, S. Lebecque, and C. Caux, "Regulation of dendritic cell trafficking: a process that involves the participation of selective chemokines," *Journal of Leukocyte Biology*, vol. 66, no. 2, pp. 252–262, 1999.

[88] J. I. Wheeler and H. R. Irving, "Plant peptide signaling: an evolutionary adaptation," in *Plant Signaling Peptides*, pp. 1–23, Springer, 2012.

[89] K. A. Lease and J. C. Walker, "The Arabidopsis unannotated secreted peptide database, a resource for plant peptidomics," *Plant Physiology*, vol. 142, no. 3, pp. 831–838, 2006.

[90] R. Hammami, J. Ben Hamida, G. Vergoten, and I. Fliss, "PhytAMP: a database dedicated to antimicrobial plant peptides," *Nucleic Acids Research*, vol. 37, no. 1, pp. D963–D968, 2009.

[91] A. Niarchou, A. Alexandridou, E. Athanasiadis, G. Spyrou, and J. Vadivelu, "C-PAmP: large scale analysis and database construction containing high scoring," *PLoS ONE*, vol. 8, no. 11, Article ID e79728, 2013.

[92] K. Oelkers, N. Goffard, G. F. Weiller, P. M. Gresshoff, U. Mathesius, and T. Frickey, "Bioinformatic analysis of the CLE signaling peptide family," *BMC Plant Biology*, vol. 8, article 1, 2008.

[93] T. Billington, M. Pharmawati, and C. A. Gehring, "Isolation and immunoaffinity purification of biologically active plant natriuretic peptide," *Biochemical and Biophysical Research Communications*, vol. 235, no. 3, pp. 722–725, 1997.

[94] M. M. Maryani, G. Bradley, D. M. Cahill, and C. A. Gehring, "Natriuretic peptides and immunoreactants modify osmoticum-dependent volume changes in *Solanum tuberosum* L. mesophyll cell protoplasts," *Plant Science*, vol. 161, no. 3, pp. 443–452, 2001.

[95] S. Rafudeen, G. Gxaba, G. Makgoke et al., "A role for plant natriuretic peptide immuno-analogues in NaCl- and drought-stress responses," *Physiologia Plantarum*, vol. 119, no. 4, pp. 554–562, 2003.

[96] M. Pharmawati, T. Billington, and C. A. Gehring, "Stomatal guard cell responses to kinetin and natriuretic peptides are cGMP-dependent," *Cellular and Molecular Life Sciences*, vol. 54, no. 3, pp. 272–276, 1998.

[97] I. N. Suwastika and C. A. Gehring, "Natriuretic peptide hormones promote radial water movements from the xylem of Tradescantia shoots," *Cellular and Molecular Life Sciences*, vol. 54, no. 10, pp. 1161–1167, 1998.

[98] M. Morse, G. Pironcheva, and C. Gehring, "AtPNP-A is a systemically mobile natriuretic peptide immunoanalogue with a role in *Arabidopsis thaliana* cell volume regulation," *FEBS Letters*, vol. 556, no. 1–3, pp. 99–103, 2004.

[99] L. Kwezi, S. Meier, L. Mungur, O. Ruzvidzo, H. Irving, and C. Gehring, "The *Arabidopsis thaliana* brassinosteroid receptor (AtBRI1) contains a domain that functions as a Guanylyl cyclase *In Vitro*," *PLoS ONE*, vol. 2, no. 5, article e449, 2007.

[100] Y. Matsubayashi, M. Ogawa, A. Morita, and Y. Sakagami, "An LRR receptor kinase involved in perception of a peptide plant hormone, phytosulfokine," *Science*, vol. 296, no. 5572, pp. 1470–1472, 2002.

[101] G. Pearce, G. Munske, Y. Yamaguchi, and C. A. Ryan, "Structure-activity studies of GmSubPep, a soybean peptide defense signal derived from an extracellular protease," *Peptides*, vol. 31, no. 12, pp. 2159–2164, 2010.

[102] L. Yu, M. Moshelion, and N. Moran, "Extracellular protons inhibit the activity of inward-rectifying potassium channels in the motor cells of Samanea saman pulvini," *Plant Physiology*, vol. 127, no. 3, pp. 1310–1322, 2001.

[103] M. R. Blatt and F. Armstrong, "K^+ channels of stomatal guard cells: abscisic-acid-evoked control of the outward rectifier mediated by cytoplasmic pH," *Planta*, vol. 191, no. 3, pp. 330–341, 1993.

[104] J. C. Fletcher, "Signaling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems," *Science*, vol. 283, no. 5409, pp. 1911–1914, 1999.

[105] M. Ogawa, H. Shinohara, Y. Sakagami, and Y. Matsubayash, "*Arabidopsis* CLV3 peptide directly binds CLV1 ectodomain," *Science*, vol. 319, no. 5861, p. 294, 2008.

[106] T. J. Strabala, P. J. O'Donnell, A.-M. Smit et al., "Gain-of-function phenotypes of many *CLAVATA3/ESR* genes, including four new family members, correlate with tandem variations in the conserved *CLAVATA3/ESR* domain," *Plant Physiology*, vol. 140, no. 4, pp. 1331–1344, 2006.

[107] T.-T. Xu, X.-F. Song, S.-C. Ren, and C.-M. Liu, "The sequence flanking the N-terminus of the CLV3 peptide is critical for its cleavage and activity in stem cell regulation in *Arabidopsis*," *BMC Plant Biology*, vol. 13, article 225, 2013.

[108] S. E. Clark, M. P. Running, and E. M. Meyerowitz, "CLAVATA1, a regulator of meristem and flower development in Arabidopsis," *Development*, vol. 119, no. 2, pp. 397–418, 1993.

[109] A. Bleckmann, S. Weidtkamp-Peters, C. A. M. Seidel, and R. Simon, "Stem cell signaling in Arabidopsis requires CRN to localize CLV2 to the plasma membrane," *Plant Physiology*, vol. 152, no. 1, pp. 166–176, 2010.

[110] R. Müller, A. Bleckmann, and R. Simon, "The receptor kinase CORYNE of Arabidopsis transmits the stem cell-limiting signal CLAVATA3 independently of CLAVATA1," *The Plant Cell*, vol. 20, no. 4, pp. 934–946, 2008.

[111] R. W. Williams, J. M. Wilson, and E. M. Meyerowitz, "A possible role for kinase-associated protein phosphatase in the Arabidopsis CLAVATA1 signaling pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 19, pp. 10467–10472, 1997.

[112] L. P. Yu, E. J. Simon, A. E. Trotochaud, and S. E. Clark, "*POLTERGEIST* functions to regulate meristem development downstream of the *CLAVATA* loci," *Development*, vol. 127, no. 8, pp. 1661–1670, 2000.

[113] H. Yue, S. Nie, and D. Xing, "Over-expression of *Arabidopsis* Bax inhibitor-1 delays methyl jasmonate-induced leaf senescence by suppressing the activation of MAP kinase 6," *Journal of Experimental Botany*, vol. 63, no. 12, pp. 4463–4474, 2012.

[114] Y. Ito, I. Nakanomyo, H. Motose et al., "Dodeca-CLE as peptides as suppressors of plant stem cell differentiation," *Science*, vol. 313, no. 5788, pp. 842–845, 2006.

[115] Y. Hirakawa, Y. Kondo, and H. Fukuda, "TDIF peptide signaling regulates vascular stem cell proliferation via the WOX4 homeobox gene in Arabidopsis," *The Plant Cell*, vol. 22, no. 8, pp. 2618–2629, 2010.

[116] G. Suzuki, N. Kai, T. Hirose et al., "Genomic organization of the S locus: Identification and characterization of genes in *SLG/SRK* region of S9 haplotype of *Brassica* campestris (syn. rapa)," *Genetics*, vol. 153, no. 1, pp. 391–400, 1999.

[117] J. B. Nasrallah, T. Nishio, and M. E. Nasrallah, "The self-incompatibility genes of Brassica: expression and use in genetic ablation of floral tissues," *Annual Review of Plant Physiology and Plant Molecular Biology*, vol. 42, no. 1, pp. 393–422, 1991.

[118] A. Kachroo, C. R. Schopfer, M. E. Nasrallah, and J. B. Nasrallah, "Allele-specific receptor-ligand interactions in Brassica self-incompatibility," *Science*, vol. 293, no. 5536, pp. 1824–1826, 2001.

[119] L. Hunt and J. E. Gray, "The signaling peptide EPF2 controls asymmetric cell divisions during stomatal development," *Current Biology*, vol. 19, no. 10, pp. 864–869, 2009.

[120] S. S. Sugano, T. Shimada, Y. Imai et al., "Stomagen positively regulates stomatal density in *Arabidopsis*," *Nature*, vol. 463, no. 7278, pp. 241–244, 2010.

[121] T. Niwa, T. Kondo, M. Nishizawa, R. Kajita, T. Kakimoto, and S. Ishiguro, "Epidermal Patterning factor like 5 peptide represses stomatal development by inhibiting meristemoid maintenance in *Arabidopsis thaliana*," *Bioscience, Biotechnology and Biochemistry*, vol. 77, no. 6, pp. 1287–1295, 2013.

[122] E. D. Shpak, J. M. McAbee, L. J. Pillitteri, and K. U. Torii, "Stomatal patterning and differentiation by synergistic interactions of receptor kinases," *Science*, vol. 309, no. 5732, pp. 290–293, 2005.

[123] T.-H. Kim, M. Böhmer, H. Hu, N. Nishimura, and J. I. Schroeder, "Guard cell signal transduction network: advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling," *Annual Review of Plant Biology*, vol. 61, pp. 561–591, 2010.

[124] L. J. Pillitteri and J. Dong, "Stomatal development in arabidopsis," in *The Arabidopsis Book*, vol. 11, American Society of Plant Biologists, 2013.

[125] A. Mannheim and M. Cheryan, "Continuous hydrolysis of milk protein in a membrane reactor," *Journal of Food Science*, vol. 55, no. 2, pp. 381–385, 1990.

[126] D. A. Clare and H. E. Swaisgood, "Bioactive milk peptides: a prospectus," *Journal of Dairy Science*, vol. 83, no. 6, pp. 1187–1195, 2000.

[127] P. K. Singh, M. R. Parsek, E. P. Greenberg, and M. J. Welsh, "A component of innate immunity prevents bacterial biofilm development," *Nature*, vol. 417, no. 6888, pp. 552–555, 2002.

[128] J. Overhage, A. Campisano, M. Bains, E. C. W. Torfs, B. H. A. Rehm, and R. E. W. Hancock, "Human host defense peptide LL-37 prevents bacterial biofilm formation," *Infection and Immunity*, vol. 76, no. 9, pp. 4176–4182, 2008.

[129] K. Lewis, "Persister cells," *Annual Review of Microbiology*, vol. 64, pp. 357–372, 2010.

[130] X. Chen, M. Zhang, C. Zhou, N. R. Kallenbach, and D. Ren, "Control of bacterial persister cells by Trp/Arg-containing antimicrobial peptides," *Applied and Environmental Microbiology*, vol. 77, no. 14, pp. 4878–4885, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 735087, 11 pages
http://dx.doi.org/10.1155/2015/735087

Review Article

Plant Antimicrobial Peptides as Potential Anticancer Agents

Jaquelina Julia Guzmán-Rodríguez,[1] Alejandra Ochoa-Zarzosa,[1] Rodolfo López-Gómez,[2] and Joel E. López-Meza[1]

[1] *Centro Multidisciplinario de Estudios en Biotecnología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Michoacana de San Nicolás de Hidalgo, Km 9.5 Carretera Morelia-Zinapécuaro, Posta Veterinaria, 58893 Morelia, MICH, Mexico*
[2] *Instituto de Investigaciones Químico Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Edif. B1, Ciudad Universitaria, 58030 Morelia, MICH, Mexico*

Correspondence should be addressed to Joel E. López-Meza; elmeza@umich.mx

Received 1 August 2014; Revised 25 September 2014; Accepted 26 September 2014

Academic Editor: Dennis K. Bideshi

Antimicrobial peptides (AMPs) are part of the innate immune defense mechanism of many organisms and are promising candidates to treat infections caused by pathogenic bacteria to animals and humans. AMPs also display anticancer activities because of their ability to inactivate a wide range of cancer cells. Cancer remains a cause of high morbidity and mortality worldwide. Therefore, the development of methods for its control is desirable. Attractive alternatives include plant AMP thionins, defensins, and cyclotides, which have anticancer activities. Here, we provide an overview of plant AMPs anticancer activities, with an emphasis on their mode of action, their selectivity, and their efficacy.

1. Introduction

Cancer is a leading cause of death worldwide. In 2012, cancer caused 8.2 million deaths, and cancers of the lungs, liver, colon, stomach, and breast are main types [1]. A hallmark of cancer is the rapid growth of abnormal cells that extend beyond their usual limits and invade adjoining parts of the body or spread to other organs, a process known as metastasis. Cancer treatment requires careful selection of one or more therapeutic modalities, such as surgery, radiotherapy, or chemotherapy. Despite progress in anticancer therapies, the chemotherapeutic drugs used in cancer treatment have the serious drawback of nonspecific toxicity. Additionally, many neoplasms eventually become resistant to conventional chemotherapy because of selection for multidrug-resistant variants [2]. These limitations have led to the search for new anticancer therapies. An attractive alternative is the use of antimicrobial peptides or AMPs, which represent a novel family of anticancer agents that avoid the shortcomings of conventional chemotherapy [3].

AMPs are amphipathic molecules produced by a wide variety of organisms as part of their first line of defense (eukaryotes) or as a competition strategy for nutrients and space (prokaryotes) [4]. Currently, over 2400 AMPs are reported in The Antimicrobial Peptide Database (URL http://aps.unmc.edu/AP/main.php) [5]. The continuous discovery of new AMP groups in diverse organisms has made these natural antibiotics the basic elements of a new generation of potential biomedical treatments against infectious diseases in humans and animals. Moreover, the broad spectrum of biological activities and the low incidence of resistance to these molecules suggest a potential benefit in cancer treatment, which reinforces the importance of their study [6].

AMPs are usually short peptides (12–100 aa residues), which mainly have a positive charge (+2 to +9), although there are also neutral and negatively charged molecules [7]. AMPs are classified into the following four groups according to their structural characteristics: (1) cysteine-rich and β-sheet AMPs (α- and β-defensins); (2) AMPs possessing α-helices (LL-37 cathelicidin, cecropins, and magainins);

(3) AMPs with extended structure (rich in glycine, proline, tryptophan, arginine, and/or histidine); (4) peptide "loop," which have a single disulfide bond (bactenecin) [8]. In recent years, several reviews on the structures, mechanisms of action, and emergence of resistance to AMPs have been published, to which the reader is referred for additional information [9–11]. Furthermore, recent reviews of the anticancer activities and selectivity and efficacy of AMPs, particularly from animals, have been reported [12–15]. The mechanisms by which AMPs kill cancerous cells are poorly understood although evidences indicate that both membranolytic and nonmembranolytic mechanisms are involved. The membranolytic activity of AMPs depends on their own characteristics as well as of the target membrane [13]. Also, the selectivity of some AMPs against cancer cells has been related with the charge of membrane, which has a net negative charge [12]. Anionic molecules (phosphatidylserine, O-glycosylated mucins, sialylated gangliosides, and heparin sulfate) confer a net negative charge to cancer cells, which contrasts with the normal mammalian cell membrane (typically zwitterionic) [14, 15]. On the other hand, the nonmembranolytic activities of AMPs involve the inhibition of processes such as angiogenesis, which is essential for the formation of tumor-associated vasculature [14].

Despite the promising characteristics of anticancer agents such as AMPs, only a few of them have been tested using *in vivo* models. Cecropin B from *Hyalophora cecropia* increases the survival time of mice bearing ascitic murine colon adenocarcinoma cells [16]. In the same way, when magainin 2 was tested against murine sarcoma tumors, animals increase its life span (45%) [17]. However, there is little information related to the anticancer effects of plant AMPs. Here, we provide an overview of plant AMP anticancer activities with an emphasis on their mode of action, selectivity, and efficacy. We focus on the anticancer activity reported only for the defensins, thionins, and cyclotides because the cytotoxic effects of these families have been widely described.

2. Plant AMPs

Plants are a major source of diverse molecules with pharmacological potential. Over 300 AMP sequences have been described [5]. Plants produce small cysteine-rich AMPs as a mechanism of natural defense, which may be expressed constitutively or induced in response to a pathogen attack. Plant AMPs are abundantly expressed in the majority species, and small cysteine-rich AMPs may represent up to 3% of the repertoire of plant genes [18]. Plant AMPs are produced in all organs and are more abundant in the outer layer, which is consistent with their role as a constitutive host defense against microbial invaders attacking from the outside [19, 20]. Plant AMPs are released immediately after the infection is initiated. AMPs are expressed by a single gene and therefore require less biomass and energy consumption [19, 20]. The majorities of plant AMPs have a molecular weight between 2 and 10 kDa, are basic, and contain 4, 6, 8, or 12 cysteines that form disulfide bonds conferring structural and thermodynamic stability [21]. Plant AMPs are classified based on the identity of their amino acid sequence and the number and position of cysteines forming disulfide bonds. Twelve families have been described, which are listed in Table 1 [21–23].

Table 1: Classification of plant AMPs[1].

Family	Disulfide bonds	Activity
Thionins	3-4	Bacteria, fungi, and cytotoxic
Defensins	3-4	Bacteria, fungi, and cytotoxic
Cyclotides	3	Bacteria, virus, insects, and cytotoxic
Knottin-like	3	Gram (+) bacteria and fungi
Shepherdins	0 (linear)	Bacteria and fungi
MBP-1	2	Bacteria and fungi
Ib-AMPs	2	Gram (+) bacteria and fungi
LTP	3-4	Bacteria and fungi
Snakins	6	Bacteria and fungi
Hevein-like	4	Gram (+) bacteria and fungi
β-Barrelins	6	Fungi
2S albumins	2	Bacteria and fungi

[1]Modified from [21–23].

The primary biological activities of plant AMPs are antifungal, antibacterial, and against oomycetes and herbivorous insects [32, 34, 35]. Additionally, plant AMPs also exhibit enzyme inhibitory activities [36] and have roles in heavy metal tolerance [37], abiotic stress [38], and development [39]. In addition, some plant AMPs show cytotoxic activity against mammalian cells and/or anticancer activity against cancer cells from different origins [25, 28, 31, 40–56]. Of the 12 plant AMP families, 3 contain members with cytotoxic and anticancer properties, the defensins, thionins, and cyclotides. Here, the cytotoxic properties of these peptides are described and the possibility of their use in cancer treatment is discussed.

3. Thionins

Thionins were the first AMP isolated from plants [57]. These AMPs belong to a rapidly growing family of biologically active peptides in the plant kingdom and are small cysteine-rich peptides (~5 kDa) with toxic and antimicrobial properties [58]. Thionins are divided into at least four different types depending on the net charge, the number of amino acids, and the disulfide bonds present in the mature protein [59]. Type 1 thionins are highly basic and consist of 45 amino acids, eight of which are cysteines, forming four disulfide bonds. Type 2 thionins consist of 46 or 47 amino acid peptides, are slightly less basic than type 1 thionins, and also have four disulfide bonds. Type 3 thionins consist of 45 or 46 amino acid peptides with three or four disulfide bonds and are as basic as type 2 thionins. Finally, type 4 thionins consist of 46 amino acid peptides with three disulfide bonds and are neutral [58].

The primary role for thionins is plant protection against pathogens [57, 59]. However, they also participate in seed maturation, dormancy, or germination [58], as well as the packaging of storage proteins into protein bodies, or in their mobilization during germination [60]. In addition, thionins

Table 2: Thionins with anticancer and cytotoxic activity.

Name	Species	Activity against	Cytotoxic activity	Anticancer activity	Reference
Pyrularia	*Pyrularia pubera*	B16, HeLa, rat hepatocytes, and lymphocytes	Yes	Yes	[24]
Viscotoxin B2	*Viscum coloratum*	Rat sarcoma cells	Not tested	Yes	[25]
Viscotoxins 1-PS, A1, A2, A3, and B	*Viscum album*	Human lymphocytes	Yes	Not tested	[26]
Viscotoxin C1	*Coloratum ohwi*	Rat sarcoma cells	Not tested	Yes	[27]
Ligatoxin B	*Phoradendron liga*	U-937-GTB ACHN	Not tested	Yes	[28]
Ligatoxin A	*Phoradendron liga*	Animal cells	Yes	Not tested	[29]
Phoratoxins A and B	*Phoradendron tomentosum*	Mice	Yes	Not tested	[30]
Phoratoxins C, D, E, and F	*Phoradendron tomentosum*	10 cancer cell lines	Not tested	Yes	[31]
Thi2.1	*Arabidopsis thaliana*	HeLa, A549, MCF-7, and bovine mammary epithelial cells	Yes	Yes	[32]
β-Purothionin	*Tricum aestivum*	p388	Not tested	Yes	[33]

may play a role in altering the cell wall upon penetration of the epidermis by fungal hyphae or act as a secondary messenger in signal transduction [61].

