Basic insight on plant defensins

REVIEW

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ABSTRACT

Defensins are small cysteine-rich peptides with antimicrobial activity. Plant defensins have a characteristic three-dimensional folding pattern that is stabilized by eight disulfide-linked cysteines. This pattern is very similar to defense peptides of mammals and insects, suggesting an ancient and conserved origin. Functionally, these proteins exhibit a diverse array of biological activities, although they all serve a common function as defenders of their hosts. Plant defensins are active against a broad range of phytopathogenic fungi and even against human pathogens, which indicates their potential in therapeutics. This review briefly describes the distribution, structure, function and putative modes of antimicrobial activity of plant defensins and reflects their potential in agriculture and medical applications.

Key works: defensin, cysteine-rich peptides, mode of action

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RESUMEN

Conocimientos básicos acerca de las defensinas de plantas. Las defensinas son pequeños péptidos ricos en cisteína con actividad antimicrobiana. Las defensinas de plantas poseen un característico patrón de plegamiento tri-dimensional estabilizado por ocho puentes disulfuro de residuo de cisteinas. Este patrón comparte una alta semejanza con los péptidos de defensa de mamíferos e insectos, sugiriendo un origen común conservado. Funcionalmente estas proteínas exhiben un conjunto diverso de actividades biológicas, aunque todas están al servicio de una función común, como defensoras de sus hospederos. Las defensinas de plantas se activan contra un amplio rango de hongos fitopatogénicos e incluso contra patógenos humanos, lo cual indica su potencial para el desarrollo de terapéuticos. El objetivo de esta revisión es describir brevemente la distribución, función y los modos de actividad antimicrobiana propuestos para las defensinas de plantas, así como reflejar las aplicaciones de las mismas en la agricultura y la medicina.

Palabras claves: defensinas, péptidos ricos en cisteína, modo de acción

*I*ntroduction

Plants are constantly exposed to environmental stresses and the attacks of pathogenic organisms. Survival under these conditions demands quick defense responses to inhibit pathogen spread after initial infection and thus to limit disease. Among these defense responses are a rapid oxidative burst, local transcriptional activation of defense-related genes, and the generation of yet unknown systemic signals that trigger and induce a state of systemic immunity [1]. Small antimicrobial peptides play an important role as part of the plants` natural defense system against infectious microorganisms, by recognizing a broad range of microbes. Hundreds of antifungal peptides and proteins are known, with more being discovered almost daily.

Genetic engineering has provided a new strategy for improving disease resistance through cellular and molecular tools [2]. Based on *in vitro* analysis, many antimicrobial peptides have inhibited the growth of a broad range of plant pathogenic fungi and some bacteria. The enhancement of disease resistance in crops has contributed significantly to increase their productivity and decrease the application of pesticides, which can affect human health and the environment.

*P*lant defensins

The main groups of antimicrobial peptides found in plants are thionins, defensins and lipid transfer proteins. Plant defensins are small (c.a. 5kDa), basic, cysteine-rich antifungal peptides ranging from 45 to 54 amino acids, and are positively charged. The first plant defensins were isolated from wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), and were initially classified as a subgroup of the thionin family called the γ-thionins since they showed a similar size and the same number of disulfide bridges as α and β-thionins [3]. Subsequent identification of other γ-thionin-like proteins in other plant families, together with structural information, revealed striking differences between γ-thionins and classical thionins [4]. This class of proteins was later renamed as "plant defensins" [5], due to structural and functional similarities with insect and mammalian defensins [4]. To date, plant defensins have been isolated from seeds of various monocot and dicot species or identified by the sequencing of cDNA clones.

The plant defensin family is quite diverse regarding amino acid composition as only the eight structure-stabilizing cysteines appear to be conserved among all plant defensins [6]. The variation in the primary sequences may account for the different biological activities reported for plant defensins. The three-dimensional structure of plant defensins comprises a triple-stranded β-sheet with an α-helix in parallel, stabilized by four disulfide bridges [7-8]. This structure resembles that of insect and mammalian defensins except that insect defensins lack the domain corresponding to the amino-terminal β-strand of plant

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1. Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD. Systemic acquired resistance. Plant Cell 1996; 8:1809-19.