3.1. Cytotoxic and Anticancer Activity of Thionins. In addition to the activities described, several plant thionins show cytotoxic and anticancer activities (Table 2). The pyrularia thionin from mistletoe (*Pyrularia pubera*) showed an anticancer activity against cervical cancer cells (HeLa) and mouse melanoma cells (B16) with an IC_{50} of 50 μg/mL (half maximal inhibitory concentration); however, the pyrularia thionin is cytotoxic because it causes hemolysis [24]. The anticancer effect is attributable to a cellular response that involves the stimulation of Ca^{2+} influx coupled to depolarization of the plasma membrane, which leads to the activation of an endogenous phospholipase A_2 and, as consequence, membrane alteration, and finally the cell death.

Another group of thionins with anticancer and cytotoxic activity are the viscotoxins from *Viscum* spp. Viscotoxin B2 showed anticancer activity against rat osteoblast-like sarcoma (IC_{50} 1.6 mg/L) [42]. On the other hand, viscotoxins A1, A2, A3, and 1-PS were cytotoxic to human lymphocytes, due the fact that they induce the production of reactive oxygen species (ROS) and cell membrane permeabilization [26]. Furthermore, a mixture of viscotoxins (50 μg/mL) induced apoptosis in human lymphocytes by activating caspase 3 [43]. Conversely, viscotoxins are far less hemolytic than other thionins. Under the same experimental conditions, pyrularia thionin (20 μg/mL) lysed 50% of human erythrocytes, whereas viscotoxin B (100 μg/mL) lysed only 10% [62]. An alignment of the amino acids sequences of both thionins shows that pyrularia has more hydrophobic amino acids compared to the viscotoxin B (Figure 1). These differences could explain the differential hemolytic activity of both thionins because greater hydrophobicity increases the hemolytic activity of AMPs [63].

Another thionin with anticancer activity is the ligatoxin B (*Phoradendron league*). This AMP (100 μg/mL) inhibited the growth of lymphoma cells (U937GTB) and human adenocarcinoma (ACHN). Ligatoxin B has a DNA binding domain, which may be related to the inhibition of nucleic acid and protein synthesis [28]. Unfortunately, the cytotoxic effects of ligatoxin B have not yet been tested on normal cells.

Several thionins (phoratoxins A–F) have been identified in *Phoradendron tomentosum*, all of which possess toxic activity. Phoratoxins A and B are toxic to rats at doses of 0.5–1 mg/kg, and their mechanism of action is related to changes in the electrical charge and the mechanical activity of the rat papillary muscle [30]. Furthermore, phoratoxins C–F showed differential anticancer activity against different types of solid tumor cells (NCI-H69, ACHN, and breast carcinoma) and hematological tumors (RPMI 8226-S and U-937 GTB). Phoratoxin C was the most toxic with an IC_{50} of 0.16 μM, whereas phoratoxin F had an IC_{50} value of 0.40 μM. Furthermore, phoratoxin C was tested on primary cultures of tumor cells from patients and showed selective activity to breast cancer cells from solid tumor samples. These cells were 18 times more sensitive to phoratoxin C than the hematological tumor cells [31]. These data suggest that these compounds are an alternative for developing a new class of anticancer agents with improved activity against solid tumor malignancies. Despite the marked differences in the activity of phoratoxins, they have a high percentage of identity (~90%) (Figure 1). The small changes in specific amino acids could be the key to the biological activity of these thionins; however, further studies are necessary.

Another thionin with anticancer activity against cancer cell lines is the Thi2.1 thionin from *Arabidopsis thaliana*, which was expressed in a heterologous system [32]. The conditioned media from cells that express Thi2.1 inhibited the viability of MCF-7 cells (94%), A549 (29%), and HeLa cells (38%); however, Thi2.1 also showed cytotoxicity against bovine mammary epithelial cells (89%) and bovine endothelium (93%). The mechanism of action of Thi2.1 has not yet been determined.

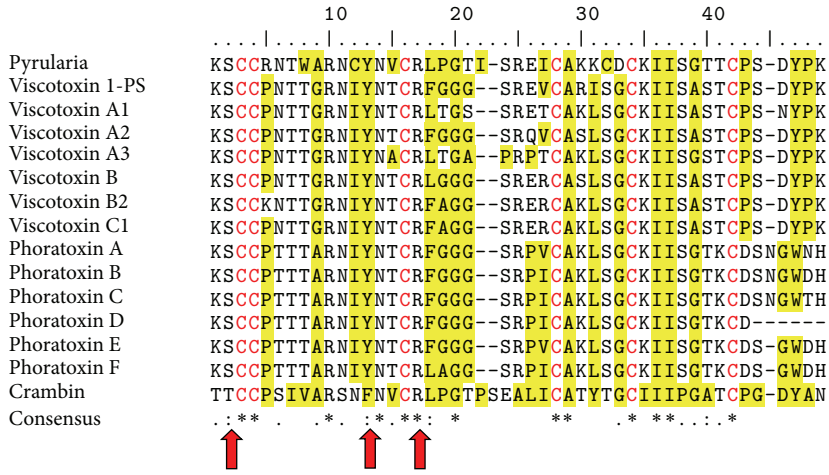


Figure 1: Alignment of amino acid sequences from thionins. The asterisk indicates amino acids conserved in all family members. The cysteine residues present in all sequences and relevant to the classification are indicated in red letters. The red arrows indicate the three residues that are essential for binding to the head regions of the membrane lipids. The hydrophobic residues are shaded in yellow. The thionin sequences included in the alignment were pyrularia (GenBank accession P07504) from *Pyrularia pubera*, viscotoxins 1-PS (GenBank accession P01537), A1 (GenBank accession 3C8P_A), A2 (GenBank accession P32880), A3 (GenBank accession VTVAA3), B (GenBank accession 1JMP_A), B2 (GenBank accession 2V9B_B), and C1 (GenBank accession P83554) from *Viscum album*, phoratoxins A (GenBank accession P01539), B, C, D, E, and F [24] from *Phoradendron tomentosum,* and crambin (GenBank accession P01542) from *Crambe hispanica.*

In summary, the cytotoxic activity of thionins is not selective; however, these peptides can be exploited for the design of new anticancer molecules. Further investigations are necessary to determine the clinical potential of this class of compounds.

4. Plant Defensins

Plant defensins are a class of plant AMPs with structural and functional properties that resemble the defense peptides produced by fungi, invertebrates, and vertebrates, called "defensins." This group of AMPs has great diversity in amino acid sequence, but its members show a clear conservation of some amino acid positions. This variation in the primary sequence is associated with the diversity of biological activities of plant defensins, which include antifungal and antibacterial activities, in addition to proteinase or amylase inhibitory activities [20]. Plant defensins can form three to four disulfide bridges that stabilize their structure [64]. Studies of the three-dimensional structure of plant defensins have shown that these peptides consist of an α-helix and three antiparallel β-sheets, arranged in the configuration $\beta\alpha\beta\beta$ [19]. These AMPs are classified into two types depending on the structure of the precursor protein from which they are derived. Type 1 defensins are the largest group, and the majority of members contain a signal peptide in the prepeptide sequence linked to the mature defensin at the N-terminus. Type 2 defensins include plant defensins for which the precursor has a signal peptide, the active domain of the defensin, and a C-terminal prodomain [20]. Recently, it was demonstrated that the C-terminal prodomain of the NaD1 defensin of *Nicotiana alata* is sufficient for vacuolar targeting and plays an important role in detoxification of the defensin [65].

Plant defensins inhibit the growth of a wide range of fungi and in a lesser extent are toxic to mammalian cells or plants [66]. The proposed mechanism of action of plant defensins is to either destabilize the cell membrane by coating its outer surface or insert themselves into the membrane to form open pores allowing vital biomolecules to leak out of the cell [34, 64].

4.1. Cytotoxic and Anticancer Activity of Plant Defensins. In addition to the antifungal activities, plant defensins exhibit anticancer and cytotoxic effects (Table 3). The first plant defensin reported with anticancer activity was the defensin sesquin from *Vigna sesquipedalis* that inhibited the proliferation of MCF-7 and leukemia M1 (2.5 mg/mL) cells [44]. Furthermore, Wong and Ng [41] reported that the defensin limenin (0.1 mg/mL), a defensin from *Phaseolus limensis*, differentially inhibited the proliferation of leukemia cells, reaching 60% inhibition for M1 and 30% inhibition for L1210 cells; however, its effect against normal cells was not evaluated. Another plant defensin with effects on cancer cell is lunatusin, a defensin purified from the seeds of the Chinese lima bean (*Phaseolus lunatus* L.), which inhibited the proliferation of MCF-7 cells (IC_{50} 5.71 μM). Unfortunately, lunatusin also possesses cell-free translation-inhibitory activity in the rabbit reticulocyte lysate system [45]. This indicates that this defensin may be cytotoxic to normal tissues and other cell types. However, from all the defensins studied, lunatusin is the only plant defensin with this effect.

Further studies identified other plant defensins that inhibit the proliferation of cancer cells, including breast and colon cancer, without cytotoxic effects on normal cells. A defensin from the purple pole bean (*Phaseolus vulgaris* cv. "Extra-long Purple Pole bean") inhibited the proliferation of the cancer cell lines HepG2, MCF-7, HT-29, and Sila (IC_{50}

Table 3: Plant defensins with anticancer and cytotoxic activity.

Name	Species	Activity against	Cytotoxic activity	Anticancer activity	Reference
Sesquin	*Vigna sesquipedalis*	MCF-7 and M1	Not tested	Yes	[44]
Limenin	*Phaseolus limensis*	L1210 and M1	Not tested	Yes	[41]
Lunatusin	*Phaseolus lunatus*	MCF-7 rabbit reticulocyte	Yes	Yes	[45]
Purple pole defensin	*Phaseolus vulgaris* cv. "Extra-long Purple Pole bean"	HepG2, MCF7, HT-29, and SiHa	No	Yes	[46]
Coccinin	*Phaseolus coccineus* cv. "Major"	HL60 and L1210	No	Yes	[47]
Phaseococcin	*Phaseolus coccineus*	L1210 and HL60	No	Yes	[48]
γ-Thionin	*Capsicum chinense*	HeLa	No	Yes	[49]
NaD1	*Nicotiana alata*	U937	Not tested	Yes	[67]
Mitogenic defensin	*Phaseolus vulgaris*	MCF-7, murine splenocytes	Yes	Yes	[68]
Vulgarinin	*Phaseolus vulgaris*	MCF-7, L1210, and M1	Not tested	Yes	[69]
Cloud bean defensin	*Phaseolus vulgaris* cv. cloud bean	L1210 and MBL2	Not tested	Yes	[70]
Nepalese	*Phaseolus angularis*	L1210, MBL2	Not tested	Yes	[71]
Gymnin	*Gymnocladus chinensis* Baill	M1, HepG2, and L1210	Not tested	Yes	[72]

4–8 μM) but did not affect human embryonic liver cells or human erythrocytes under the same conditions [46]. By contrast, coccinin from small scarlet runner beans (*Phaseolus coccineus* cv. "Major"), a peptide of 7 kDa and an N-terminal sequence resembling those of defensins, inhibited the proliferation of HL60 and L1210 cells (IC_{50} 30–40 μM); however, it did not affect the proliferation of mouse splenocytes [47]. Similarly, phaseococcin from *P. coccineus* cv. "Minor" inhibited the proliferation of HL60 and L1210 cells (IC_{50} 30–40 μM). This defensin did not affect the proliferation of mouse splenocytes or protein synthesis in a cell-free rabbit reticulocyte lysate system [48]. The lack of adverse effects of both of these defensins on the proliferation of isolated mouse splenocytes indicates that these molecules are selective. Finally, the conditioned media from bovine endothelial cells that express the cDNA of the defensin γ-thionin from *Capsicum chinense* inhibited 100% of the viability of HeLa cells but did not affect immortalized bovine endothelial cells [49]. Data from our laboratory indicate that this chemically synthetized defensin has a similar effect on both cells (data not published).

In general, the anticancer activity mechanism of plant defensins is poorly understood. However, Poon et al. [67] described the mechanism of the NaD1 defensin on the monocytic lymphoma cells U937. Interestingly, this effect was produced by a novel mechanism of cell lysis in which NaD1 acts via direct binding to the plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2).

Thus, the anticancer activities of plant defensins suggest that these AMPs may be an alternative therapy for cancer treatment. The isolation and characterization of these peptides has increased, which allows for the identification of sequences that exhibit desirable characteristics against cancer cells.

5. Cyclotides

Cyclotides are macrocyclic peptides (~30 amino acids) with diverse biological activities, isolated from the Rubiaceae and Violaceae plant families. These molecules constitute a family of plant AMPs, members of which contain six conserved cysteines that stabilize the structure by the formation of disulfide bonds [74]. Cyclotides have a cystine knot with an embedded ring in the structure formed by two disulfide bonds and connecting backbone segments threaded by a third disulfide bond. These combined features of the cyclic cystine knot produce a unique protein fold that is topologically complex and has exceptional chemical and biological stability with pharmaceutical and medicinal significance for drug design [75].

Cyclotides are biosynthesized ribosomally as a precursor protein that encodes one or more cyclotide domains. The arrangement of a typical cyclotide precursor protein is an endoplasmic reticulum signal sequence, a prodomain, a mature cyclotide domain, and a C-terminal region [76]. Although the excision and cyclization processes that yield cyclic mature peptides from these precursors are not fully understood, it has been suggested that asparaginyl endoproteinase enzyme activity plays an important role in this process [77]. This hypothesis is consistent with the presence of a conserved Asn (or Asp) residue at the C-terminus of the cyclotide domain within the precursor proteins (Figure 2(a)). It is also supported by studies of the expression of mutated cyclotides in transgenic plants, in which substitution of the conserved Asn by Ala abolished the production of cyclic peptides *in planta* [78].

The main role attributable to cyclotides is host defense, and there are molecules that are expressed in large quantities in the plant (up to 1 g/kg of leaf material) [75]. Furthermore,

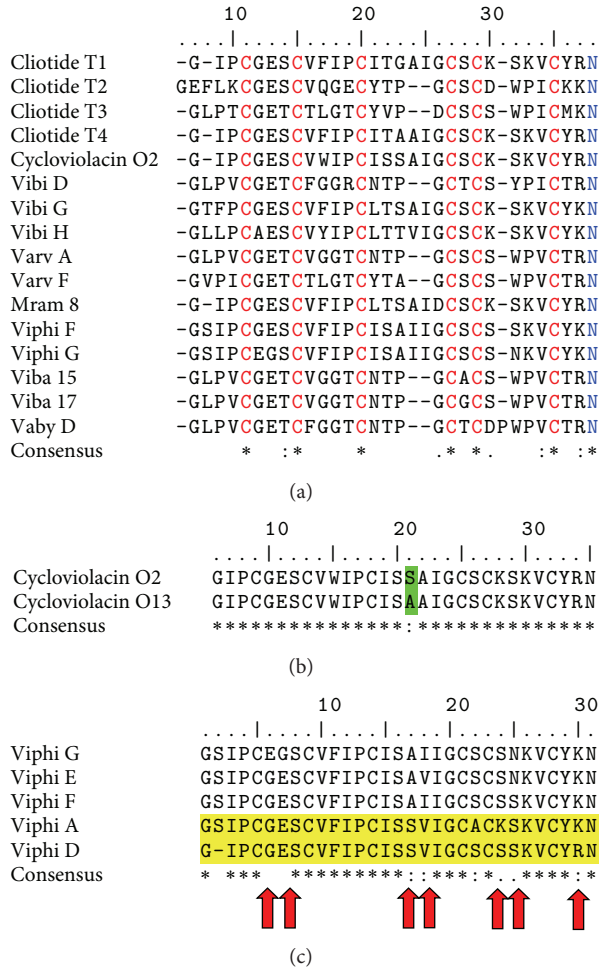


Figure 2: Alignment of amino acid sequences from cytotoxic cyclotides. (a) The cysteine residues present in all sequences and relevant to the classification are indicated in red letters. The asparagine residues present in all sequences and relevant to the cyclization process are indicated in blue letters. (b) Amino acid sequence alignment of cycloviolacins O2 and 13. The replacement of serine by alanine (shaded in green) increases the hemolytic effect by more than 3-fold. (c) Amino acid sequence alignment of Viphi G, Viphi E, Viphi F, Viphi A, and Viphi D cyclotides. Shaded in yellow are the sequences with no-toxic effects; the red arrows indicate the residues with specific variations. The sequences included in the alignment were cliotides T1 (GenBank accession AEK26402), T2 (GenBank accession AEK26403), T3 (GenBank accession AEK26404), and T4 (GenBank accession AEK26405) from *Clitoria ternatea*, cycloviolacins O2 (GenBank accession P58434) and O13 (GenBank accession Q5USNB) from *Viola odorata*, Vibi D (GenBank accession P85242), Vibi G (GenBank accession P85245), and Vibi H (GenBank accession P85246) from *Viola biflora*, Varv A (GenBank accession Q5USN7) and Varv F (GenBank accession 3E4H_A) from *Viola odorata*, Mram 8, Viphi A, Viphi D, Viphi E, Viphi F, and Viphi G [73] from *Viola philippica,* and Vaby D [55] from *Viola abyssinica*.

cyclotides display a wide range of biological and pharmacological activities, including anti-HIV, anthelmintic, insecticidal, antimicrobial, and cytotoxic effects [79]. Therefore, there is increasing interest in exploring the plant kingdom to identify new cyclotides.

5.1. Cytotoxic and Anticancer Activity of Cyclotides. One of the first activities reported for cyclotides was hemolytic activity, which only occurs in the cyclic condition. Cyclotides lose their hemolytic activity when they are linearized [80], demonstrating that the cyclic backbone is important for this activity, which also appears to be important for the other activities of cyclotides. A directed mutational analysis of cyclotide kalata B1, in which all 23 noncysteine residues were replaced with alanine, shows that both the insecticidal and hemolytic activities are dependent on a well-defined cluster of hydrophilic residues on one face of the cyclotide. Interestingly, these molecules retain the characteristic stability of the framework [73]. In addition, it has been suggested that the hemolytic activity of the cyclotides depends on the amino acid sequence. The cyclotides cycloviolacins O2 and O13 from *Viola odorata* have different hemolytic activities. Both molecules differ only in one residue (Figure 2(b)). Cycloviolacin O2 has a serine residue, whereas cycloviolacin O13 has an alanine in the same position. The loss of the hydroxyl group changes the hemolytic activity by more than 3-fold [50].

Table 4: Cyclotides with anticancer and cytotoxic activity.

Name	Species	Activity against	Cytotoxic activity	Anticancer activity	Reference
Cycloviolacin O2	*Viola odorata*	U-937, HeLa	Yes	Yes	[54]
Viphi A, Viphi F, and Viphi G	*Viola philippica*	MM96L, HeLa, BGC-823, and HFF-1	Yes	Yes	[51]
MCoTI-I	*Momordica cochinchinensis*	LNCaP and HCT116	Not tested	Yes	[81]
HB7	*Hedyotis biflora*	Capan2 and PANC1	Not tested	Yes	[82]
Vaby A and Vaby D	*Viola abyssinica*	U-937	Not tested	Yes	[83]
Cliotides T1–T4	*Clitoria ternatea*	HeLa and human erythrocytes	Yes	Yes	[84]
Psyle A, Psyle C, and Psyle E	*Psychotria leptothyrsa*	U-937	Not tested	Yes	[85]
Vibi G and Vibi H	*Viola biflora*	U-937	Not tested	Yes	[86]
Varv A and Varv F	*Viola arvensis*	10 cancer cell lines	Not tested	Yes	[87]
Viba 15, Viba 17, and Mram 8	*Viola philippica*	HFF1, MM96L, HeLa, BGC-823, and HFF-1	Yes	Yes	[51]
CT-2, CT-4, CT-7, CT-10, CT-12, and CT-19	*Clitoria ternatea*	A549	Not tested	Yes	[88]
Kalata B1 and kalata B2	*Oldenlandia affinis*	U-937 GTB HT-29 Ht116	Yes	Yes	[89]

In general, cyclotides also show anticancer activity against human cancer cells (Table 4); however, two cyclotides from *Viola philippica* (Viphi D and Viphi E) did not show activity against the human gastric cancer BGC-823 cell line [51]. These peptides have similar sequences to the cyclotides Viphi F and Viphi G (Figure 2(c)), indicating that even minimal sequence changes can significantly influence the bioactivity. It has been suggested that the potency and selectivity of cyclotides is dependent on their primary structure. For example, a single glutamic acid plays a key role in the anticancer activity of cycloviolacin O2, and when this residue is methylated, a 48-fold decrease in potency is observed [52].

Cycloviolacin O2 from *Viola odorata* is particular promising because of its selective toxicity to cancer cell lines relative to normal cells, which indicates the possibility of its use as an anticancer agent [53]. Analysis of the proposed mechanism of action of this cyclotide shows that the disruption of cell membranes plays a crucial role in the cytotoxicity of cycloviolacin O2 because the damage to cancer cells (human lymphoma) can be morphologically distinguished within a few minutes, indicating necrosis [54]. However, this activity was not detected when this cyclotide was tested in a mouse tumor model. The reasons of this discrepancy are not fully understood, although high clearance rates or poor distribution to the site of action may be involved. Cycloviolacin O2 was also lethal to mice (2 mg/kg), but no signs of discomfort to the animals were observed at 1.5 mg/kg [55]. Recently the cyclotide MCoTI-I was engineering and the resulting cyclotide MCo-PMI showed activity *in vivo* in a murine xenograft model with prostate cancer cell; treatment (40 mg/kg) significantly suppressed tumor growth [81]. In the same way, HB7 cyclotide from *Hedyotis biflora* in an *in vivo* xenograft model significantly inhibited the tumor weight and size compared to control [82]. These results suggest that cyclotides may have a good anticancer bioactivity.

With respect to the action mechanism of cyclotides, a study showed that cycloviolacin O2 and kalatas B1–B9 target membranes through binding to phospholipids containing phosphatidylethanolamine headgroups [90]. Therefore, the biological potency of these cyclotides may be correlated with their ability to target and disrupt cell membranes. The knowledge of their membrane specificity could be useful to design novel drugs based on the cyclotide framework, allowing the targeting of specific peptide drugs to different cell types.

6. Small Cationic Peptides Isolated from Plants with Anticancer Activity

In addition to plant AMPs, other small linear and cyclic peptides (2–10 aa) with anticancer activity have been reported in plants. For example, the linear peptide *Cn*-AMP1, isolated and purified from coconut water (*Cocos nucifera*), was tested against Caco-2, RAW264.7, MCF-7, HCT-116 cells, and human erythrocytes and showed a reduction of cell viability in cancer cells without causing hemolysis [91]. Other examples are the peptides Cr-ACP, isolated from *Cycas revoluta*, and the acetylated-modified Cr-AcACP1, both repressors of cell proliferation of human epidermoid cancer (Hep2) and colon carcinoma. These peptides induce cell cycle arrest at the G0-G1 phase of Hep2 cells [92]. Moreover, four small cyclic peptides, dianthins C–F, have anticancer activity against Hep G2, Hep 3B, MCF-7, A-549, and MDA-MB-231 cancer cell lines (IC_{50} 20 μg/mL) [93]. Furthermore, the cyclic heptapeptide cherimolacyclopeptide C, obtained from a methanol extract of the seeds of *Annona cherimola*, exhibited significant

in vitro cytotoxicity against KB cells (IC_{50} 0.072 μM) [94]. Other examples of small cyclic peptides are RA-XVII and RA-XVIII from the roots of *Rubia cordifolia* L., which have cytotoxicity against P-388 cells at 0.0030 μg/mL and 0.012 μg/mL, respectively; however, it was not determined whether these peptides are effective against normal cells [95]. Recently, an antiproliferative cyclic octapeptide (cyclosaplin) was purified from *Santalum album* L. The anticancer activity from this peptide was tested against human breast cancer (MDA-MB-231) cells and exhibited significant growth inhibition in a dose and time dependent manner (IC_{50} 2.06 μg/mL). Additionally, cytotoxicity on normal fibroblast cell line at concentrations up to 1000 μg/mL was not detected [56].

7. Conclusion and Future Perspectives

The identification and development of plant AMPs with anticancer properties will provide good opportunities for cancer treatment. AMPs with anticancer activities, including plant-derived peptides, show many therapeutic challenges that must be considered before they can be developed commercially. Strategies to solve their poor stability and susceptibility to proteolytic digestion, such as amino acid substitution, structural fusion of functional peptides, and conjugation with chemotherapeutic drugs, must be evaluated. Despite these limitations, AMPs are an important source of molecules useful for the design of new drugs. In this sense, cationic peptides from plants have great potential as anticancer agents, particularly because of their selectivity towards cancer cells, as has been demonstrated to coccinin and phaseococcin. The number of plant AMPs with anticancer activity is increasing and is expected to rise in the next years, particularly when the remaining plant AMP families are assessed. A crucial step in the studies of plant AMPs as anticancer agents is the identification of their mechanisms of action to discover new targets. Furthermore, the development of novel synthetic analogs of these natural molecules could enhance their activities, facilitating the development of new drugs. With the rapid development in proteomics, bioinformatics, peptide libraries, and modification strategies, these plant AMPs emerge as novel promising anticancer drugs in future clinical applications.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

A grant from CIC-UMSNH to Joel E. López-Meza (CIC14.5) supported this publication. J. J. Guzmán-Rodríguez was supported by a scholarship from CONACyT.

References

[1] J. Ferlay, I. Soerjomataram, M. Ervik et al., *GLOBOCAN 2012: Cancer Incidence And Mortality Worldwide*, vol. 1.0 of *IARC Cancer Base no. 11*, International Agency for Research on Cancer, Lyon, France, 2013, http://globocan.iarc.fr.

[2] H. Zahreddine and K. L. B. Borden, "Mechanisms and insights into drug resistance in cancer," *Frontiers in Pharmacology*, vol. 4, article 28, 2013.

[3] S. Al-Benna, Y. Shai, F. Jacobsen, and L. Steinstraesser, "Oncolytic activities of host defense peptides," *International Journal of Molecular Sciences*, vol. 12, no. 11, pp. 8027–8051, 2011.

[4] E. Guaní-Guerra, T. Santos-Mendoza, S. O. Lugo-Reyes, and L. M. Terán, "Antimicrobial peptides: general overview and clinical implications in human health and disease," *Clinical Immunology*, vol. 135, no. 1, pp. 1–11, 2010.

[5] G. Wang, X. Li, and Z. Wang, "APD2: the updated antimicrobial peptide database and its application in peptide design," *Nucleic Acids Research*, vol. 37, no. 1, pp. D933–D937, 2009.

[6] M.-D. Seo, H.-S. Won, J.-H. Kim, T. Mishig-Ochir, and B.-J. Lee, "Antimicrobial peptides for therapeutic applications: a review," *Molecules*, vol. 17, no. 10, pp. 12276–12286, 2012.

[7] M. Zasloff, "Antimicrobial peptides of multicellular organisms," *Nature*, vol. 415, no. 6870, pp. 389–395, 2002.

[8] R. E. W. Hancock and H.-G. Sahl, "Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies," *Nature Biotechnology*, vol. 24, no. 12, pp. 1551–1557, 2006.

[9] M. Pushpanathan, P. Gunasekaran, and J. Rajendhran, "Antimicrobial peptides: versatile biological properties," *International Journal of Peptides*, vol. 2013, Article ID 675391, 15 pages, 2013.

[10] F. Guilhelmelli, N. Vilela, P. Albuquerque, L. D. S. Derengowski, I. Silva-Pereira, and C. M. Kyaw, "Antibiotic development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance," *Frontiers in Microbiology*, vol. 4, article 353, pp. 1–12, 2013.

[11] J. L. Anaya-López, J. E. López-Meza, and A. Ochoa-Zarzosa, "Bacterial resistance to cationic antimicrobial peptides," *Critical Reviews in Microbiology*, vol. 39, no. 2, pp. 180–195, 2013.

[12] D. Gaspar, A. S. Veiga, and M. A. R. B. Castanho, "From antimicrobial to anticancer peptides. A review," *Frontiers in Microbiology*, vol. 4, article 294, 2013.

[13] K. C. Mulder, L. A. Lima, V. J. Miranda, S. C. Dias, and O. L. Franco, "Current scenario of peptide-based drugs: the key roles of cationic antitumor and antiviral peptides," *Frontiers in Microbiology*, vol. 4, article 321, 23 pages, 2013.

[14] F. Schweizer, "Cationic amphiphilic peptides with cancer-selective toxicity," *European Journal of Pharmacology*, vol. 625, no. 1–3, pp. 190–194, 2009.

[15] D. W. Hoskin and A. Ramamoorthy, "Studies on anticancer activities of antimicrobial peptides," *Biochimica et Biophysica Acta—Biomembranes*, vol. 1778, no. 2, pp. 357–375, 2008.

[16] A. J. Moore, D. A. Devine, and M. C. Bibby, "Preliminary experimental anticancer activity of cecropins," *Peptide Research*, vol. 7, no. 5, pp. 265–269, 1994.

[17] M. A. Baker, W. L. Maloy, M. Zasloff, and L. S. Jacob, "Anticancer efficacy of Magainin2 and analogue peptides," *Cancer Research*, vol. 53, no. 13, pp. 3052–3057, 1993.

[18] K. A. Silverstein, W. A. Moskal Jr., H. C. Wu et al., "Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants," *Plant Journal*, vol. 51, no. 2, pp. 262–280, 2007.

[19] B. P. H. J. Thomma, B. P. A. Cammue, and K. Thevissen, "Plant defensins," *Planta*, vol. 216, no. 2, pp. 193–202, 2002.

[20] F. T. Lay and M. A. Anderson, "Defensins—components of the innate immune system in plants," *Current Protein & Peptide Science*, vol. 6, no. 1, pp. 85–101, 2005.

[21] F. García-Olmedo, P. Rodríguez-Palenzuela, A. Molina et al., "Antibiotic activities of peptides, hydrogen peroxide and peroxynitrite in plant defence," *FEBS Letters*, vol. 498, no. 2-3, pp. 219–222, 2001.

[22] J. P. Marcus, K. C. Goulter, J. L. Green, S. J. Harrison, and J. M. Manners, "Purification, characterisation and cDNA cloning of an antimicrobial peptide from *Macadamia integrifolia*," *European Journal of Biochemistry*, vol. 244, no. 3, pp. 743–749, 1997.

[23] E. de Souza Cândido, M. F. S. Pinto, P. B. Pelegrini et al., "Plant storage proteins with antimicrobial activity: novel insights into plant defense mechanisms," *The FASEB Journal*, vol. 25, no. 10, pp. 3290–3305, 2011.

[24] J. Evans, Y. D. Wang, K. P. Shaw, and L. P. Vernon, "Cellular responses to Pyrularia thionin are mediated by Ca^{2+} influx and phospholipase A_2 activation and are inhibited by thionin tyrosine iodination," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 15, pp. 5849–5853, 1989.

[25] J. L. Kong, X. B. Du, C. X. Fan et al., "Purification and primary structure determination of a novel polypeptide isolated from mistletoe *Viscum coloratum*," *Chinese Chemical Letters*, vol. 15, no. 11, pp. 1311–1314, 2004.

[26] A. Büssing, G. M. Stein, M. Wagner et al., "Accidental cell death and generation of reactive oxygen intermediates in human lymphocytes induced by thionins from *Viscum album* L," *European Journal of Biochemistry*, vol. 262, no. 1, pp. 79–87, 1999.

[27] S. Romagnoli, F. Fogolari, M. Catalano et al., "NMR solution structure of viscotoxin C1 from viscum album species *Coloratum ohwi*: Toward a structure-function analysis of viscotoxins," *Biochemistry*, vol. 42, no. 43, pp. 12503–12510, 2003.

[28] S.-S. Li, J. Gullbo, P. Lindholm et al., "Ligatoxin B, a new cytotoxic protein with a novel helix-turn-helix DNA-binding domain from the mistletoe *Phoradendron liga*," *Biochemical Journal*, vol. 366, no. part 2, pp. 405–413, 2002.

[29] F. Thunberg and G. Samuelsson, "Isolation and properties of ligatoxin A, a toxic protein from the mistletoe *Phoradendron liga*," *Acta Pharmaceutica Suecica*, vol. 19, no. 4, pp. 285–292, 1982.

[30] M. P. Sauviat, J. Berton, and C. Pater, "Effect of phoratoxin B on electrical and mechanical activities of rat papillary muscle," *Acta Pharmacologica Sinica*, vol. 6, no. 2, pp. 91–93, 1985.

[31] S. Johansson, J. Gullbo, P. Lindholm et al., "Small, novel proteins from the mistletoe *Phoradendron tomentosum* exhibit highly selective cytotoxicity to human breast cancer cells," *Cellular and Molecular Life Sciences*, vol. 60, no. 1, pp. 165–175, 2003.

[32] H. Loeza-Ángeles, E. Sagrero-Cisneros, L. Lara-Zárate, E. Villagómez-Gómez, J. E. López-Meza, and A. Ochoa-Zarzosa, "Thionin Thi2.1 from *Arabidopsis thaliana* expressed in endothelial cells shows antibacterial, antifungal and cytotoxic activity," *Biotechnology Letters*, vol. 30, no. 10, pp. 1713–1719, 2008.

[33] P. Hughes, E. Dennis, M. Whitecross, D. Llewellyn, and P. Gage, "The cytotoxic plant protein, β-purothionin, forms ion channels in lipid membranes," *The Journal of Biological Chemistry*, vol. 275, no. 2, pp. 823–827, 2000.

[34] P. Barbosa Pelegrini, R. P. del Sarto, O. N. Silva, O. L. Franco, and M. F. Grossi-De-Sa, "Antibacterial peptides from plants: what they are and how they probably work," *Biochemistry Research International*, vol. 2011, Article ID 250349, 9 pages, 2011.

[35] H. U. Stotz, F. Waller, and K. Wang, "Innate immunity in plants: the role of antimicrobial peptides," in *Antimicrobial Peptides and Innate Immunity*, P. S. Hiemstra and S. A. J. Zaat, Eds., pp. 29–51, Springer Science & Business Media, Broken Arrow, Okla, USA, 2013.

[36] P. H. K. Ngai and T. B. Ng, "A napin-like polypeptide from dwarf Chinese white cabbage seeds with translation-inhibitory, trypsin-inhibitory, and antibacterial activities," *Peptides*, vol. 25, no. 2, pp. 171–176, 2004.

[37] M. Mirouze, J. Sels, O. Richard et al., "A putative novel role for plant defensins: a defensin from the zinc hyper-accumulating plant, *Arabidopsis halleri*, confers zinc tolerance," *The Plant Journal*, vol. 47, no. 3, pp. 329–342, 2006.

[38] M. Koike, T. Okamoto, S. Tsuda, and R. Imai, "A novel plant defensin-like gene of winter wheat is specifically induced during cold acclimation," *Biochemical and Biophysical Research Communications*, vol. 298, no. 1, pp. 46–53, 2002.

[39] A. Allen, A. K. Snyder, M. Preuss, E. E. Nielsen, D. M. Shah, and T. J. Smith, "Plant defensins and virally encoded fungal toxin KP4 inhibit plant root growth," *Planta*, vol. 227, no. 2, pp. 331–339, 2008.

[40] L. Carrasco, D. Vázquez, C. Hernández-Lucas, P. Carbonero, and F. García-Olmedo, "Thionins: plant peptides that modify membrane permeability in cultured mammalian cells," *European Journal of Biochemistry*, vol. 116, no. 1, pp. 185–189, 1981.

[41] J. H. Wong and T. B. Ng, "Limenin, a defensin-like peptide with multiple exploitable activities from shelf beans," *Journal of Peptide Science*, vol. 12, no. 5, pp. 341–346, 2006.

[42] J. L. Kong, X. B. Du, C. X. Fan, J. F. Xu, and X. J. Zheng, "Determination of primary structure of a novel peptide from mistletoe and its antitumor activity," *Acta Pharmaceutica Sinica*, vol. 39, no. 10, Article ID 0513-4870(2004)10-0813-05, pp. 813–817, 2004.

[43] A. Büssing, W. Vervecken, M. Wagner, B. Wagner, U. Pfüller, and M. Schietzel, "Expression of mitochondrial Apo2.7 molecules and caspase-3 activation in human lymphocytes treated with the ribosome-inhibiting mistletoe lectins and the cell membrane permeabilizing viscotoxins," *Cytometry*, vol. 37, no. 2, pp. 133–139, 1999.

[44] J. H. Wong and T. B. Ng, "Sesquin, a potent defensin-like antimicrobial peptide from ground beans with inhibitory activities toward tumor cells and HIV-1 reverse transcriptase," *Peptides*, vol. 26, no. 7, pp. 1120–1126, 2005.

[45] J. H. Wong and T. B. Ng, "Lunatusin, a trypsin-stable antimicrobial peptide from lima beans (*Phaseolus lunatus* L.)," *Peptides*, vol. 26, no. 11, pp. 2086–2092, 2005.

[46] P. Lin, J. H. Wong, and T. B. Ng, "A defensin with highly potent antipathogenic activities from the seeds of purple pole bean," *Bioscience Reports*, vol. 30, no. 2, pp. 101–109, 2010.

[47] P. H. K. Ngai and T. B. Ng, "Coccinin, an antifungal peptide with antiproliferative and HIV-1 reverse transcriptase inhibitory activities from large scarlet runner beans," *Peptides*, vol. 25, no. 12, pp. 2063–2068, 2004.

[48] P. H. K. Ngai and T. B. Ng, "Phaseococcin, an antifungal protein with antiproliferative and anti-HIV-1 reverse transcriptase activities from small scarlet runner beans," *Biochemistry and Cell Biology*, vol. 83, no. 2, pp. 212–220, 2005.

[49] J. L. Anaya-López, J. E. López-Meza, V. M. Baizabal-Aguirre, H. Cano-Camacho, and A. Ochoa-Zarzosa, "Fungicidal and cytotoxic activity of a *Capsicum chinense* defensin expressed by endothelial cells," *Biotechnology Letters*, vol. 28, no. 14, pp. 1101–1108, 2006.

[50] D. C. Ireland, M. L. Colgrave, and D. J. Craik, "A novel suite of cyclotides from *Viola odorata*: sequence variation and the implications for structure, function and stability," *The Biochemical Journal*, vol. 400, no. 1, pp. 1–12, 2006.

[51] W. He, L. Y. Chan, G. Zeng, N. L. Daly, D. J. Craik, and N. Tan, "Isolation and characterization of cytotoxic cyclotides from *Viola philippica*," *Peptides*, vol. 32, no. 8, pp. 1719–1723, 2011.

[52] A. Herrmann, E. Svangård, P. Claeson, J. Gullbo, L. Bohlin, and U. Göransson, "Key role of glutamic acid for the cytotoxic activity of the cyclotide cycloviolacin O2," *Cellular and Molecular Life Sciences*, vol. 63, no. 2, pp. 235–245, 2006.

[53] S. L. Gerlach, R. Rathinakumar, G. Chakravarty et al., "Anticancer and chemosensitizing abilities of cycloviolacin O_2 from *Viola odorata* and psyle cyclotides from *Psychotria leptothyrsa*," *Biopolymers*, vol. 94, no. 5, pp. 617–625, 2010.

[54] E. Svangård, R. Burman, S. Gunasekera, H. Lövborg, J. Gullbo, and U. Göransson, "Mechanism of action of cytotoxic cyclotides: cycloviolacin O2 disrupts lipid membranes," *Journal of Natural Products*, vol. 70, no. 4, pp. 643–647, 2007.

[55] R. Burman, E. Svedlund, J. Felth et al., "Evaluation of toxicity and antitumor activity of cycloviolacin O2 in mice," *Biopolymers*, vol. 94, no. 5, pp. 626–634, 2010.

[56] A. Mishra, S. S. Gauri, S. K. Mukhopadhyay et al., "Identification and structural characterization of a new pro-apoptotic cyclic octapeptide cyclosaplin from somatic seedlings of *Santalum album* L," *Peptides*, vol. 54, pp. 148–158, 2014.