2. Kawata M, Nakajima T, Yamamoto T, Mori K, Oikawa T, Fukumoto F, Kuroda S. Genetic Engineering for Disease Resistance in Rice (*Oryzasativa* L.) using antimicrobial Peptides. JARQ 2003;37:71-6.

3. Colilla FJ, Rocher R, Mendez E. γ-Purothionins: amino acid sequence of two polypeptides of a new family of thionins from wheat endosperm. FEBS Lett 1990; 270:191-4.

4. Terras FRG, Eggermont K, Kovaleva V, Raikhel NV, Osborn RW, Kester A, Ress SB, Torrekens S, Van Leuven F, Vanderleyden J. Small cysteine-rich antifungal proteins from radish: their role in host defense. Plant Cell 1995;7:573-88.

5. Broekaert WF, Terras FRG, Cammue BPA, Osborn RW. Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant Physiol 1995;108:1353-8.

6. Thomma BPHJ, Thevissen K, Cammue BP. Plants defensins. Planta 2002;216: 193-202.

7. Almeida MS, Cabral KM, Kurtenbach E, Almeida FC, Valente APJX. Solutionstructure of *Pisum sativum* defensin 1 by high resolution NMR: plant defensins, identical backbone with different me-chanisms of action. Mol Biol 2002;315:749-57.

defensins (Figure 1). Mammalian defensins, on the other hand, not only have a triple-stranded, antiparallel β-sheet, which is roughly comparable in size and spatial orientation to that occurring in plant defensins, but also the α-helix appears to be approximately in the same position relative to the β-sheet [9-10]. The fact that plant defensins, belong to a superfamily of similarly folded antimicrobial peptides that has representati-ves in vertebrates, invertebrates, and plants, suggests that these defense molecules predate the evolutionary divergence of animals and plants.

Structure-activity relationships and modes of action

The structure-activity relationships and modes of action for most of the plant defensins remain unknown. Not all plant defensins have the same mode of action. Some of them exhibit potent antifungal activity *in vitro* at micromolar concentrations against a broad spectrum of filamentous fungi. Morphogenic antifungal defensins reduce hyphal elongation and induce hyperbranching, whereas non-morphogenic defensins reduce hyphal elongation without causing any morphological distortions [5-11-12]. The plant defensins Rs-AFP1 and Rs-AFP2 from radish (*Raphanus sativus*), and alfAFP isolated from seeds of the *Medicago sativa* (alfalfa) plants [13], are examples of potent antifungal proteins, causing morphological distortions of the fungal hyphae, resulting in hyperbranched fungal structures. The antifungal activity of plant defensins, whether morphogenic or not, is reduced by increasing the ionic strength of the fungal growth assay medium. However, the antagonistic effect of ions is strongly dependent on the fungus and thus on the conformation of the putative target site. Ionic strength antagonism was found to be due to cations, with divalent cations being at least one order of magnitude more potent than monovalent cations. In general, the antifungal activity of plant defensins is slightly more reduced by Ca^{2+} than by Mg^{2+} [14]. Alfalfa seed defensin, MsDef1, strongly inhibits the growth of *Fusarium graminearum in vitro*, and its antifungal activity is markedly reduced in the presence of Ca^{2+}. This antagonistic effect of cations was also observed in the case of plant defensin-induced membrane permeabilization through specific interaction with high-affinity binding sites on fungal cells but no ion-permeable pores are formed in artificial lipid bilayers, nor are there changes in their electrical properties [15-16].

Antifungal plant defensin, Rs-AFP2, appears to act primarily at the cell membrane [17] and induces rapid Ca^{2+} uptake and K^{+} efflux from *Neurospora crassa* hyphae and it may thus inhibit the growth of filamentous fungi by disrupting cytosolic Ca^{2+} gradients essential for hyphal tip growth. Thesivven and colleagues [16-17] have suggested that defensin initiates this response by its interaccion with a membrane-bound receptor rather than by permeabilizing the membrane by the direct defensin-lipid interaction.