[57] R. Fernandez de Caleya, B. Gonzalez-Pascual, F. García-Olmedo, and P. Carbonero, "Susceptibility of phytopathogenic bacteria to wheat purothionins *in vitro*," *Applied Microbiology*, vol. 23, no. 5, pp. 998–1000, 1972.

[58] D. E. A. Florack and W. J. Stiekema, "Thionins: properties, possible biological roles and mechanisms of action," *Plant Molecular Biology*, vol. 26, no. 1, pp. 25–37, 1994.

[59] H. Bohlmann and K. Apel, "Thionins," *Annual Review of Plant Physiology and Plant Molecular Biology*, vol. 42, no. 1, pp. 227–240, 1991.

[60] M. J. Carmona, C. Hernández-Lucas, C. San Martin, P. González, and F. García-Olmedo, "Subcellular localization of type I thionins in the endosperms of wheat and barley," *Protoplasma*, vol. 173, no. 1-2, pp. 1–7, 1993.

[61] B. Stec, "Plant thionins—the structural perspective," *Cellular and Molecular Life Sciences*, vol. 63, no. 12, pp. 1370–1385, 2006.

[62] A. Coulon, E. Berkane, A.-M. Sautereau, K. Urech, P. Rougé, and A. López, "Modes of membrane interaction of a natural cysteine-rich peptide: viscotoxin A3," *Biochimica et Biophysica Acta*, vol. 1559, no. 2, pp. 145–159, 2002.

[63] Y. Chen, M. T. Guarnieri, A. I. Vasil, M. L. Vasil, C. T. Mant, and R. S. Hodges, "Role of peptide hydrophobicity in the mechanism of action of α-helical antimicrobial peptides," *Antimicrobial Agents and Chemotherapy*, vol. 51, no. 4, pp. 1398–1406, 2007.

[64] A. F. Lacerda, É. A. R. Vasconcelos, P. B. Pelegrini, and M. F. Grossi de Sa, "Antifungal defensins and their role in plant defense," *Frontiers in Microbiology*, vol. 5, no. 116, pp. 1–10, 2014.

[65] F. T. Lay, S. Poon, J. A. McKenna et al., "The C-terminal propeptide of a plant defensin confers cytoprotective and subcellular targeting functions," *BMC Plant Biology*, vol. 14, no. 1, article 41, 2014.

[66] K. Vriens, B. P. A. Cammue, and K. Thevissen, "Antifungal plant defensins: mechanisms of action and production," *Molecules*, vol. 19, no. 8, pp. 12280–12303, 2014.

[67] I. K. H. Poon, A. A. Baxter, F. T. Lay et al., "Phosphoinositide-mediated oligomerization of a defensin induces cell lysis," *eLife*, vol. 3, Article ID e01808, 27 pages, 2014.

[68] J. H. Wong, X. Q. Zhang, H. X. Wang, and T. B. Ng, "A mitogenic defensin from white cloud beans (*Phaseolus vulgaris*)," *Peptides*, vol. 27, no. 9, pp. 2075–2081, 2006.

[69] H. W. Jack and B. N. Tzi, "Vulgarinin, a broad-spectrum antifungal peptide from haricot beans (*Phaseolus vulgaris*)," *International Journal of Biochemistry and Cell Biology*, vol. 37, no. 8, pp. 1626–1632, 2005.

[70] X. Wu, J. Sun, G. Zhang, H. Wang, and T. B. Ng, "An antifungal defensin from *Phaseolus vulgaris* cv. 'Cloud Bean'," *Phytomedicine*, vol. 18, no. 2-3, pp. 104–109, 2011.

[71] D. Z. Ma, H. X. Wang, and T. B. Ng, "A peptide with potent antifungal and antiproliferative activities from Nepalese large red beans," *Peptides*, vol. 30, no. 12, pp. 2089–2094, 2009.

[72] J. H. Wong and T. B. Ng, "Gymnin, a potent defensin-like antifungal peptide from the Yunnan bean (*Gymnocladus chinensis* Baill)," *Peptides*, vol. 24, no. 7, pp. 963–968, 2003.

[73] S. M. Simonsen, L. Sando, K. J. Rosengren et al., "Alanine scanning mutagenesis of the prototypic cyclotide reveals a cluster of residues essential for bioactivity," *The Journal of Biological Chemistry*, vol. 283, no. 15, pp. 9805–9813, 2008.

[74] C. K. Wang, H. Shu-Hong, J. L. Martin et al., "Combined x-ray and NMR analysis of the stability of the cyclotide cystine knot fold that underpins its insecticidal activity and potential use as a drug scaffold," *The Journal of Biological Chemistry*, vol. 284, no. 16, pp. 10672–10683, 2009.

[75] D. J. Craik, N. L. Daly, T. Bond, and C. Waine, "Plant cyclotides: a unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif," *Journal of Molecular Biology*, vol. 294, no. 5, pp. 1327–1336, 1999.

[76] J. L. Dutton, R. F. Renda, C. Waine et al., "Conserved structural and sequence elements implicated in the processing of gene-encoded circular proteine," *The Journal of Biological Chemistry*, vol. 279, no. 45, pp. 46858–46867, 2004.

[77] I. Saska, A. D. Gillon, N. Hatsugai et al., "An asparaginyl endopeptidase mediates in vivo protein backbone cyclization," *The Journal of Biological Chemistry*, vol. 282, no. 40, pp. 29721–29728, 2007.

[78] A. D. Gillon, I. Saska, C. V. Jennings, R. F. Guarino, D. J. Craik, and M. A. Anderson, "Biosynthesis of circular proteins in plants," *Plant Journal*, vol. 53, no. 3, pp. 505–515, 2008.

[79] D. J. Craik, "Host-defense activities of cyclotides," *Toxins*, vol. 4, no. 2, pp. 139–156, 2012.

[80] D. G. Barry, N. L. Daly, R. J. Clark, L. Sando, and D. J. Craik, "Linearization of a naturally occurring circular protein maintains structure but eliminates hemolytic activity," *Biochemistry*, vol. 42, no. 22, pp. 6688–6695, 2003.

[81] Y. Ji, S. Majumder, M. Millard et al., "*In vivo* activation of the p53 tumor suppressor pathway by an engineered cyclotide," *Journal of the American Chemical Society*, vol. 135, no. 31, pp. 11623–11633, 2013.

[82] X. Ding, D. Bai, and J. Qian, "Novel cyclotides from Hedyotis biflora inhibit proliferation and migration of pancreatic cancer cell *in vitro* and *in vivo*," *Medicinal Chemistry Research*, vol. 23, no. 3, pp. 1406–1413, 2014.

[83] M. Y. Yeshak, R. Burman, K. Asres, and U. Göransson, "Cyclotides from an extreme habitat: characterization of cyclic peptides from viola abyssinica of the ethiopian highlands," *Journal of Natural Products*, vol. 74, no. 4, pp. 727–731, 2011.

[84] G. K. T. Nguyen, S. Zhang, N. T. K. Nguyen et al., "Discovery and characterization of novel cyclotides originated from chimeric precursors consisting of albumin-1 chain a and cyclotide domains in the fabaceae family," *The Journal of Biological Chemistry*, vol. 286, no. 27, pp. 24275–24287, 2011.

[85] S. L. Gerlach, R. Burman, L. Bohlin, D. Mondal, and U. Göransson, "Isolation, characterization, and bioactivity of cyclotides from the micronesian plant *Psychotria leptothyrsa*," *Journal of Natural Products*, vol. 73, no. 7, pp. 1207–1213, 2010.

[86] A. Herrmann, R. Burman, J. S. Mylne et al., "The alpine violet, *Viola biflora*, is a rich source of cyclotides with potent cytotoxicity," *Phytochemistry*, vol. 69, no. 4, pp. 939–952, 2008.

[87] P. Lindholm, U. Göransson, S. Johansson et al., "Cyclotides: a novel type of cytotoxic agents," *Molecular Cancer Therapeutics*, vol. 1, no. 6, pp. 365–369, 2002.

[88] S. Zhang, K. Z. Xiao, J. Jin, Y. Zhang, and W. Zhou, "Chemosensitizing activities of cyclotides from *Clitoria ternatea* in paclitaxel-resistant lung cancer cells," *Oncology Letters*, vol. 5, no. 2, pp. 641–644, 2013 (Chinese).

[89] R. Burman, A. A. Strömstedt, M. Malmsten, and U. Göransson, "Cyclotide-membrane interactions: defining factors of membrane binding, depletion and disruption," *Biochimica et Biophysica Acta*, vol. 1808, no. 11, pp. 2665–2673, 2011.

[90] S. T. Henriques, Y.-H. Huang, M. A. R. B. Castanho et al., "Phosphatidylethanolamine binding is a conserved feature of cyclotide-membrane interactions," *The Journal of Biological Chemistry*, vol. 287, no. 40, pp. 33629–33643, 2012.

[91] O. N. Silva, W. F. Porto, L. Migliolo et al., "Cn-AMP1: a new promiscuous peptide with potential for microbial infections treatment," *Biopolymers*, vol. 98, no. 4, pp. 322–331, 2012.

[92] S. M. Mandal, L. Migliolo, S. Das, M. Mandal, O. L. Franco, and T. K. Hazra, "Identification and characterization of a bactericidal and proapoptotic peptide from cycas revoluta seeds with DNA binding properties," *Journal of Cellular Biochemistry*, vol. 113, no. 1, pp. 184–193, 2012.

[93] P. W. Hsieh, F. R. Chang, C. C. Wu et al., "New cytotoxic cyclic peptides and dianthramide from *Dianthus superbus*," *Journal of Natural Products*, vol. 67, no. 9, pp. 1522–1527, 2004.

[94] A. Wélé, Y. Zhang, I. Ndoye, J.-P. Brouard, J.-L. Pousset, and B. Bodo, "A cytotoxic cyclic heptapeptide from the seeds of *Annona cherimola*," *Journal of Natural Products*, vol. 67, no. 9, pp. 1577–1579, 2004.

[95] J.-E. Lee, Y. Hitotsuyanagi, I.-H. Kim, T. Hasuda, and K. Takeya, "A novel bicyclic hexapeptide, RA-XVIII, from *Rubia cordifolia*: structure, semi-synthesis, and cytotoxicity," *Bioorganic and Medicinal Chemistry Letters*, vol. 18, no. 2, pp. 808–811, 2008.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 714580, 9 pages
http://dx.doi.org/10.1155/2015/714580

Research Article

Ascorbic Acid, Ultraviolet C Rays, and Glucose but not Hyperthermia Are Elicitors of Human β-Defensin 1 mRNA in Normal Keratinocytes

Luis Antonio Cruz Díaz,[1] María Guadalupe Flores Miramontes,[1] Paulina Chávez Hurtado,[1] Kirk Allen,[2,3] Marisela Gonzalez Ávila,[4] and Ernesto Prado Montes de Oca[1,2]

[1] *Molecular Biology Laboratory, Biosecurity Area, Pharmaceutical and Medical Biotechnology Unit (UBMF), Research Center in Technology and Design Assistance of Jalisco State (CIATEJ, AC), National Council of Science and Technology (CONACYT), Guadalajara, JAL, Mexico*
[2] *In silico Laboratory, UBMF, CIATEJ AC, CONACYT, Guadalajara, JAL, Mexico*
[3] *Lancaster Medical School, Lancaster University, Lancaster, UK*
[4] *Human Digestive Tract Simulator, UBMF, CIATEJ, AC, CONACYT, Guadalajara, JAL, Mexico*

Correspondence should be addressed to Ernesto Prado Montes de Oca; eprado@ciatej.mx

Received 31 July 2014; Revised 2 October 2014; Accepted 24 October 2014

Academic Editor: Alejandra Ochoa-Zarzosa

Hosts' innate defense systems are upregulated by antimicrobial peptide elicitors (APEs). Our aim was to investigate the effects of hyperthermia, ultraviolet A rays (UVA), and ultraviolet C rays (UVC) as well as glucose and ascorbic acid (AA) on the regulation of human β-defensin 1 (*DEFB1*), cathelicidin (*CAMP*), and interferon-γ (*IFNG*) genes in normal human keratinocytes (NHK). The indirect *in vitro* antimicrobial activity against *Staphylococcus aureus* and *Listeria monocytogenes* of these potential APEs was tested. We found that AA is a more potent APE for *DEFB1* than glucose in NHK. Glucose but not AA is an APE for *CAMP*. Mild hypo- (35°C) and hyperthermia (39°C) are not APEs in NHK. AA-dependent *DEFB1* upregulation below 20 mM predicts *in vitro* antimicrobial activity as well as glucose- and AA-dependent *CAMP* and *IFNG* upregulation. UVC upregulates *CAMP* and *DEFB1* genes but UVA only upregulates the *DEFB1* gene. UVC is a previously unrecognized APE in human cells. Our results suggest that glucose upregulates *CAMP* in an IFN-γ-independent manner. AA is an elicitor of innate immunity that will challenge the current concept of late activation of adaptive immunity of this vitamin. These results could be useful in designing new potential drugs and devices to combat skin infections.

1. Introduction

Keratinocytes control skin microbial colonization/infection in part by synthesizing human β-defensin 1 (hBD-1) and cathelicidin LL-37, both of which are wide-spectrum antimicrobial peptides [1–4]. Antimicrobial peptide elicitors (APEs) are defined as physical (class I), chemical (class II), and biological (class III) agents that promote upregulation of endogenous antimicrobial peptides (APs) [5–8].

IFNG is a relevant gene in innate and adaptive responses; specifically, its product, IFN-γ, is an APE for both *CAMP* (codes for LL-37) and *DEFB1* (codes for hBD-1) in monocytes and gingival keratinocytes, respectively [6]. In this report we wanted to know if keratinocytes upregulate *IFNG* in response to APE, independently of the *IFNG* mRNA provided by lymphocytes in adaptive immunity. Probable APEs with potential application in skin therapy against infections in humans are hyperthermia, ultraviolet A rays (UVA), ultraviolet C rays (UVC), ascorbic acid (AA or vitamin C), and glucose.

In a mice model of influenza infection, hyperthermia, a potential class I APE, is beneficial because it increases leukocyte count and diminishes proinflammatory cytokines, presumably avoiding damage to infected tissue [9]. In humans, hyperthermia is beneficial in treating several diseases such as

neurosyphilis, some forms of chronic arthritis, and cancer. Furthermore, many infections cause fever during certain phases, including fever caused by *Rickettsia* sp., *Chlamydia* sp., viruses, or parasites. When this fever is associated with endogen pyrogen, it leads to the activation of T cells which enhances the host's defense system [10].

UVA (320–400 nm) are APEs of *DEFB1* in scleroderma skin lesions [11] but they have not been tested in normal keratinocytes. UVC (180–280 nm) have been suggested as a prophylactic approach in a mice model of infection against *Pseudomonas aeruginosa* and *Staphylococcus aureus* [12] but the function as an APE of this wavelength has not been described. *S. aureus* is a very common human pathogen susceptible to LL-37 and hBD-1 [6]. In the case of *Listeria* sp., the pathogen is susceptible to LL-37. These APEs have a therapeutic potential against this pathogen in epithelial cells [13, 14]. In this study, we indirectly assume that microbicidal activity is due the increment of the level of synthesis of these peptides by the APEs as suggested by the mRNA levels and inhibition zones of the antimicrobial assays.

AA has been widely used to prevent and treat the common cold, malaria, and diarrhea infections and pneumonia. AA improves the efficacy of antimicrobial and natural killer cell (NK) activities [15]. Whether this antimicrobial activity is dependent on induction and/or upregulation of APs is unknown. Glucose is an APE of *DEFB1* in kidney and colon cells [16] but its effect on both *DEFB1* and *CAMP* in keratinocytes is unknown.

In this study we assessed the capacity of UVA, UVC, AA, glucose, and hyperthermia to act as APEs of *DEFB1* and *CAMP* genes and as inductors of *IFNG* in a cell line of neonate normal human keratinocytes (NHK).

2. Materials and Methods

2.1. Cell Culture and Exposure to Potential APEs. Normal human neonate keratinocytes (ATCC PCS-200-010) were maintained in K-SFM cell culture media (Gibco) at 37°C in a humidified incubator with 5% CO_2. We exposed 1×10^6 cells to D-(+)-glucose (5.5, 15.5, 25.5, and 45.5 mM, Sigma, number of catalog: G8644) or L-ascorbic acid (0, 5, 10, and 20 mM, Sigma, number catalog: A4544) for 24, 48, and 72 h. The incubation temperature was also assessed as potential APE mimicking human mild hypothermia (35°C), normothermia (37°C), and hyperthermia (39°C) *in vitro*. Additionally, for the ascorbic acid at 20 mM, the interaction between time (24, 48, and 72 h) and temperature (35, 37, and 39°C) was assessed. The exposition to UV rays (UVA at 365 nm, UVC at 254 nm) was performed with a square source of irradiation of 4 Watts (UV lamp UVP-UVGL-25, UVP, Cambridge, UK, number of catalog: 95-0021-12) at 9.2 cm from the adhered cells.

2.2. Quantitative Real-Time PCR (qPCR). Nucleic acids were extracted in a MagNA Pure LC 2.0 Instrument (Roche) with the MagNA Pure LC total nucleic acid kit (Roche, catalog number 03038505001). Purified RNA was DNAse-treated and reverse transcribed with a mix containing random primers (Invitrogen) and ArrayScript enzyme (Applied Biosystems). Quantitative real-time PCR was performed with 33 ng cDNA with Fast SYBR Green Master Mix (Applied Biosystems). The reactions were run in a Step One Thermocycler (Applied Biosystems) using the following primers sequences designed with Primer Express v. 3.0 software (Applied Biosystems) and tested for specificity in PRIMER-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/): for *HPRT* (endogenous control suggested for normal human keratinocytes according to Allen et al. [17]), F 5′-TGTTCAAATTATTACCAGTGAATCTTTGTC-3′, R 5′ TTTTAAATTTTTGGGAATTTATTGATTTG-3′; for *IFNG* F 5′-GCTGACTAATTATTCGGTAACTGACTTG-3′, R 5′-TAGCTGCTGGCGACAGTTCA-3′; for *DEFB1* F 5′-GAGAACTTCCTACCTTCTGCTGTTTAC-3′, R 5′-AAAGTTACCACCTGAGGCCATCT-3′; for *CAMP* F 5′-ACCCAGACACGCCAAAGC-3′, R 5′-TTCACCAGCCCGTCCTTCT-3′. The PCR program was 95°C for 20 s, 40 cycles of denaturing at 95°C for 3 s, and annealing/extension at 60°C for 30 s. Melting curves were performed at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Amplification efficiencies for each gene were calculated and the relative quantitative expression was obtained according to the Pfaffl method [18]. The normalization of gene expression included the input RNA and input cell count. The controls were samples with the vehicle at 24 h, or in the case of temperatures the normothermic sample (37°C) for each day.

2.3. Antimicrobial Assays. Diffusion assays were performed according to Kirby et al. [19]. We tested culture supernatants samples with a volume of 50 μL poured into 6 mm diameter wells. The negative control was the supernatant of the culture medium without the potential APE and the positive control was 2.25 μM (1 mg/mL) of tetracycline. Three biological replicas of each were performed with strains of *Listeria monocytogenes* (ATCC 19114, susceptible to LL-37 [13, 14]) and *Staphylococcus aureus* (ATCC 25923, susceptible to LL-37 and hBD-1) [6].

2.4. Cytotoxicity Assays. In the experimental conditions where gene upregulation was observed, experiments were repeated and cytotoxicity assays were performed in increasing concentrations of the APEs. This was assessed by applying 0.05% trypsin by 12 min and neutralizing with DMEM (10% fetal bovine serum). After centrifugation (400 ×g, 5 min), three 10 μL aliquots of homogeneous precipitated cells were quantified in a Neubauer chamber. The percentage of viable cells adhered to the plate was compared with the control without elicitor, considered as being 100% viable. Quantifications of each day were compared with the control of the corresponding days (Cruz Díaz et al., in process).

2.5. Statistical Analysis. Statistical comparisons were done in Microsoft Excel 2010. To evaluate if variances were different or equal among treatments, we performed F tests. Depending of the results of F tests, Student's t-tests for equal or nonequal variances were performed. Results are shown as mean + standard error of the mean (SEM). All experiments were

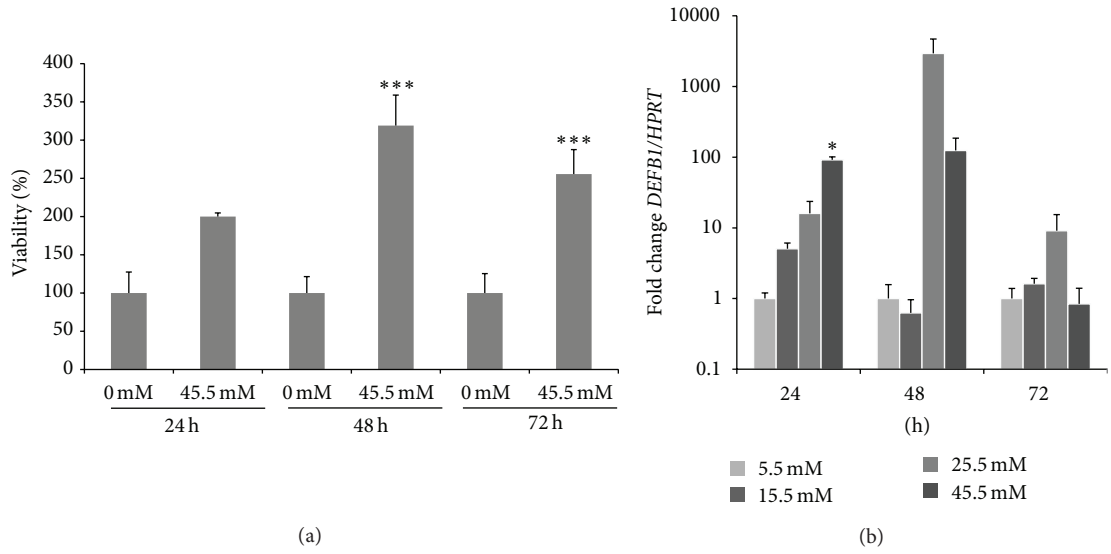


Figure 1: Effect of glucose in normal human keratinocytes. (a) Viability percentage of keratinocytes to glucose exposition by 24 h, 48 h, and 72 h in comparison with their controls for each exposure time. (b) *DEFB1* expression at 24, 48, and 72 h of keratinocytes exposed to glucose. Reference group was 5.5 mM (normal concentration of culture medium) at each time. Error bars represent SEM and significant values of Student's t tests are depicted as $^{*}P < 0.05$; $^{***}P < 0.001$.

performed at least in triplicate, and a P value <0.05 was considered statistically significant.

3. Results

When we exposed the dermal cells to increasing levels of glucose, simulating increased absorption and distribution of glucose after feeding, the viability of keratinocytes increased at 24 h and was found to be highly significant at 48 h and 72 h (Figure 1(a)). At 24 h of glucose exposure (45.5 mM), *DEFB1* expression was significantly upregulated (Figure 1(b)).

The viability of keratinocytes is not affected at 10 mM of ascorbic acid (AA, Figure 2(a)) but begins to be affected at 20 mM and was highly significantly affected at 40 mM (Figure 2(b)). When keratinocytes were exposed to 10 and 20 mM of ascorbic acid, *DEFB1* was upregulated by almost 5 logs after 72 h (Figure 2(c)).

At 72 h rather than at 24 h, glucose also upregulated *IFNG* and *CAMP* genes (Figure 3). AA also upregulated *IFNG* (Figure 3), but AA did not affect *CAMP* expression at any time or concentration (data not shown). *DEFB1* (3 logs), *CAMP* (1 log), and *IFNG* (1 log) were upregulated in both hypothermia and hyperthermia conditions mainly at 72 h, although the results were not significant (data not shown). We then assessed the effect of temperature at 20 mM of AA by 72 h (highest significant upregulation of *DEFB1*, Figure 2(c)). Compared with normothermia, *DEFB1* was upregulated at 24 h at 35°C but downregulated at 72 h at both 35 and 39°C (Figure 2(d)). Interestingly, at hypothermia and 20 mM of AA both *IFNG* and *CAMP* were downregulated at 72 h (Figure 3). Furthermore, the expressions of *DEFB1* and *IFNG* correlate linearly with 20 mM at 35°C ($r^2 = 0.9$).

UVC upregulated *CAMP* at 5 and 10 min of exposure (Figure 4(a)) as well as upregulating *DEFB1* (Figure 4(b)) and *IFNG* genes (Figure 4(c)). Surprisingly, UVA slightly upregulated *DEFB1* at 5 min of exposure and downregulated it at 10 and 20 min of exposure (Figure 4(d)). UVA do not impact *CAMP* or *IFNG* regulation (data not shown).

The supernatant of NHK in the condition 5 mM AA, 24 h, 37°C, that shows upregulation of both *CAMP* (2.56-fold) and *IFNG* (3.31-fold) showed an increased antimicrobial activity against both *L. monocytogenes* and *S. aureus* (Figure 5) in spite the diminished expression of *DEFB1* (0.5-fold). As expected due to diminished viability, both conditions 37°C, 20 mM AA, 72 h, and 35°C, 20 mM AA, 24 h, diminish the activity against *S. aureus* significantly even at the former condition which had the highest *DEFB1* expression (4 logs).

Both samples from 45.5 mM glucose, 24 h and 72 h, showed increased antimicrobial activity against *S. aureus* and *L. monocytogenes* respectively, as expected, presumably because in these samples *DEFB1* (90-fold) and *CAMP* (6.43-fold) genes were upregulated, respectively.

4. Discussion

We demonstrate for the first time that AA is an APE for *DEFB1* but not for *CAMP*. We therefore propose that the indirect antimicrobial effect of ascorbic acid in NHK could be at least partially dependent on *DEFB1* induction which challenges the traditional view regarding AA only as being considered a player in the later activation of the adaptive immunity [20]. This also could explain why AA acts as an antitumor agent [21–23], since *DEFB1* is considered a tumor suppressor gene [24, 25].

Regarding cytotoxicity assay, Trypan Blue Exclusion (TBE) Assay overestimates viable cells [26] and assignment of trypan-stained cells to viable or nonviable categories

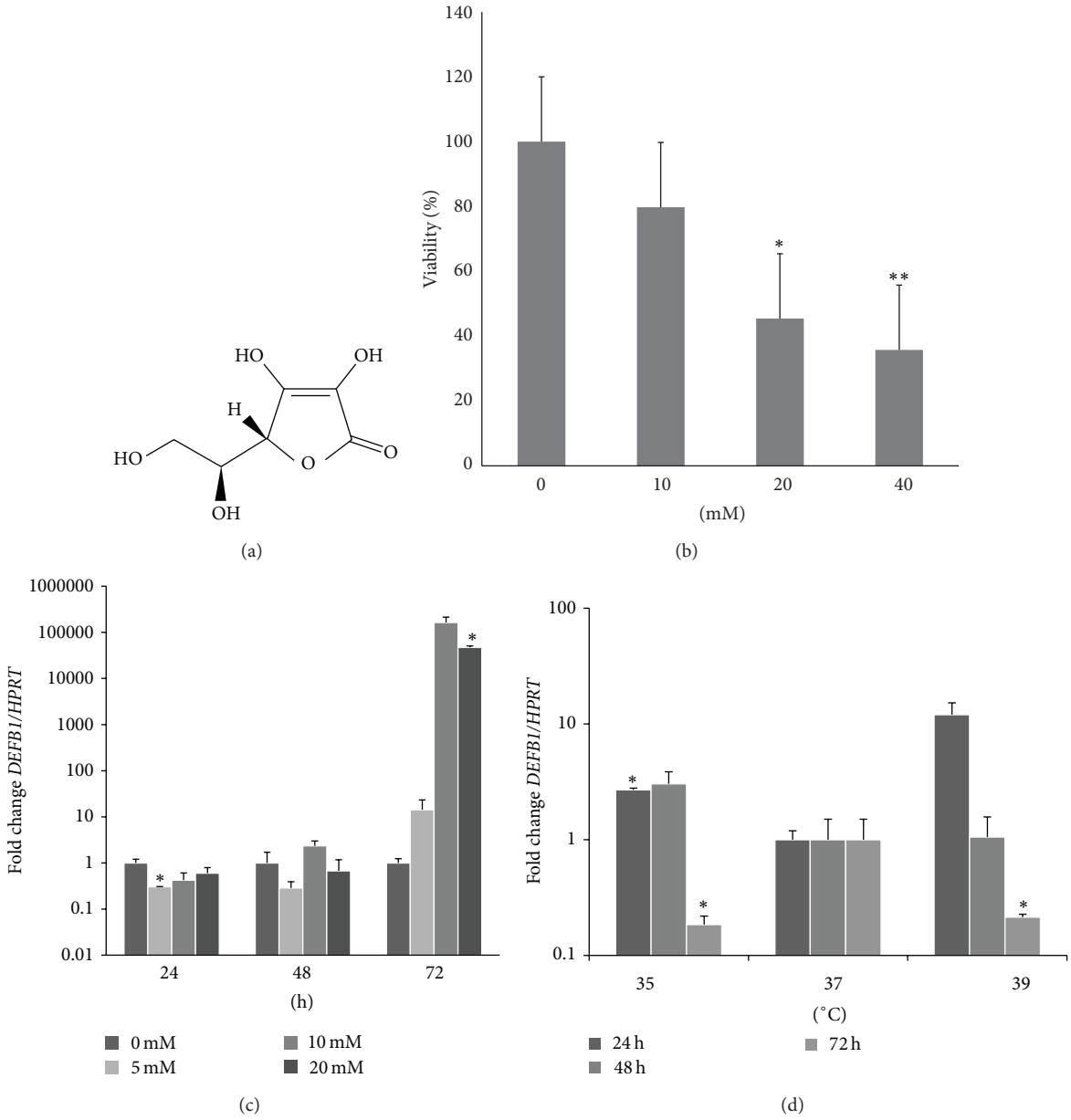


Figure 2: Effect of ascorbic acid alone or in combination with hypothermia or hyperthermia in normal human keratinocytes. (a) Structure of L-ascorbic acid. (b) Cytotoxicity assays exposed 24 h with ascorbic acid. (c) Effect of different concentrations of ascorbic acid on *DEFB1* expression at 24, 48, and 72 h. The reference group was 0 mM at each time. (d) Effect of hypothermia, normothermia, and hyperthermia, all plus interaction with 20 mM ascorbic acid on *DEFB1* expression. Reference group was normothermia (37°C) at each time. Error bars represent SEM and significant values of Student's t tests are depicted as $^{*}P < 0.05$; $^{**}P < 0.01$.

was found to be subjective and arbitrary [27]. Regarding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a lineal relationship exists between reduced tetrazolium dye and cell number only up to 2×10^4 cells/well with no relationship at higher cell numbers and/or absorbance values of greater than 0.8 [27]. Also MTT was designed to test cytotoxic drugs and the recommended set of concentrations must include the highest concentration that kills most of the cells and the lowest concentration that kills none of the cells [28]. The main advantages of the proposed protocol is that in adherent cultures it permits to obtain a number of viable cells that are already attached, because the nonattached cells, if any, are removed by aspirating the supernatant. With our method, there is less ambiguity assigning viability as in TBE and chemical reactions even in the absence of viable cells. This does not affect the results as in MTT (Cruz Díaz et al., in process).

It has been reported that short-term hypothermia (taken as 30–34°C, 2–4 hours,) in cell lines and murine models increases the level of anti-inflammatory cytokines (IL-4

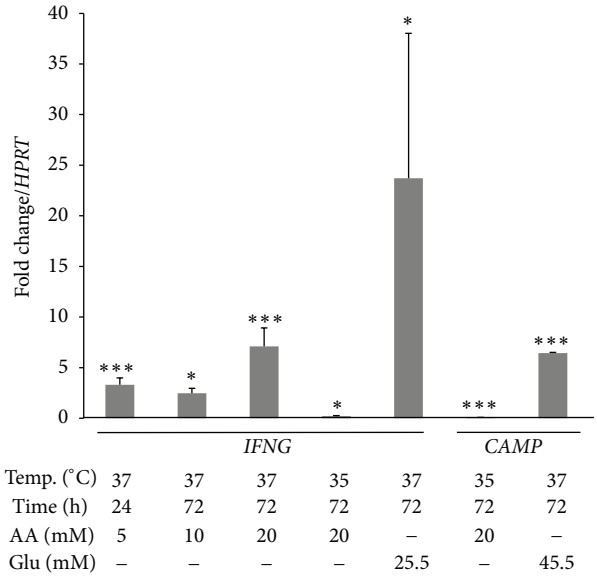


Figure 3: Statistically significant results of the effect of ascorbic acid, hypothermia, and/or glucose exposure in *IFNG* and *CAMP* expression in normal human keratinocytes. Error bars represent SEM and significant values of Student's t tests are depicted as $^{*}P < 0.05$; $^{***}P < 0.001$.

and IL-10) and decreases proinflammatory cytokines (IL-1, IL-2, IL-6, and TNF-α), inhibiting lymphocyte proliferation and decreasing HLA-DR expression associated with cell activation. Long term (>24 hours) hypothermia, however, increases proinflammatory cytokine levels [29]. The hyperthermic conditioning (41°C) has been an effective treatment in mice with sepsis in combination with LL-37. In rats with hypothermia (32°C) prior to sepsis, the IL-10 levels were significantly increased compared to normothermic rats, altering the cytokines profile, survival, and recruitment of granulocytes suggesting immunosuppression [30]. Human hypothermia (32°C) is associated with elevated frequency of infectious complications; dysfunction of the immune response caused by hypothermia has been demonstrated in both clinical and animal studies [31]. Contrary to our hypothesis, hyperthermia does not upregulate the expression of *DEFB1* or *CAMP* in a significant manner; nevertheless the strong tendency to upregulate mainly *DEFB1* (data not shown) deserves further investigation.

On the contrary, we observed that hypothermia improved the potential of AA (20 mM) both acting as APE at 24 h. However, in this combination, both extreme conditions (hypo- and hyperthermia) diminished *DEFB1* expression at 72 h. This could be because in these two scenarios the keratinocytes are exposed to a prolonged oxidative stress probably requiring more AA to counteract the adverse environment. Notably, the upregulation of *DEFB1* with AA does not predict antimicrobial activity *in vitro* probably because in an amount of equal to or higher than 20 mM we found that AA is cytotoxic. Also, high oral doses of AA *in vivo* cause hyperoxaluria, which is the excessive urinary excretion of oxalate and often the formation of kidney stones [10]. Clinical trials must uncover the optimal doses of AA in fever episodes of diverse infections to reach the expected efficacy of AA as APE of *DEFB1*. The role of AA as APE was anticipated because AA reduces the expression of IL-10 in a dose-dependent way [32] and this cytokine inhibits the expression of hBD-1 [33].

According to our results, *IFNG* mRNA levels probably provided the keratinocyte with an innate immunity against skin pathogens, independent of the late lymphocyte-derived IFN-γ (adaptive immunity) in an established infection [34]. Also we found that IFN-γ is upregulated earlier than *DEFB1* suggesting that the former could act as an APE in NHK of foreskin as is true in gingival keratinocytes [35, 36], macrophages [37], and monocytes [38]. The condition where *IFNG* and *CAMP* were upregulated was the only one that predicts increment antimicrobial activity in the two tested Gram-positive pathogens, suggesting that this *IFNG*-dependent response is a stronger APE than *IFNG*-independent response (e.g., TNF-α-mediated) [6]. The antimicrobial *in vitro* prediction of *CAMP* upregulation is in concordance with a recent demonstration that transfecting *CAMP* mRNA increases resistance to *Listeria* sp. in oral epithelial cells [14].

In diabetic and obese rat models, the expression of β-defensin 1 (BD-1) was lower than that of slim rat controls [39] and only β-defensin 2 was found to be increased in diabetic rats, most likely as a result of a proinflammatory response [40]. Glucose has been described as an APE of *DEFB1* in human embryonic kidney HEK-293 [16, 41] and colon adenocarcinoma cells HCT-116 [16] but not for keratinocytes. Our results suggest an impact of intracellular glucose deficiency in susceptibility to skin infections due to *DEFB1* downregulation, for example, ulcers in diabetic patients. We also demonstrate that glucose induces a faster and higher expression of *DEFB1* compared to *CAMP* in normal keratinocytes. The glucose-dependent expression of *DEFB1* and *CAMP* has a biological relevance because they show constitutive skin expression, probably explained by the constant supply of glucose through blood in a non-starved state and/or normal internalization of glucose in a nondiabetic host. Interestingly, Barnea et al. did not find dose-dependent response above 10 mM of glucose in HEK-293 cells [16] as we found in NHK, revealing a possible higher tissue-specific response. Furthermore, some authors consider that *CAMP* is an inducible gene. However in our system of NHK from neonate foreskin it always showed a constitutive expression as previously suggested in squamous epithelia of mouth, tongue, esophagus, vagina, and cervix [42].

UVA makes up 95% of UV light that reaches the earth's surface [43]. UVA inhibits cell proliferation due to the arrest of the S-phase [44] and also acts as an inhibitor of *DEFB1* in scleroderma lesional skin, with no effect in unaffected skin in these patients [11]. On the contrary, we found that in NHK UVA is a modest APE of *DEFB1* but an antimicrobial peptide inhibitor (API) at longer exposures. These suggest that in scleroderma lesions the potential of this APE could be affected by an alteration of *DEFB1* overexpression pathways [7, 45] probably due to a unbalanced cytokine expression profile [6]. The lack of upregulation of *IFNG* in the exposure

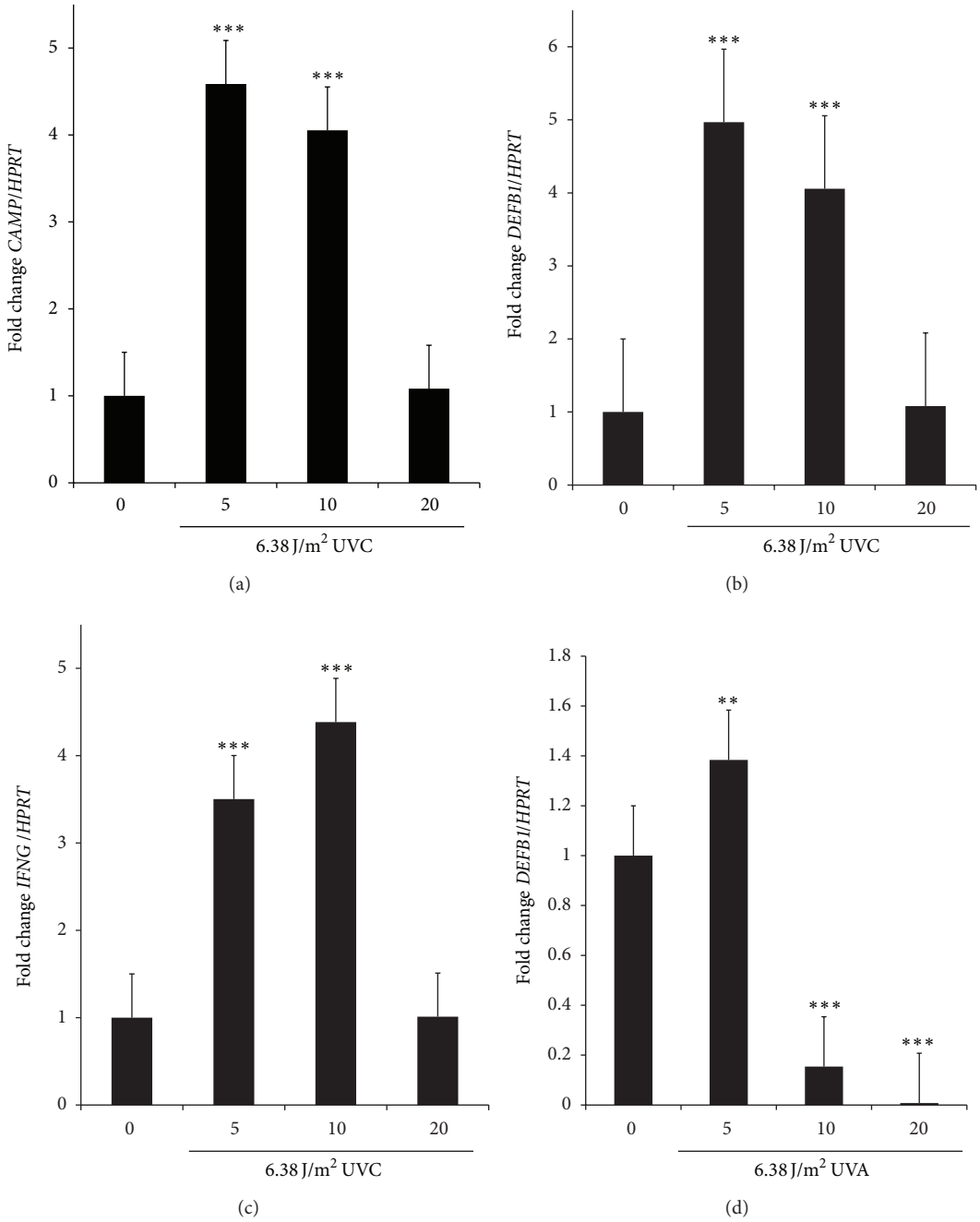


Figure 4: Relative gene expression in keratinocytes exposed to UVC (254 nm) and UVA (365 nm). (a) *CAMP* expression in UVC exposure. (b) *DEFB1* expression in UVC exposure. (c) *IFNG* expression in UVC exposure. (d) *DEFB1* expression in UVA exposure. Exposure is expressed in minutes. In all cases reference group was 0 min (not exposed). Error bars represent SEM and significant values of Student's *t* tests are depicted as $^{**}P < 0.01$; $^{***}P < 0.001$.

to UVA suggests that the route of *DEFB1* expression with this APE is IFN-γ-independent, but this deserves further investigation.