Membrane permeabilization induced by plant defensins in *Neurospora crassa* is biphasic, depending on the plant defensin dose. At high defensin levels (10 to 40 μM), strong permeabilization is detected that can be suppressed by cations in the medium. At lower defensin levels (0.1 to 1 μM), a weaker, but more cation-resistant permeabilization correlates with the inhibition of fungal growth. Reduction of the antimicrobial activity in the presence of divalent cations, especially Ca^{2+}, has also been observed for insect and mammalian defensins [18]. A partial explanation for this effect could be that an increase in ionic strength weakens the electrostatic charge interactions required for the initial interaction between the peptides and the membrane.

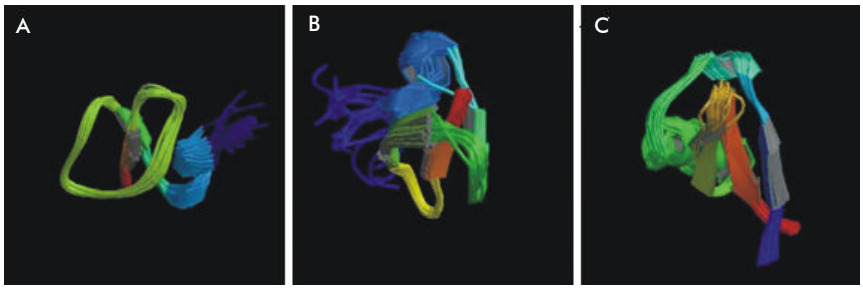

Figure 1. Three-dimensional structure of some known defensins isolated from different organisms. Structures were downloaded from the protein data bank, PDB accession ID numbers: A. Human b defensin HBD-1 (PDB 1E4S) (ref 9). B. Mouse defensin MBD-8 (PDB 1E4R) (ref 9). C. Plant Defensin (PDB 1TI5) (ref 10).

For most plant defensins, molecular components involved in signaling and putative intracellular targets remain unknown. At present there are two tentative models for the mode of action of plant defensins in relation to sphingolipids, that are one of the three major types of lipids found in eukaryotic membranes, along with sterols and phosphoglycerolipids. They associate with sterols in the plasma membrane to form patches or rafts that are highly enriched in glycosyl-phosphatidyl-inositol (GPI)-anchored membrane proteins [19]. The interaction facilitates the insertion of these peptides in the plasma and leads to fungal growth arrest. Alternatively, GPI-anchored proteins could act as docking sites for these defensins and facilitate their insertion into the fungal plasma membrane, leading to alterations in membrane permeability. Likewise, mammalian GPI-anchored proteins have been shown to act as receptors for a bacterial toxin, aerolysin, that causes pore formation [20]. Recently, the theory is gaining support in that many cationic peptides exert their anti-microbial activity not only through the permeabilization of the membrane, but also through cytoplasmic targets [21].

Plant defensins are active against a broad range of phytopathogenic fungi and even against human pathogens. Certain members within the plant defensin family also display other biological activities, including proteinase [22-23] and á-amylase [24] inhibitory activities and the inhibition of protein translation [3], which may contribute to their role in defense.

Applications

To date, several types of antimicrobial peptides have been isolated and characterized and the applications of plant defensins are diverse. They can be used as resistance traits in transgenic plants, resulting in an enhanced protection against pathogen attack (Figure 2). The constitutive expression of a novel alfalfa defensin gene in potato provides high levels of field resistance against *dahliae*, the causal agent of the agrono-mically important "early dying" disease of

8. Fant F, Vranken WF, Borremans FAM. The three-dimensional solution structure of *Aesculus hippocastanum* antimicrobial protein 1 determined by 1H nuclear magnetic resonance. Proteins 1999;37: 388-403.

9. Bauer F, Schweimer K, Kluver E, Conejo JR, Forssmann WG, Rosch P, Adermann K, Sticht H. .Structure determination of human and murine beta-defensins reveals structural conservation in the absence of significant sequence similarity. Protein Sci 2001;10:2470-9.

10. Liu YJ, Cheng CS, Liu YN, Hsu MP, Chen CS, Lyu PC. Solution structure of plant defensin (unpublished).

11. Thomma BP, Cammue BP, Thevissen K. Mode of action of plant defensins suggests therapeutic potential. Curr Drug Targets Infect Disord 2003;3:1-8.

12. Spelbrink RG, Nejmi AA, Thomas JS, Dilip MS, Hockerman GH. Differential Antifungal and Calcium Channel-Blocking Activity among Structurally Related Plant Defensins. Plant Physiology 2004; 135:2055-67.