Our results reinforce and expand the notion that UV light (beyond UVA and UVB) stimulates APs gene expression. Furthermore, we were able to induce upregulation of *DEFB1* and *CAMP* genes at 4.25% of energy and 0.347% of exposure time required to upregulate *DEFB4, DEFB103*, and *S100A7* genes [46]. In spite of the fact that in nature most of UVC is usually blocked by the stratospheric ozone layer [43], compact fluorescent light bulbs expose our skin to UVC as well as UVA [44]. UVC causes degradation of IκBα and nuclear entry of p65/RelA thus activating the NF-κB pathway (independent of IκB kinase-IKK-activation) [47]. This nuclear factor is a probable regulator of *DEFB1* expression as suggested by our group [48]. We demonstrate for the first time that UVC in

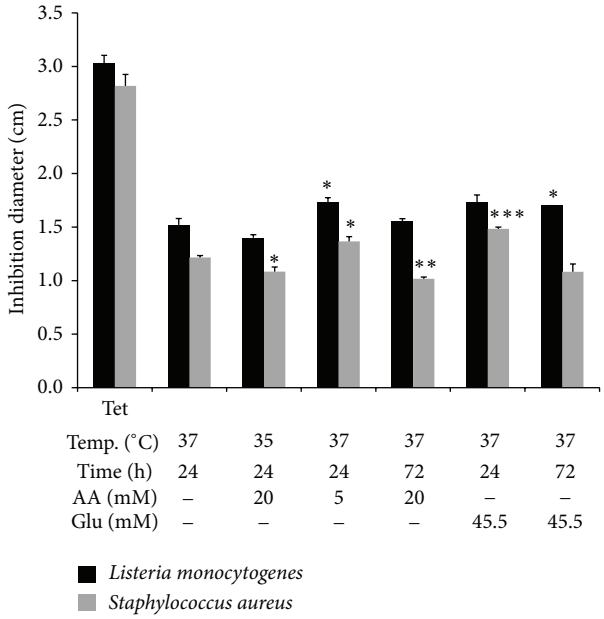


Figure 5: Antimicrobial diffusion assays with the supernatants of APEs-exposed keratinocytes. Tet, tetracycline, was included as a positive control (2.25 μM, 1 mg/mL). The negative control was supernatants of cells without elicitor (37°C, 24 h). Error bars represent SEM and significant values of Student's t tests are depicted as $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

short exposures can be useful as APE of *CAMP* and *DEFB1*, even though it is well known that longer exposure to UVC causes DNA damage [44].

5. Conclusion

Mild hypo- (35°C) and hyperthermia (39°C) are not APEs in NHK *in vitro*, even when hyperthermia shows a tendency to act as an APE with probable biological significance, in spite of its not being statistically significant. Moderate exposure to UVC upregulates *CAMP* and *DEFB1* genes, but UVA only upregulates the *DEFB1* gene in HNK at 5 min of exposure. UVA is an API of *DEFB1* at longer exposures. The later apparently downregulation could be explained because mRNA of *DEFB1* start degrading after 5 min of exposure to UVA. But this requires further investigation. These results suggest alternative expression pathways or different induction thresholds for *DEFB1* and *CAMP*. UVC is a previously unrecognized APE in human cells. These findings uncover the potential of novel therapeutics in skin infectious diseases.

Our results also suggest that glucose upregulates *CAMP* in an IFN-γ-independent manner and thus probably in an inflammation-independent manner. AA is a more potent APE for *DEFB1* than glucose in NHK. Glucose but not AA is an APE for *CAMP*. AA-dependent *DEFB1* upregulation below 20 mM predicts *in vitro* antimicrobial activity as well as glucose- and AA-dependent *CAMP* and *IFNG* upregulation. Levels of serum glucose in diabetic patients could be useful to predict susceptibility to infections due to diminished APs expression, but this requires further clinical studies. AA as APE will challenge the current concept of late activation of adaptive immunity of this vitamin probably acting at the keratinocyte level independent of adaptive immunity. This knowledge could be useful to develop new potential drugs and devices to combat skin infections based on these APEs. Future research in classes I and II APEs holds great promise.

Conflict of Interests

The authors declare that they are pursuing patents for the methods and uses and, when applicable, the elicitors, inhibitors, and derivatives or their combination generated in the name of CIATEJ AC, with E. Prado Montes de Oca et al., as inventors. The primers sequences also will be protected and should be used only for academic purposes.

Acknowledgments

This work was supported by Fondos Sectoriales de Ciencia Básica SEP grant CB-2008-01-105813 from the National Council of Science and Technology (CONACYT, Mexico). Ernesto Prado Montes de Oca and Marisela Gonzalez Ávila are Fellows of the Sistema Nacional de Investigadores (SNI, CONACYT). Thanks are due to Dr. Jim Rheinwald (Harvard Medical School) for provision of his lab's protocols to cultivate keratinocytes.

References

[1] J.-M. Schröder, "The role of keratinocytes in defense against infection," *Current Opinion in Infectious Diseases*, vol. 23, no. 2, pp. 106–110, 2010.

[2] M. H. Braff, M. Zaiou, J. Fierer, V. Nizet, and R. L. Gallo, "Keratinocyte production of cathelicidin provides direct activity against bacterial skin pathogens," *Infection and Immunity*, vol. 73, no. 10, pp. 6771–6781, 2005.

[3] M. H. Braff, A. Di Nardo, and R. L. Gallo, "Keratinocytes store the antimicrobial peptide cathelicidin in lamellar bodies," *Journal of Investigative Dermatology*, vol. 124, no. 2, pp. 394–400, 2005.

[4] J. M. Schröder and J. Harder, "Antimicrobial skin peptides and proteins," *Cellular and Molecular Life Sciences*, vol. 63, no. 4, pp. 469–486, 2006.

[5] E. Prado Montes de Oca, "The potential of antimicrobial peptide inhibitors (APIs) and elicitors (APEs) in leprosy treatment," in *Leprosy: Epidemiology, Treatment Strategies and Current Challenges in Research*, R. S. Koop, Ed., Nova Publishers, Hauppage, NY, USA, 2014.

[6] E. Prado Montes de Oca, "Antimicrobial peptide elicitors: new hope for the post-antibiotic era," *Innate Immunity*, vol. 19, no. 3, pp. 227–241, 2013.

[7] E. Prado Montes de Oca, "Human β-defensin 1: a restless warrior against allergies, infections and cancer," *The International Journal of Biochemistry & Cell Biology*, vol. 42, no. 6, pp. 800–804, 2010.

[8] E. Prado-Montes de Oca, "Antimicrobial peptide elicitors: a potential strategy against infections," *Gaceta Medica de Mexico*, vol. 145, pp. 241–243, 2009.

[9] K. A. Jhaveri, R. A. Trammell, and L. A. Toth, "Effect of environmental temperature on sleep, locomotor activity, core

body temperature and immune responses of C57BL/6J mice," *Brain, Behavior, and Immunity*, vol. 21, no. 7, pp. 975–987, 2007.

[10] R. G. Petersdorf, R. D. Adams, E. Braunwald, K. J. Isselbacher, J. B. Martin, and J. D. Wilson, Eds., *Principles of Internal Medicine*, Mc Graw Hill, New York, NY, USA, 1983.

[11] A. Kreuter, J. Hyun, M. Skrygan et al., "Ultraviolet A1-induced downregulation of human β-defensins and interleukin-6 and interleukin-8 correlates with clinical improvement in localized scleroderma," *The British Journal of Dermatology*, vol. 155, no. 3, pp. 600–607, 2006.

[12] T. Dai, B. Garcia, C. K. Murray, M. S. Vrahas, and M. R. Hamblin, "UVC light prophylaxis for cutaneous wound infections in mice," *Antimicrobial Agents and Chemotherapy*, vol. 56, no. 7, pp. 3841–3848, 2012.

[13] J. Turner, Y. Cho, N.-N. Dinh, A. J. Waring, and R. I. Lehrer, "Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils," *Antimicrobial Agents and Chemotherapy*, vol. 42, no. 9, pp. 2206–2214, 1998.

[14] X. Zou, B. S. Sorenson, K. F. Ross, and M. C. Herzberg, "Augmentation of epithelial resistance to invading bacteria by using mRNA transfections," *Infection and Immunity*, vol. 81, no. 11, pp. 3975–3983, 2013.

[15] E. S. Wintergerst, S. Maggini, and D. H. Hornig, "Immune-enhancing role of Vitamin C and zinc and effect on clinical conditions," *Annals of Nutrition and Metabolism*, vol. 50, no. 2, pp. 85–94, 2006.

[16] M. Barnea, Z. Madar, and O. Froy, "Glucose and insulin are needed for optimal defensin expression in human cell lines," *Biochemical and Biophysical Research Communications*, vol. 367, no. 2, pp. 452–456, 2008.

[17] D. Allen, E. Winters, P. F. Kenna, P. Humphries, and G. J. Farrar, "Reference gene selection for real-time rtPCR in human epidermal keratinocytes," *Journal of Dermatological Science*, vol. 49, no. 3, pp. 217–225, 2008.

[18] M. W. Pfaffl, "A new mathematical model for relative quantification in real-time RT-PCR," *Nucleic Acids Research*, vol. 29, no. 9, article e45, 2001.

[19] W. M. Kirby, D. M. Perry, and A. W. Bauer, "Treatment of staphylococcal septicemia with vancomycin: report of thirty-three cases," *The New England Journal of Medicine*, vol. 262, pp. 49–55, 1960.

[20] Y. B. Shaik-Dasthagirisaheb, G. Varvara, G. Murmura et al., "Role of vitamins D, e and C in immunity and inflammation," *Journal of Biological Regulators and Homeostatic Agents*, vol. 27, no. 2, pp. 291–295, 2013.

[21] H. Kawada, M. Kaneko, M. Sawanobori et al., "High concentrations of L-ascorbic acid specifically inhibit the growth of human leukemic cells via downregulation of HIF-1alpha transcription," *PLoS ONE*, vol. 8, no. 4, Article ID e62717, 2013.

[22] J. Kim, S.-D. Lee, B. Chang et al., "Enhanced antitumor activity of vitamin C via p53 in cancer cells," *Free Radical Biology & Medicine*, vol. 53, no. 8, pp. 1607–1615, 2012.

[23] R. Asada, K. Kageyama, H. Tanaka, M. Kimura, Y. Saitoh, and N. Miwa, "Carcinostatic effects of diverse ascorbate derivatives in comparison with aliphatic chain moiety structures: promotion by combined hyperthermia and reduced cytotoxicity to normal cells," *Oncology Letters*, vol. 3, no. 5, pp. 1042–1046, 2012.

[24] C. D. Donald, C. Q. Sun, S. D. Lim et al., "Cancer-specific loss of β-defensin 1 in renal and prostatic carcinomas," *Laboratory Investigation*, vol. 83, no. 4, pp. 501–505, 2003.

[25] C. Q. Sun, R. Arnold, C. Fernandez-Golarz et al., "Human β-defensin-1, a potential chromosome 8p tumor suppressor: control of transcription and induction of apoptosis in renal cell carcinoma," *Cancer Research*, vol. 66, no. 17, pp. 8542–8549, 2006.

[26] S. A. Altman, L. Renders, and G. Rao, "Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations," *Biotechnology Progress*, vol. 9, no. 6, pp. 671–674, 1993.

[27] J. A. Plumb, R. Milroy, and S. B. Kaye, "Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay," *Cancer Research*, vol. 49, no. 16, pp. 4435–4440, 1989.

[28] R. I. Freshney, *Culture of Animal Cells: A Manual of Basic Technique*, John Wiley & Sons, Hoboken, NJ, USA, 5th edition, 2005.

[29] V. Pongor, G. Toldi, M. Szabó, and B. Vásárhelyi, "Systemic and immunomodulatory effects of whole body therapeutic hypothermia," *Orvosi Hetilap*, vol. 152, no. 15, pp. 575–580, 2011.

[30] A. Torossian, E. Gurschi, R. Bals, T. Vassiliou, H. F. Wulf, and A. Bauhofer, "Effects of the antimicrobial peptide LL-37 and hyperthermic preconditioning in septic rats," *Anesthesiology*, vol. 107, no. 3, pp. 437–441, 2007.

[31] S. Russwurm, I. Stonans, K. Schwerter, E. Stonane, W. Meissner, and K. Reinhart, "Direct influence of mild hypothermia on cytokine expression and release in cultures of human peripheral blood mononuclear cells," *Journal of Interferon & Cytokine Research*, vol. 22, no. 2, pp. 215–221, 2002.

[32] M. Bergman, H. Salman, M. Djaldetti, L. Fish, I. Punsky, and H. Bessler, "In vitro immune response of human peripheral blood cells to vitamins C and E," *The Journal of Nutritional Biochemistry*, vol. 15, no. 1, pp. 45–50, 2004.

[33] T. Gambichler, M. Skrygan, N. S. Tomi et al., "Differential mRNA expression of antimicrobial peptides and proteins in atopic dermatitis as compared to psoriasis vulgaris and healthy skin," *International Archives of Allergy and Immunology*, vol. 147, no. 1, pp. 17–24, 2008.

[34] K. Schroder, P. J. Hertzog, T. Ravasi, and D. A. Hume, "Interferon-γ: an overview of signals, mechanisms and functions," *Journal of Leukocyte Biology*, vol. 75, no. 2, pp. 163–189, 2004.

[35] S. Joly, C. C. Organ, G. K. Johnson, P. B. McCray Jr., and J. M. Guthmiller, "Correlation between β-defensin expression and induction profiles in gingival keratinocytes," *Molecular Immunology*, vol. 42, no. 9, pp. 1073–1084, 2005.

[36] A. A. Kalus, L. P. Fredericks, B. M. Hacker et al., "Association of a genetic polymorphism (-44 C/G SNP) in the human DEFB1 gene with expression and inducibility of multiple -defensins in gingival keratinocytes," *BMC Oral Health*, vol. 9, article 21, 2009.

[37] M. Fabri, S. Stenger, D.-M. Shin et al., "Vitamin D is required for IFN-gamma-mediated antimicrobial activity of human macrophages," *Science Translational Medicine*, vol. 3, no. 104, Article ID 104ra102, 2011.

[38] G. M. Klug-Micu, S. Stenger, A. Sommer, and eatl, "CD40 ligand and interferon-γ induce an antimicrobial response against *Mycobacterium tuberculosis* in human monocytes," *Immunology*, vol. 139, no. 1, pp. 121–128, 2013.

[39] T. Hiratsuka, M. Nakazato, Y. Date, H. Mukae, and S. Matsukura, "Nucleotide sequence and expression of rat β-defensin-1: its significance in diabetic rodent models," *Nephron*, vol. 88, no. 1, pp. 65–70, 2001.

[40] O. Froy, A. Hananel, N. Chapnik, and Z. Madar, "Differential effect of insulin treatment on decreased levels of beta-defensins and Toll-like receptors in diabetic rats," *Molecular Immunology*, vol. 44, no. 5, pp. 796–802, 2007.

[41] A. N. Malik and G. Al-Kafaji, "Glucose regulation of β-defensin-1 mRNA in human renal cells," *Biochemical and Biophysical Research Communications*, vol. 353, no. 2, pp. 318–323, 2007.

[42] M. F. Nilsson, B. Sandstedt, O. Sørensen, G. Weber, N. Borregaard, and M. Ståhle-Bäckdahl, "The human cationic antimicrobial protein (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6," *Infection and Immunity*, vol. 67, no. 5, pp. 2561–2566, 1999.

[43] V. Muthusamy and T. J. Piva, "The UV response of the skin: a review of the MAPK, NFκB and TNFα signal transduction pathways," *Archives of Dermatological Research*, vol. 302, no. 1, pp. 5–17, 2010.

[44] T. Mironava, M. Hadjiargyrou, M. Simon, and M. H. Rafailovich, "The effects of UV emission from compact fluorescent light exposure on human dermal fibroblasts and keratinocytes in vitro," *Photochemistry and Photobiology*, vol. 88, no. 6, pp. 1497–1506, 2012.

[45] E. Prado Montes de Oca, "DEFB1 (defensin, beta 1)," in *Atlas of Genetics and Cytogenetics in Oncology and Haematology 15*, 2011.

[46] R. Gläser, F. Navid, W. Schuller et al., "UV-B radiation induces the expression of antimicrobial peptides in human keratinocytes in vitro and in vivo," *The Journal of Allergy and Clinical Immunology*, vol. 123, no. 5, pp. 1117–1123, 2009.

[47] N. Li and M. Karin, "Ionizing radiation and short wavelength UV activate NF-κB through two distinct mechanisms," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 22, pp. 13012–13017, 1998.

[48] E. Prado-Montes de Oca, J. S. Velarde-Felix, J. J. Rios-Tostado, V. J. Picos-Cardenas, and L. E. Figuera, "SNP 668C (-44) alters a NF-kappaB1 putative binding site in non-coding strand of human beta-defensin 1 (DEFB1) and is associated with lepromatous leprosy, Infection, genetics and evolution," *Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases*, vol. 9, pp. 617–625, 2009.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 430436, 8 pages
http://dx.doi.org/10.1155/2015/430436

Research Article

Effect of Recombinant Prophenin 2 on the Integrity and Viability of *Trichomonas vaginalis*

J. L. Hernandez-Flores,[1] M. C. Rodriguez,[2] A. Gastelum Arellanez,[3] A. Alvarez-Morales,[1] and E. E. Avila[2]

[1] *Unidad Irapuato, Departamento de Ingeniería Genética, Centro de Investigación y de Estudios Avanzados del IPN, P.O. Box 629, 36500 Irapuato, GTO, Mexico*
[2] *Division de Ciencias Naturales y Exactas, Departamento de Biologia, Universidad de Guanajuato, Col. Noria Alta, 36040 Guanajuato, GTO, Mexico*
[3] *Universidad Politécnica del Mar y la Sierra, Carretera a La Cruz km 15.5, Col. Arroyitos, La Cruz, 82700 Elota, SIN, Mexico*

Correspondence should be addressed to E. E. Avila; edilia@ugto.mx

Received 31 July 2014; Accepted 26 September 2014

Academic Editor: J. Eleazar Barboza-Corona

Trichomonas vaginalis is the causal agent of trichomoniasis, which is associated with preterm child delivery, low birth weight, and an increased risk of infection by human papilloma virus and human immunodeficiency virus following exposure. Several reports have established increasing numbers of trichomoniasis cases resistant to metronidazole, the agent used for treatment, and it is therefore important to identify new therapeutic alternatives. Previously, our group reported the effect of tritrpticin, a synthetic peptide derived from porcine prophenin, on *T. vaginalis*; however, the hemolytic activity of this small peptide complicates its possible use as a therapeutic agent. In this study, we report that the propeptide and the processed peptide of prophenin 2 (cleaved with hydroxylamine) affected the integrity and growth of *T. vaginalis* and that pro-prophenin 2 displays some resistance to proteolysis by *T. vaginalis* proteinases at 1 h. Its effect on *T. vaginalis* as well as its low hemolytic activity and short-time stability to parasite proteinases makes prophenin 2 an interesting candidate for synergistic or alternative treatment against *T. vaginalis*.

1. Introduction

Antimicrobial peptides (AMPs) are natural antibiotics synthesized by all known organisms, from bacteria to vertebrates; they have antimicrobial and immunoregulatory functions. AMPs are active against several infectious agents, including viruses, bacteria, fungi, and parasites [1–3]. Most studies have examined the effects of AMPs on bacteria, and fewer reports exist regarding their effects on protozoa [3–8]. In mammals, defensins and cathelicidins are AMPs that are widely expressed in phagocytic immune cells that migrate to infection sites [9].

Pigs have the most diverse collection of cathelicidins of any mammalian species, among which are the proline-phenylalanine-rich prophenin-1 (PF-1), prophenin-2 (PF-2), proline-arginine-rich PR-39, and cysteine-rich protegrins 1 to 5 (PG-1 to PG-5), which have been purified from neutrophils [10].

The protozoan flagellate *Trichomonas vaginalis* is the causal agent of trichomoniasis, the most common nonviral sexually transmitted disease. Trichomoniasis is associated with membrane rupture in pregnant women and, therefore, preterm child delivery, low birth weight, and augmented risk of infection with human papilloma virus or human immunodeficiency virus type 1 [11–14] following exposure. Trichomoniasis is treated with metronidazole or tinidazole, but several reports describe increasing numbers of cases resistant to these compounds [15–19]. AMPs are potential alternatives for trichomoniasis treatment, but *T. vaginalis* trophozoites are rich in cysteine proteinases that might

degrade the peptides; some of these enzymes are surface associated, excreted/secreted, and involved in parasite adherence and cytopathogenicity [20–22].

We reported an interesting trichomonacidal effect of tritrpticin, a small peptide derived from the porcine prophenin 2 [23], but the complete prophenin 2 peptide has not been tested for activity against *T. vaginalis* or other protozoan pathogens. In addition, prophenin 2 is interesting because it should theoretically be resistant to the attack by *T. vaginalis* proteinases due to its high proline content, which may prevent cleavage of the peptide bonds [10, 24]. In this study, we demonstrated that pro-prophenin 2 is partially resistant to proteolysis by *T. vaginalis* proteinases at 1 h and that the complete propeptide and the processed prophenin 2 peptide diminished the integrity and growth of *T. vaginalis*.

2. Materials and Methods

2.1. Strains and Plasmids. Plasmids were propagated and maintained using *E. coli* DH5α and purified using a Plasmid Midi Kit 100 (Qiagen, Cat. 12145). For protein expression, *E. coli* BL21 STAR was used. Both strains were grown at 37°C in Luria-Bertani medium supplemented with 100 mg/L carbenicillin (LB/Cb) when necessary. The plasmid pQE-TriSystem (Qiagen, Cat. 33903) was used to express pro-prophenin 2.

T. vaginalis strain RFC-1 (ATCC) was grown in 8 mL screw-cap tubes with 5 mL of TYI-S-33 medium, pH 7.0 [25], supplemented with 6% bovine serum. *T. vaginalis* was cultured for 72 h at 36.5°C to maintain trophozoites and for 24 h before assays.

2.2. Prophenin 2 Propeptide Cloning and Expression. The nucleotide sequence of the open reading frame corresponding to porcine pro-prophenin 2 [26] was optimized for *E. coli* codon usage. The optimized open reading frame was fused to an 8-amino acid FLAG tag [27] (Figure 1) followed by Western blot analysis and purification of the recombinant protein by affinity chromatography. The optimized pro-prophenin 2::FLAG tag was synthesized (Epoch Biolabs), cloned into the SmaI-EcoRI sites of the pQE-TriSystem expression vector and transformed into *E. coli* DH5α. Putative recombinant clones were confirmed by restriction analysis and sequencing. Confirmed clones were propagated using *E. coli* DH5α, and plasmid DNA was isolated; one clone, pUG2, was selected. To express pro-prophenin 2, *E. coli* BL21 STAR was transformed with the pUG2 construct and grown in LB/Cb. Overnight cultures were used to inoculate fresh LB/Cb medium supplemented with 0.4% glucose and incubated at 37°C until the OD600 reached 0.5. IPTG was then added to a final concentration of 0.25 mM, and the cells were further incubated for 4 h at 37°C. Cultures were centrifuged at 5,000 ×g for 20 minutes at 4°C. Cell pellets were washed twice with buffer A (100 mM Tris pH 7.5, 150 mM NaCl) and were stored at −80°C until used.

2.3. Pro-Prophenin 2 Purification. Bacterial pellets were thawed and kept on ice, then 1 mg/mL lysozyme and 25 μg/mL DNase I were added and incubated 30 minutes at 37°C and disrupted with a VirTis sonicator (model VirSonic 60) using 6 pulses of 30 seconds each with 30-second intervals and a 5-watt output. The soluble phase was recovered by centrifugation at 14,500 ×g for 20 minutes and further clarified by filtering through a 0.2 μm syringe filter (Nalgene, Cat. 190-9920). A total of 300 μL of anti-Flag M2 affinity gel (Sigma, Cat. A2220) was applied to the supernatant and incubated overnight at 4°C with gentle agitation. Anti-Flag M2 affinity gel was recovered and washed 3 times with 2 mL of TBS (50 mM Tris-HCl, 150 mM NaCl pH 7.5)/0.02% NaN_3, and pro-prophenin 2 was eluted with three 6 mL aliquots of 0.1 M glycine-HCl, pH 3.5, into Eppendorf tubes containing 20 μL of 1 M Tris-HCl, pH 8.0. Anti-Flag M2 affinity gel was reequilibrated to neutral pH using 30 volumes of TBS. Pro-prophenin 2 was dialyzed using 14 kDa cutoff dialysis tubing (Spectra/Por, Cat. 132678) and concentrated using a Concentrator plus (Eppendorf, Cat. 5305 000.304).

2.4. Hydroxylamine Cleavage of Pro-Prophenin 2. To release the processed peptide, the purified pro-prophenin 2 was incubated 4 h at 45°C in a hydroxylamine reaction mixture (0.22 M Tris, 1.7 M hydroxylamine hydrochloride, 4 M guanidine hydrochloride, and pH 9). After incubation, the reaction mixture was incubated in an ice bath for 20 minutes and desalted using an Excellulose GF-5 Desalting Column (Pierce, Cat. 1851850); 10 fractions of 1 mL each were collected and concentrated using a Concentrator plus (Eppendorf, Cat. 5305 000.304). Because hydroxylamine cleavage is incomplete, uncleaved propeptide and cathelin were removed by affinity chromatography, using anti-Flag M2 affinity gel as described for pro-prophenin 2 purification.

2.5. Hemolytic Assay. To determine the hemolytic activity of the peptides, fresh human red blood cells were used; peripheral blood was collected from healthy volunteer donors into Vacutainer tubes containing heparin (Cat. 366480) or EDTA (Cat. 366450). Erythrocytes were washed three times with phosphate-buffered saline (PBS, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 150 mM NaCl, and pH 7.0) and resuspended in PBS at the originally collected blood volume (erythrocyte concentration of 100%). The assay was performed in sterile 96-well microtiter plates. The final reaction volume was 200 μL containing 1% erythrocytes and different concentrations of pro-prophenin 2, processed peptide, and amidated tritrpticin. Plates were incubated for 1 h at 37°C and centrifuged at 1000 ×g for 5 min. Released hemoglobin was determined by measuring absorbance at 540 nm in the supernatant. Erythrocyte integrity was calculated according to the hemoglobin released from a 100% hemolysis control (red cells lysed with 0.1% Triton X-100) and a negative control (red cells incubated with PBS), using the following formula: % hemolysis = $[(\lambda_{540}$ in peptide solution − λ_{540} PBS)/(λ_{540} 0.1% Triton X-100 − λ_{540} PBS)] × 100.

2.6. Peptide-Trichomonas Interactions. To determine the effect of the propeptide and processed prophenin 2 on the integrity and growth of *T. vaginalis*, peptide-trichomonas

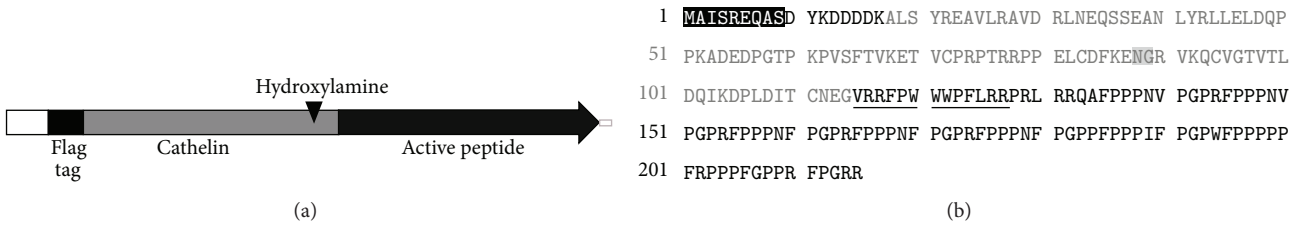


Figure 1: Amino acid sequence of pro-prophenin 2. (a) Graphical representation of the DNA fragment cloned into the pQE-TriSystem. (b) Amino acid sequence. Amino acids derived from the vector (white), Flag tag (black), cathelin (light gray), and processed peptide (dark gray). In (b) the hydroxylamine cleavage site (shaded) and the tritrpticin sequence (underlined) are shown.

assays were performed, as previously described [23]. Briefly, *T. vaginalis* RFC-1 was grown for 24 h in screw-cap tubes containing 5 mL of TYI-S-33. Cell were collected by centrifugation at 1000 ×g for 5 min and washed with 5 mL of TYI. The *T. vaginalis*-peptide interaction took place in 96-well sterile microtiter plates (Corning Inc., USA) containing 40,000 trophozoites and different concentrations of peptide in a final volume of 100 μL. The medium for the 4 h interaction assay was TYI supplemented with 25 mM sodium bicarbonate and 100 mM HEPES, pH 7.0. Complete TYI-S-33 culture medium was used for the 24 h interaction assays. The cultures were incubated at 36.5°C in a microaerophilic atmosphere. After incubation, the trophozoite number was calculated using a Neubauer chamber and compared with a control group that did not interact with the peptides. The effect of the different peptide concentrations on *T. vaginalis* was evaluated by ANOVA and a mean comparison using the least significant difference (LSD) test ($\alpha = 0.05$). Using the data obtained, we estimated the LD_{50} (lethal dosage 50) for the 24 h interaction, adjusting the model as follows: PERCENT INHIBITION/MORTALITY = A ∗ log (DOSAGE) + B, using the lm function in the R environment.

2.7. Stability of Pro-Prophenin 2 to T. vaginalis Proteinases. The stability of pro-prophenin 2 was determined at 1, 2, and 4 h of interaction with *T. vaginalis* in TYI supplemented with 25 mM sodium bicarbonate and 100 mM HEPES, pH 7.0, as described above. A 5 μM final concentration of pro-prophenin 2 was added to 40,000 *T. vaginalis* trophozoites in a volume of 100 μL; after incubation at 37°C, samples were analyzed by Western blot using an anti-Flag antibody. A similar set of samples was incubated in the presence of the cysteine proteinase inhibitor E64.

3. Results

3.1. Pro-Prophenin 2 Expression and Purification. To express pro-prophenin 2, a synthetic gene was obtained and cloned into the pQE-TriSystem vector. We verified the correct in-phase insertion of the pro-prophenin 2 open reading frame into the expression vector by restriction analysis and sequencing (LANGEBIO, Irapuato, Mexico). Transformed *E. coli* induced with 1 mM IPTG expressed a protein with the expected molecular weight of the FLAG pro-prophenin 2 fusion protein; however, all of the recombinant protein was found in the pellet. Soluble pro-prophenin 2 was obtained by inducing the cultures with 0.25 mM IPTG, followed by 4 h incubation at 37°C after induction (Figure 2(a), lane 1).

FLAG pro-prophenin 2 was purified by affinity chromatography with an anti-FLAG M2 antibody using a wash buffer containing 300 mM NaCl and 3% Tween 20 and exhaustively washing the column to avoid copurification of contaminant proteins (Figure 2(b)). Pro-prophenin 2 yield was 325 μg per liter of culture on average.

To obtain the processed portion of prophenin 2, the propeptide was cleaved with hydroxylamine; the cleavage site is located within the cathelin region, 26 amino acid residues upstream the predicted mature peptide (Figure 1(b), shaded residues). Pro-prophenin 2 cleavage with hydroxylamine had an efficiency of approximately 60% (data not shown). Using an anti-FLAG immunoaffinity column, processed prophenin 2 eluted in the void volume as a single band without binding to the antibody (Figure 2(c)), whereas uncleaved pro-prophenin 2 as well as the cathelin region was retained.

3.2. Hemolytic Assays. To determine the hemolytic effect of pro-prophenin 2 and the processed peptide, we performed interaction experiments using the peptides pro-prophenin 2, processed prophenin 2, and tritrpticin with human red blood cells. Figure 3 shows the integrity of the erythrocytes after 1 h of interaction with different concentrations of peptides. We observed almost no effect of either the processed peptide or pro-prophenin 2 on human erythrocytes compared with erythrocytes treated with 0.1% Triton X-100 (100% hemolysis control). Hemolytic activity of NH_2-tritrpticin is shown as control.

3.3. Peptide-Trichomonas Interactions. The effect of prophenin 2 on *T. vaginalis* was estimated using a 4 h-interaction assay to examine trophozoite integrity, and a 24 h assay was used to assess parasite growth. After 4 h incubation with prophenin 2, refringent trophozoites were decreased by 3% at 6.25 μM to 58% at 100 μM of the processed peptide, showing a dose-dependent effect (Figure 4). Surprisingly, pro-prophenin 2 also had a deleterious effect on *T. vaginalis* integrity, and the effect was dose dependent, similar to the processed peptide (Figure 4).

T. vaginalis grew less at 24 h in the presence of pro-prophenin 2 or processed peptide compared with the culture in the absence of the peptides. Growth was reduced by

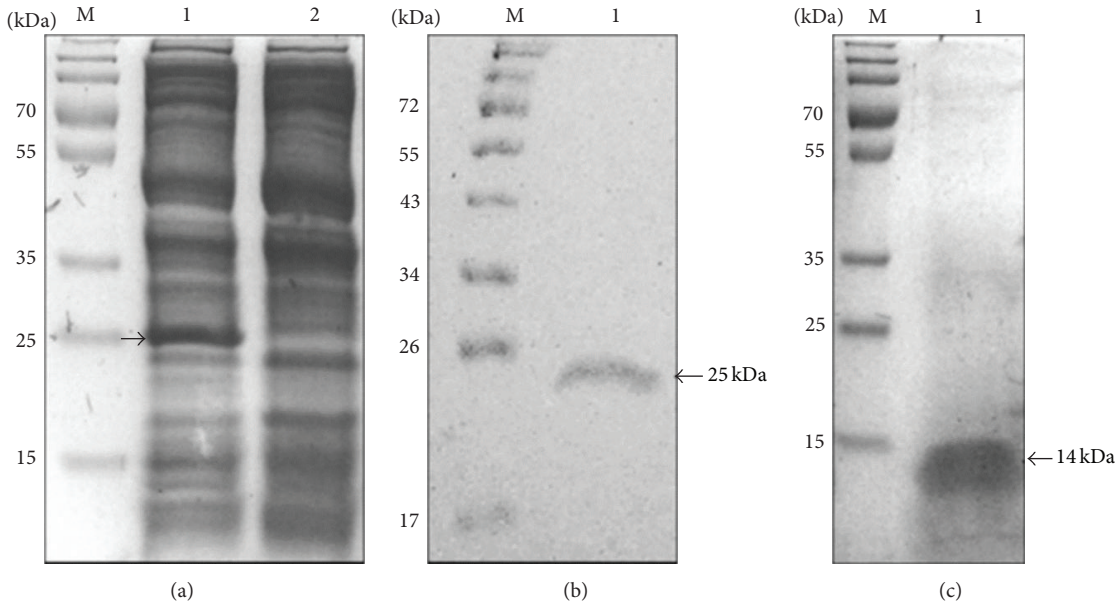


Figure 2: Electrophoretic analysis of pro-prophenin 2 expression, purification, and cleavage with hydroxylamine. Twelve percent polyacrylamide gels (20 : 1 acrylamide : bis-acrylamide) were used and proteins were stained with Coomassie blue. (a) Pro-prophenin 2 expression: lane 1, crude extract from *E. coli* BL21 STAR induced 4 h with 0.25 mM IPTG; lane 2, crude extract from uninduced *E. coli* BL21 STAR, otherwise grown as induced. (b) Pro-prophenin 2 purified by affinity chromatography (lane 1). (c) Processed prophenin 2 after hydroxylamine cleavage and purification (lane 1). M: molecular weight markers.

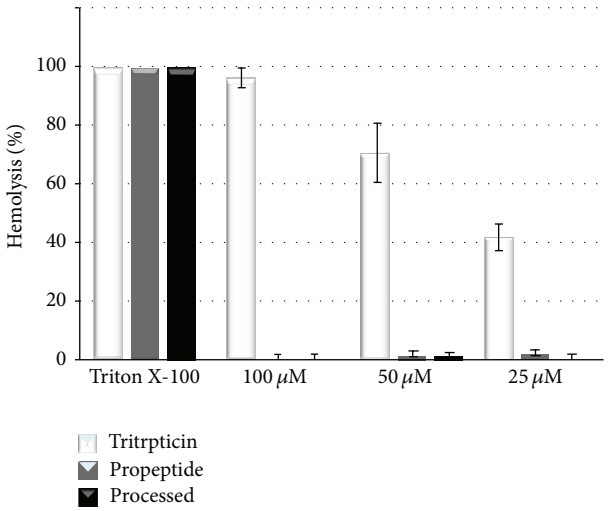


Figure 3: Hemolytic activity of prophenin 2. The percentage of hemolysis was calculated from the absorbance at 540 nm of the supernatants after 1 h of interaction of red blood cells with pro-prophenin 2, processed peptide, and NH_2-tritrpticin. Hemolysis of 100% was obtained by the treatment of erythrocytes with 0.1% Triton X-100.

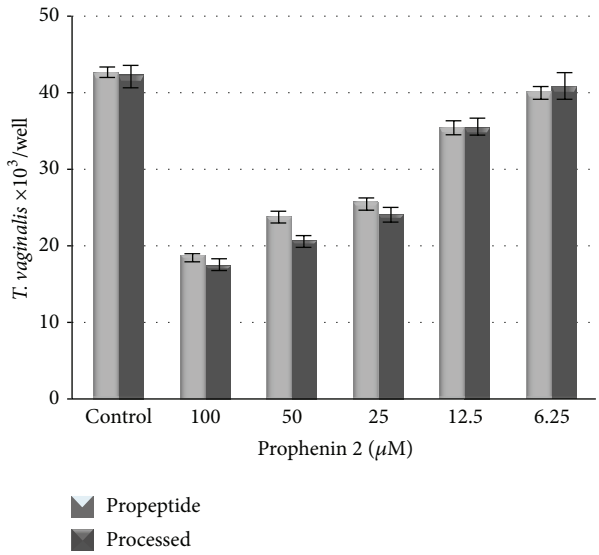


Figure 4: Effect of prophenin 2 on *T. vaginalis* integrity. Trophozoites were inoculated in TYI-HEPES-sodium bicarbonate at 40,000 cells/well; serial dilutions of prophenin 2 were added and incubated at 36.5°C for 4 h. After incubation, each well was sampled and trophozoites were counted. The size of the sample for every treatment was $n = 44$–48, and the standard errors observed were 1.18 or less.

12% at 6.25 μM and by 60% at 100 μM of the processed peptide (Figure 5(a)). The results demonstrated high reproducibility because the standard error was low (11.24). Comparing the means of every treatment, we observed a dose-dependent effect on the integrity and growth of *T. vaginalis* and found that the LD_{50} is approximately 50 μM of the processed peptide. Based on the linear regression analysis of the equation described in Section 2, we determined that the LD_{50} for the processed peptide was 47.66 μM (Figure 5(b)). The significance level of the regression was $P = 2.2e - 16$, indicating an effect on the growth of *T. vaginalis*.