13. Gao AG, Hakimi SM, Mittanck CA, Wu Y, Woerner BM, Stark DM, Shah DM, Shah DM, Liang J, Rommens CM. Fungal pathogen protection in potato by expression of a plant defensin peptide. Nat Biotechnol 2000;18:1307-10.

14. Osborn RW, De Samblanx GW, Thevissen K, Goderis I, Torrekens S, Van Leuven F, Attenborough S, Ress SB, Broekaert WF. Isolation and characterisation of plant defensins from seeds of *Asteraceae, Fabaceae, Hippocastanaceae* and *Saxifragaceae*. FEBS Lett 1995;368:257-62.

15. Thevissen K, Osborn RW, Acland DP, Broekaert WF. Specific, high affinity binding sites for an antifungal plant defensin on *Neurospora crassa* hyphae and microsomal membranes. J Biol Chem 1997;272:32176-81.

16. Thevissen K, Osborn RW, Acland DP, Broekaert WF. Specific binding sites for an antifungal plant defensin from dahlia (*Dahlia merckii*) on fungal cells are required for antifungal activity. Mol Plant Microbe Interact 2000;13:54-61.

17. Hevissen K, Ghazi A, De Samblanx GW, Brownlee C, Osborn RW, Broekaert WF. Fungal membrane responses induced by plant defensins and thionins. J Biol Chem 1996;271:15018-25.

Fig 2. A cartoon showing how a plant has acquire a pathogen resistance state after spraying defensins in it while not treated plants died.

potato. This protein also inhibits the growth of other plant pathogens such as *Alternaria solani* and *Fusarium culmorum*, the causal agents of potato early blight and wheat head scab, respectively [13]. On the other hand, plants that constitutively expressed a plant defensin gene from *B. oleracea* and *B. campestris* were modified to substitute a single amino acid at each position, and were individually introduced into rice since rice and food production have experienced a critical damage from fungal rice blast caused by *Magnaporthe grisea*, the main problem for crop yield and bacterial leaf blight caused by *Xanthomonas oryzae*, a serious rice disease in subtropical and tropical countries. These two plant defensins from *B. oleracea* and *B. campestris* conferred an effective resistance to both rice blast and bacterial leaf blight, and the modification of the defensin genes led to an increase in the broad disease resistance spectrum in transgenic rice [2].

The constitutive expression of plant defensins in peripheral cells of the seeds in radish [25], the flower organs in tobacco [26], the leaves in pea [5], or the tubers in potato [27] is consistent with the first-line defense role of vulnerable tissues. Similarly, enhanced resistance has been obtained in transgenic oil rapeseed, chinese cabbage and tomato plants by constitutive plant defensin expression [28]. These demonstrate that plant defensins are important components of host defense. Moreover, they can be used to generate transgenic crops with improved pathogen resistance.

The application of plant defensins to crops to reduce losses caused by fungal pathogens provides two primary advantages over the conventional spray of chemical fungicides. First, plant defensins are derived from plant seeds, roots and tubers, and are thus natural products. They have minimal harmful effects on human beings. Secondly, like any other proteins, plant defensins quickly degrade to natural elements. Essentially no "residues" are left after the anti-fungal effectiveness expires. One drawback of plant defensins is their relatively lower potency compared to chemical fungicides. But the apparent environmental safety of plant defensins can justify using them as alternative fungicides. This disadvantage can also be reverted by a more thorough understanding of the fungal disease process and by the application of multiple plant defensins (a "cocktail" of defensins) for a synergistic effectiveness at the right place and time. Since plant defensins are protein based, it is presently not feasible to synthesize large amounts of plant defensins as in the large-scale syntheses of chemical fungicides in a factory. One stumbling block preventing the application of plant defensins is the lack of an efficient way of producing large amounts of these natural antifungal proteins for commercial use.

On the other hand, the control of eukaryotic pathogens constitutes one of the most challenging problems in medicine. It is widely accepted among clinicians, medical researchers, microbiologists and pharmacologist, that antibiotic resistance will, in the very near future, leave healthcare professionals without effective therapies for bacterial infections. As an example, it is now estimated that about half of all *Staphylococcus aureus* strains found in many medical institutions are resistant to antibiotics such as methicillin [29]. The emergence among enterococci of resistance to another useful and widely effective antibiotic, vancomycin [30], might accelerate the spread of vancomycin-resistant genes, via plasmids, throughout other species, eventually limiting the efficacy of this drug. Consequently, the priority for the next decades should be focused on the development of alternative drugs and/or the recovery of natural molecules that would allow a consistent and appropriate control of pathogen-caused diseases. Ideally, these molecules should be as natural as possible, with a wide range of action over several pathogens, easy to produce, and not prone to induce resistance.

Antimicrobial peptides including defensins, isolated from a full range of organisms and species from bacteria to man, seem to fit this description. They can be used as the leading molecules for the development of natural antibiotics, the basic element of a novel generation of drugs for the treatment of bacterial and fungal infections [31-34]. In addition, the wide spectrum of antimicrobial activities reported for these molecules suggests their potential benefit in the treatment of cancer [35] and viral [36-38] or parasitic infections [39].

Different therapeutic applications of these compounds, from topical administration to the systemic treatment of infections, have been developed by several biotechnological companies [32]. Various insect and mammalian defensins are currently being tested in clinical trials to examine their use in bacterial and fungal infections. Micrologix biotech Inc. developed different defensin variants which are currently evaluated in a late-stage clinical trial to prevent catheter-related infections caused by bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and for their potential to cure severe acne and to prevent acute *S. aureaus* infections [40]. The French company EntoMed is conducting preclinical studies with a defensin-like antifungal insect peptide to combat *Candida albicans* and *Aspergillus fumigatus*, which often cause fatal infections in immunocompromised patients [41]. Plant defensins, in contrast to insect and mammalian defensins, interact with specific structures in the fungal membrane, such as phosphorylinositol containing

18.De Samblanx GW, Goderis IJ, Thevissen K, Raemaekers R, Fant F, Borremans F, Acland D P, Osborn R W, Patel S, Broekaert WF. Mutational analysis of a plant defensin from Radish (*Raphanus sativus* L.) reveals two adjacent sites important for antifungal activity. J Biol Chem 1997; 272:1171-9.

19.Bagnat M, Keranen S, Shevchenko A, Simons K. Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. Proc Natl Acad Sci (USA) 2000;97: 3254-9.

20.Abrami L, Velluz MC, Hong Y, Ohishi K, Mehlert A, Ferguson M, Kinoshita T, Gisou van der Goot F. The glycan core of GPI-anchored proteins modulates aerolysin binding but is not sufficient: the polypeptide moiety is required for the toxin-receptor interaction. FEBS lett 2002;512: 249-54.

21.Xiong YQ, Yeaman MR, Bayer AS. *In vitro* antibacterial activities of platelet microbicidal protein and neutrophil defensin against *Staphylococcus aureus*are influenced by antibiotics differing in mechanism of action. Antimicrob Agents Chemother 1999;43:1111-7.

22.Wijaya R, Neumann GM, Condron R, Hughes AB, Polya GM. Defense proteins from seed of *Cassia fistula* include a lipid transfer protein homologue and a protease inhibitory plant defensin. Plant Sci 2000;159:243-55.

23.Melo FR, Rigden DJ, Franco OL, Mello LV, Ary MB, Crossi de Sa MF, Bloch C. Inhibition of trypsin by cowpea thionin: characterization, molecular modeling, and docking. Proteins Struct Funct Genet 2002;48:311-9.

24.Zhang NY, Jones BL, Tao P. Purifica-tion and characterisation of a new class of insect α-amylase inhibitors from barley. Cereal Chem 1997;74:119-22.

25.Terras FR, Eggermont K, Kovaleva V, Raikhel NV, Osborn RW, Kester A, Ress SB, Torrekens S, Van Leuven F, Vanderleyden J. Small cysteine-rich antifungal proteins from radish: their role in host defense. Plant Cell 1995;7:573-88.

26.Gu Q, Kawata EE, Morse MJ, Wu HM, Cheung AY. A flower-specific cDNA encoding a novel thionin in tobacco. Mol Gen Genet 1992;234:89-96.

27.Moreno M, Segura A, García-Olmedo F. Pseudothionin-St1, a potato peptide active against potato pathogens. Eur J Biochem 1994;223:135-9.