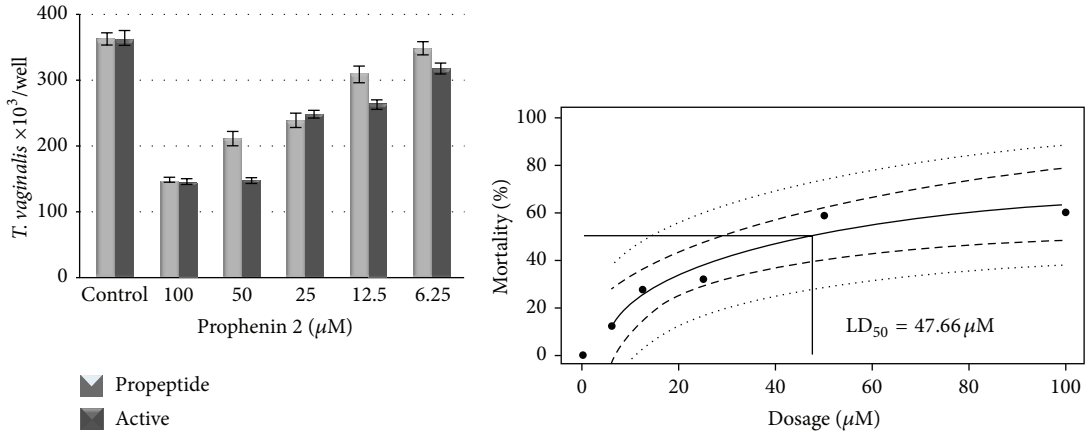
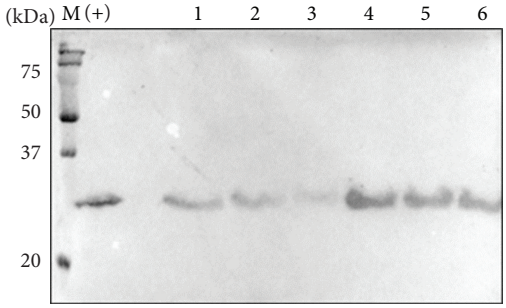


Figure 5: Effect of prophenin 2 on *T. vaginalis* growth. (a) Trophozoites were inoculated in TYI-S-33 at 40,000 cells/well; serial dilutions of pro-prophenin 2 were added and incubated at 36.5°C for 24 h. After incubation, each well was sampled, and trophozoites were counted. The size of the sample for every treatment was $n = 44–48$, and the standard errors observed were 1.18 or less. (b) Estimated LD_{50} for prophenin 2. The results from 24 h interaction assays were used to construct a linear regression model, and the LD_{50} was estimated to be 47.66 μM.



Sample	(+)	1	2	3	4	5	6
Signal intensity	5.74	2.66	1.21	0.92	7.71	5.67	3.79
Relative intensity	1	0.46	0.21	0.16	1.34	0.98	0.66

(b)

Figure 6: Stability of pro-prophenin 2 after interaction with *T. vaginalis*. (a) After parasite-peptide interaction at several incubation times, contents of wells (cells + supernatant) were analyzed by Western blot using anti-FLAG M2 antibodies and signal developed with luminol. Lane M: molecular weight markers; lane (+): 5 μM pro-prophenin 2 as a positive control. Lanes 1 to 3: *T. vaginalis* with 5 μM pro-prophenin 2 incubated 1, 2, and 4 h, respectively. Lanes 4 to 6: same as lanes 1 to 3 but in the presence of 10 μM E64. (b) Signal intensity analysis is done with Image Lab software (BioRad).

3.4. Stability of Pro-Prophenin 2 to T. vaginalis Proteinases. Previous work in our group demonstrated that human cathelicidin LL-37 is very sensitive to proteases from *E. histolytica*, being degraded in just one hour [28]. We decided to test the stability of pro-prophenin 2 by Western blot of the propeptide after 1, 2, and 4 h of interaction with *T. vaginalis*, using the anti-FLAG M2 antibody. We detected the presence of pro-prophenin 2 in all lanes (Figure 6), even in samples that were not treated with the cysteine proteinase inhibitor E64; although after 4 h interaction, without E-64, the signal was very weak (lane 3).

4. Discussion

This study demonstrated that recombinant prophenin 2, both the propeptide and the processed peptide, diminished the integrity and growth of *T. vaginalis*. Prophenin 2 belongs to the porcine cathelicidin family, which are host defense peptides sharing a highly conserved N-terminal cathelin region homologous to the cathepsin L inhibitor [29]. Pigs possess the largest number of cathelicidins described for a single species, with 11 members [10, 30]. Originally, the sequence of prophenin was described by Pungercar et al. in 1993 from a cDNA clone [31]. Later, the protein prophenin-1 was purified and characterized as a 79 aa fragment from porcine leukocytes. It is rich in proline and phenylalanine and contains 5 nearly perfect tandem repeats of a proline-rich decamer, FPPPNFPGPR [32]. Additionally, Zhao et al. [26] reported a closely related sequence named prophenin 2. To date, much of the work performed with prophenin has focused on a small derivative named tritrpticin, which is 13 amino acid residues long and possesses 3 consecutive tryptophan residues. Most studies have used bacteria [33, 34], and only our group has reported the effect of tritrpticin on a protozoa [23]. In this work we expressed the recombinant pro-prophenin 2; although the yield was low (about

300 μg/L), this can likely be circumvented using vectors that fuse pro-prophenin 2 to thioredoxin or glutathione S transferase; we are currently pursuing this line of research.

The use of vital staining to determine viability of *T. vaginalis* in studies involving interaction with antimicrobial peptides is not a suitable methodology, since these molecules alter the cell membrane making it more permeable to the colorant [4]. In a previous report, we demonstrate that after treatment with tritrpticin, the number of refringent *T. vaginalis* trophozoites correlates with their ability to growth [23]. Additionally we observed that tritrpticin at 105 μM reduced *T. vaginalis* viability by approximately 72% after 3 h of interaction and that it reduced growth by 58% after 24 h of interaction [23]. In this study, we reported similar results, with a reduction of 58% in *T. vaginalis* integrity at 100 μM of processed prophenin 2 in 4 h interaction assays and a decrease of 60% in *T. vaginalis* growth using 100 μM of processed peptide in 24 h interaction assays.

A major concern regarding the possible therapeutic use of C-terminus amidated tritrpticin is the hemolytic activity, as it demonstrates ~50% hemolysis at 30 μM [35]. Our work demonstrated that prophenin 2 presents very low hemolytic activity against human red blood cells. This hemolytic activity has to be determined on an individual case basis because it depends not only on the surface selectivity of the target membrane based on the lipid composition [36], but also on the composition of the peptide itself [37]. We demonstrated that prophenin 2 presented good trichomonacidal activity and very low hemolytic activity, which makes this molecule an attractive candidate for studying its possible use as a synergistic or alternative therapy.

In nature, it is accepted that prophenin 2 is processed by neutrophil elastase [38, 39]; the proteolytic cleavage site is predicted between Gly114 and Val115 to release a 101 aa mature peptide [26]. In this study we used as the processed prophenin 2, the portion obtained by cleavage of the recombinant propeptide at a naturally occurring hydroxylamine site (cleavage site Asp88-Gly89), which contained 26 extra amino acids at the N-terminus of the predicted mature peptide. We do not know if these extra amino acids affect the activity of the peptide because the antimicrobial activity of the mature peptide of prophenin 2 is unknown. However, the 79-80 and 17-18 aa prophenin peptides isolated from porcine leukocytes [32, 40, 41] have antibacterial activity. The predicted site for processing the propeptide includes 18 amino acids more than the 79 aa peptide isolated from leukocytes [26]; these fragments and the 17-18 aa long peptide may be produced by additional processing of the mature peptide.

In porcine leukocytes, prophenins are amidated at the C-terminus [40, 41], and this extra positive charge may contribute to the binding of negatively charged targets; therefore, it is expected that the amidated version of the peptide tested in this study will be active at lower concentrations against *T. vaginalis*.

Interestingly, our results demonstrate similar anti-*T. vaginalis* activity of recombinant pro-prophenin as the processed peptide, which is in accordance with the observation that the human pro-cathelicidin HCap18 has similar antimicrobial activity than the C-terminal LL-37 active peptide [42].

T. vaginalis secretes cysteine proteinases as virulence factors, and these enzymes may negatively affect the integrity of most antimicrobial peptides. It is proposed that the proline-rich sequences of some mature cathelicidins are naturally resistant to serine proteases because these types of sequences are very poor substrates for proteolysis [10, 24]. In this study, we demonstrated that pro-prophenin 2 displays an acceptable stability after 1 h of interaction with *T. vaginalis* because protein degradation is not as evident as in our previous work with peptides derived from human cathelicidin LL-37 [28]. We did not test the stability of processed prophenin 2 after interaction with *T. vaginalis* because no antibody targeting the mature peptide was available.

5. Conclusions

We conclude that *T. vaginalis* integrity and growth were significantly diminished in the presence of prophenin 2. Although *T. vaginalis* secretes very active cysteine proteinases, pro-prophenin 2 partially overcomes this limitation, which is inherent to antimicrobial peptides, making prophenin 2 an interestingly candidate for alternative *T. vaginalis* treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This study was supported by grants from Consejo Nacional de Ciencia y Tecnologia, Mexico (Grant no. CB-2012-01 182671) and Universidad de Guanajuato (Grant no. 2013-310).

References

[1] K. A. Brogden, M. Ackermann, P. B. McCray Jr., and B. F. Tack, "Antimicrobial peptides in animals and their role in host defences," *International Journal of Antimicrobial Agents*, vol. 22, no. 5, pp. 465–478, 2003.

[2] G. Maróti Gergely, A. Kereszt, É. Kondorosi, and P. Mergaert, "Natural roles of antimicrobial peptides in microbes, plants and animals," *Research in Microbiology*, vol. 162, no. 4, pp. 363–374, 2011.

[3] A. A. Bahar and D. Ren, "Antimicrobial peptides," *Pharmaceuticals*, vol. 6, no. 12, pp. 1543–1575, 2013.

[4] G. K. Mutwiri, W. G. Henk, F. M. Enright, and L. B. Corbeil, "Effect of the antimicrobial peptide, D-hecate, on Trichomonads," *Journal of Parasitology*, vol. 86, no. 6, pp. 1355–1359, 2000.

[5] J. Vizioli and M. Salzet, "Antimicrobial peptides versus parasitic infections?" *Trends in Parasitology*, vol. 18, no. 11, pp. 475–476, 2002.

[6] A. J. Mason, W. Moussaoui, T. Abdelrahman et al., "Structural determinants of antimicrobial and antiplasmodial activity and selectivity in histidine-rich amphipathic cationic peptides," *The Journal of Biological Chemistry*, vol. 284, no. 1, pp. 119–133, 2009.

[7] C.-Y. Pan, J.-Y. Chen, T.-L. Lin, and C.-H. Lin, "In vitro activities of three synthetic peptides derived from epinecidin-1 and an

anti-lipopolysaccharide factor against *Propionibacterium acnes*, *Candida albicans*, and *Trichomonas vaginalis*," *Peptides*, vol. 30, no. 6, pp. 1058–1068, 2009.

[8] L. Rivas, J. R. Luque-Ortega, and D. Andreu, "Amphibian antimicrobial peptides and Protozoa: lessons from parasites," *Biochimica et Biophysica Acta*, vol. 1788, no. 8, pp. 1570–1581, 2009.

[9] B. Rivas-Santiago, C. J. Serrano, and J. A. Enciso-Moreno, "Susceptibility to infectious diseases based on antimicrobial peptide production," *Infection and Immunity*, vol. 77, no. 11, pp. 4690–4695, 2009.

[10] Y. Sang and F. Blecha, "Porcine host defense peptides: expanding repertoire and functions," *Developmental & Comparative Immunology*, vol. 33, no. 3, pp. 334–343, 2009.

[11] M. F. Cotch, J. G. Pastorek II, R. P. Nugent et al., "*Trichomonas vaginalis* associated with low birth weight and preterm delivery. The Vaginal Infections and Prematurity Study Group," *Sexually Transmitted Diseases*, vol. 24, no. 6, pp. 353–360, 1997.

[12] M. Laga, A. Manoka, M. Kivuvu et al., "Non-ulcerative sexually transmitted diseases as risk factors for HIV-1 transmission in women: Results from a cohort study," *AIDS*, vol. 7, no. 1, pp. 95–102, 1993.

[13] G. G. G. Donders, C. E. Depuydt, J.-P. Bogers, and A. J. Vereecken, "Association of Trichomonas vaginalis and cytological abnormalities of the cervix in low risk women," *PLoS ONE*, vol. 8, no. 12, Article ID e86266, 2013.

[14] P. Kissinger and A. Adamski, "Trichomoniasis and HIV interactions: a review," *Sexually Transmitted Infections*, vol. 89, no. 6, pp. 426–433, 2013.

[15] S. L. Cudmore, K. L. Delgaty, S. F. Hayward-McClelland, D. P. Petrin, and G. E. Garber, "Treatment of infections caused by metronidazole-resistant Trichomonas vaginalis," *Clinical Microbiology Reviews*, vol. 17, no. 4, pp. 783–793, 2004.

[16] D. H. H. Robertson, R. Heyworth, C. Harrison, and W. H. R. Lumsden, "Treatment failure in *Trichomonas vaginalis* infections in females. I. Concentrations of metronidazole in plasma and vaginal content during normal and high dosage," *Journal of Antimicrobial Chemotherapy*, vol. 21, no. 3, pp. 373–378, 1988.

[17] R. L. Dunne, L. A. Dunn, P. Upcroft, P. J. O'Donoghue, and J. A. Upcroft, "Drug resistance in the sexually transmitted protozoan Trichomonas vaginalis," *Cell Research*, vol. 13, no. 4, pp. 239–249, 2003.

[18] J. R. Schwebke and F. J. Barrientes, "Prevalence of Trichomonas vaginalis isolates with resistance to metronidazole and tinidazole," *Antimicrobial Agents and Chemotherapy*, vol. 50, no. 12, pp. 4209–4210, 2006.

[19] J. D. Sobel, V. Nagappan, and P. Nyirjesy, "Metronidazole-resistant vaginal trichomoniasis—an emerging problem," *The New England Journal of Medicine*, vol. 341, no. 4, pp. 292–293, 1999.

[20] H. Hernández, I. Sariego, G. Garber, R. Delgado, O. López, and J. Sarracent, "Monoclonal antibodies against a 62 kDa proteinase of *Trichomonas vaginalis* decrease parasite cytoadherence to epithelial cells and confer protection in mice," *Parasite Immunology*, vol. 26, no. 3, pp. 119–125, 2004.

[21] R. Hernández-Gutiérrez, L. Avila-González, J. Ortega-López, F. Cruz-Talonia, G. Gómez-Gutierrez, and R. Arroyo, "Trichomonas vaginalis: characterization of a 39-kDa cysteine proteinase found in patient vaginal secretions," *Experimental Parasitology*, vol. 107, no. 3-4, pp. 125–135, 2004.

[22] A. F. Garcia and J. F. Alderete, "Characterization of the *Trichomonas vaginalis* surface-associated AP65 and binding domain interacting with trichomonads and host cells," *BMC Microbiology*, vol. 7, article 116, 2007.

[23] V. V. Infante, A. D. Miranda-Olvera, L. M. de Leon-Rodriguez, F. Anaya-Velazquez, M. C. Rodriguez, and E. E. Avila, "Effect of the antimicrobial peptide tritrpticin on the in vitro viability and growth of trichomonas vaginalis," *Current Microbiology*, vol. 62, no. 1, pp. 301–306, 2011.

[24] A. E. Shinnar, K. L. Butler, and H. J. Park, "Cathelicidin family of antimicrobial peptides: proteolytic processing and protease resistance," *Bioorganic Chemistry*, vol. 31, no. 6, pp. 425–436, 2003.

[25] L. S. Diamond, D. R. Harlow, and C. C. Cunnick, "A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 72, no. 4, pp. 431–432, 1978.

[26] C. Zhao, T. Ganz, and R. I. Lehrer, "Structures of genes for two cathelin-associated antimicrobial peptides: prophenin-2 and PR-39," *FEBS Letters*, vol. 376, no. 3, pp. 130–134, 1995.

[27] T. P. Hopp, K. S. Prickett, V. L. Price et al., "A short polypeptide marker sequence useful for recombinant protein identification and purification," *Nature Biotechnology*, vol. 6, no. 10, pp. 1204–1210, 1988.

[28] R. Rico-Mata, L. M. De Leon-Rodriguez, and E. E. Avila, "Effect of antimicrobial peptides derived from human cathelicidin LL-37 on Entamoeba histolytica trophozoites," *Experimental Parasitology*, vol. 133, no. 3, pp. 300–306, 2013.

[29] A. Ritonja, M. Kopitar, R. Jerala, and V. Turk, "Primary structure of a new cysteine proteinase inhibitor from pig leucocytes," *FEBS Letters*, vol. 255, no. 2, pp. 211–214, 1989.

[30] G. Zhang, C. R. Ross, and F. Blecha, "Porcine antimicrobial peptides: new prospects for ancient molecules of host defense," *Veterinary Research*, vol. 31, no. 3, pp. 277–296, 2000.

[31] J. Pungercar, B. Strukelj, G. Kopitar et al., "Molecular cloning of a putative homolog of proline/arginine-rich antibacterial peptides from porcine bone marrow," *FEBS Letters*, vol. 336, no. 2, pp. 284–288, 1993.

[32] S. S. Harwig, V. N. Kokryakov, K. M. Swiderek, G. M. Aleshina, C. Zhao, and R. I. Lehrer, "Prophenin-1, an exceptionally proline-rich antimicrobial peptide from porcine leukocytes," *FEBS Letters*, vol. 362, pp. 65–69, 1995.

[33] C. Lawyer, S. Pai, M. Watabe et al., "Antimicrobial activity of a 13 ammo acid tryptophan-rich peptide derived from a putative porcine precursor protein of a novel family of antibacterial peptides," *The FEBS Letters*, vol. 390, no. 1, pp. 95–98, 1996.

[34] O. Cirioni, A. Giacometti, C. Silvestri et al., "In vitro activities of tritrpticin alone and in combination with other antimicrobial agents against *Pseudomonas aeruginosa*," *Antimicrobial Agents and Chemotherapy*, vol. 50, no. 11, pp. 3923–3925, 2006.

[35] L. T. Nguyen, L. de Boer, S. A. J. Zaat, and H. J. Vogel, "Investigating the cationic side chains of the antimicrobial peptide tritrpticin: hydrogen bonding properties govern its membrane-disruptive activities," *Biochimica et Biophysica Acta: Biomembranes*, vol. 1808, no. 9, pp. 2297–2303, 2011.

[36] Y. Ishitsuka, D. S. Pham, A. J. Waring, R. I. Lehrer, and K. Y. C. Lee, "Insertion selectivity of antimicrobial peptide protegrin-1 into lipid monolayers: effect of head group electrostatics and tail group packing," *Biochimica et Biophysica Acta*, vol. 1758, no. 9, pp. 1450–1460, 2006.

[37] S.-T. Yang, S. Yub Shin, Y.-C. Kim, Y. Kim, K.-S. Hahm, and J. I. Kim, "Conformation-dependent antibiotic activity of tritrpticin, a cathelicidin-derived antimicrobial peptide," *Biochemical*

and Biophysical Research Communications, vol. 296, no. 5, pp. 1044–1050, 2002.

[38] A. M. Cole, J. Shi, A. Ceccarelli, Y.-H. Kim, A. Park, and T. Ganz, "Inhibition of neutrophil elastase prevents cathelicidin activation and impairs clearance of bacteria from wounds," *Blood*, vol. 97, no. 1, pp. 297–304, 2001.

[39] J. Shi and T. Ganz, "The role of protegrins and other elastase-activated polypeptides in the bactericidal properties of porcine inflammatory fluids," *Infection and Immunity*, vol. 66, no. 8, pp. 3611–3617, 1998.

[40] Y. Wang, W. J. Griffiths, T. Curstedt, and J. Johansson, "Porcine pulmonary surfactant preparations contain the antibacterial peptide prophenin and a C-terminal 18-residue fragment thereof," *The FEBS Letters*, vol. 460, no. 2, pp. 257–262, 1999.

[41] Y. Wang, J. Johansson, and W. J. Griffiths, "Characterisation of variant forms of prophenin: mechanistic aspects of the fragmentation of proline-rich peptides," *Rapid Communications in Mass Spectrometry*, vol. 14, no. 23, pp. 2182–2202, 2000.

[42] M. Pazgier, B. Ericksen, M. Ling et al., "Structural and functional analysis of the pro-domain of human cathelicidin, LL-37," *Biochemistry*, vol. 52, no. 9, pp. 1547–1558, 2013.