28.Park HC, KangYH, Chun HJ, Koo JC, Cheong YH, Kim CY, Kim MC, Chung WS, Kim JC, Yoo JH, Koo YD, Koo SC, Lim CO, Lee SY, Cho M J. Characterization of a stamen-specific cDNA encoding a novel plant defensin in Chinese cabbage. Plant Mol Biol 2002;50:59-69.

29.Roder BL, Wandall DA, Frimodt-Moller N, Espersen F, Skinhoj P, Rosdahl VT. Clinical features of *Staphylococcus aureus* endocarditis: a 10-year experience in Denmark. Archives Internal Medicine 1999;159:462-9.

30.Novak R, Henriques B, Charpentier E, Normark S, Tuomanen E. Emergente of vancomycin tolerante in *Streptococcus pneumoniae*. Nature 1999;399:590-1.

31.De Lucca AJ. Antifungal peptides: potential candidates for the treatment of fungal infections. Expert Opinion on Investigational Drugs 2000;9:273-99.

sphingolipids or glucosyl ceramides, which points towards a high selectivity and hence, interesting perspectives for the treatment of fungal infections. Importantly, various plant defensins such a Dm-AMP1, from *Dahlia merckii* seeds Hs-AFP1 from *Heuchera sanguinea* seeds and Rs-AFP2 were found to be active against the human pathogen *Candida albicans* at micromolar concentrations, which indicates the potential of plant defensins for the development of therapeutics. These make the antibiotic peptides a powerful mine of molecules that could become antimicrobial drugs in this new century.

Concluding remarks

Since genetic engineering using antimicrobial peptides is becoming a powerful tool for the introduction of new traits in plants, many studies are in progress to improve plant breeding for disease resistance by transgenic approaches. The precise mode of action of plant defensins is still unclear and most plant defensin molecular components involved in signaling and putative intracellular targets remain unknown. Not all plant defensins act through the same mode of action. The fact that some defensins have been found to interact with fungal-specific components in the plasma membrane resulting in membrane permeabilization, makes them an attractive potential therapeutic source to treat fungal infections and can be used as the leading molecules for the development of antifungal medicines. On the other hand, among other diverse applications they can be used as resistance traits in transgenic crops, resulting in enhanced protection against pathogen attack.

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32. Hancock REW. Cationic antimicrobial peptides: towards clinical applications. Expert Opinion on Investigational Drugs 2000; 9:1723-9.

33. Welling MM, Paulusma-Annema A, Balter HS, Pauwels EK, Nibbering PH. Tenehtium-99m labeled antimicrobial peptide discriminate between bacterial infection and sterile inflammations. European Journal of Nuclear Medicine 2000;27:292-301.

34. Selitrennikoff CP. Antifungal proteins. Applied and Environmental Microbiology 2001;67:2883-94.

35. Tanaka K. P-13- kinase p85 is a target molecule of proline-rich antimicrobial peptide to suppress proliferation of ras- transformed cells. Japanese Journal of Cancer Research 2001;92:959-67.

36. Chinchar VG, Wang J, Murti G, Carey C, Rollins-Smith L. Inactivation of frog virus 3 and channel catfish virus by esculentin-2P and ranatuerin-2P, two antimicrobial peptides isolated from frog skin. Virology 2001;288: 352-7.

37. Andersen J, Osbakk S, Vorland L, Traavik T, Gutteberg T. Lactoferrin and cyclic lactoferricin inhibit the entry of human cytomegalovirus into human fibroblasts. Antiviral Research 2001;51:141-9.

38. Chernysh S, Kim SI, Beckeer G, Pleskach VA, Filatova NA, Anikin VB, Platonov VG, Bulet P. Antiviral and antitumor peptides from insects. Proceeding of the National Academy of Science (USA) 2002;99:12628-32.

39. Marshall SH, Arenas G. Antimicrobial peptides: A natural alternative to chemical antibiotics and a potential for applied biotechnology 2003;6:271-84.

40. Gura T. Innate Immunity. Ancient System Gets New Respect. Science 2001;291:2068-71.

41. Zasloff M. Antimicrobial peptides of multi-cellular organisms. Nature 2002; 415:389-95.

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