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Defensins and Bioinformatics: In Silico Approaches for Novel Therapeutic Antimicrobial Peptides

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Abstract

Defensins are antimicrobial proteins that help the immune system of animals and plants on avoiding pathogens and maintaining the body integrity. According to recent literature, humanity is in need of a new golden age of antibiotics as resistant bacterial strains are turning simple infections into lethal weapons. Therefore, defensins may help as biotechnological prototypes with an antibacterial profile to generate new antimicrobial drugs. In this work we present the in silico perspective of the molecular modeling, a subarea of Bioinformatic, as a tool to be used in comparative analysis of defensins to identify important molecular features that modulate their biological activity profile. Parameters such as electrostatic potential map, RMS and volume can be used in the structural analysis of mutants, helping to understand the defensins mechanism of action. Considering the absence of more effective, selective and low-toxicity antimicrobial treatments, this knowledge may help in designing these and other new molecules also contributing to the beginning of this new golden age of antibiotics so currently in need.

Keywords: Antimicrobial peptide; Defensins; Molecular modeling

Introduction

Antimicrobial peptides (APs) are molecules produced by several systems involved in animal and plant defense against various pathogens. Some of these pathogens are clinically important and resistant to conventional antibiotics [1-4].

According to the literature, APs are directly related to the innate immune response [5] characterized by a lower specificity, but rapid and efficient biological activity profile [6] APs are described with significant antifungal, antibacterial and/or antiviral activities. They can be divided into families based on structural features. Among them, defensin family is widely distributed in animal and plant kingdoms.

Lately the literature has described serious problems with the current antibiotics due to resistant bacterial strains. Depending on the multiresistant strain involved, the absence of efficient treatments turned simple bacterial infections into lethal weapons with no safer treatment options whatsoever. Thus, there is an urgent need of a new golden age of antibiotics and supporting of new researches on planning and discovering new antimicrobial drugs [7,8].

Considering this urgent need for antibiotics, the in silico tools from Bioinformatics may help in planning new molecules based on proteins and peptides such as APs. Molecular modeling tools may hugely contribute to designing of these new structures targeting on a more efficient and lower toxicity antimicrobial profile.

Therefore, in this paper, we aim to review briefly not only the structural and functional aspects of APs and their biotechnological potential, but also the feasibility of using molecular modeling tools to contribute to the beginning of this new golden age of antibiotics.

Antimicrobial peptides

Several biological systems involve the participation of proteins and peptides whose function is directly related to defense mechanisms against pathogenic microorganisms. Antimicrobial peptides and proteins were identified as antibacterial agents as they affect bacterial critical membrane functions[9] This opens for discovering new alternatives to fight these microorganisms and may enable advances in medical, veterinarian and agriculture areas [10] Interestingly, emerging evidence has demonstrated additional functions for these molecules such as chemotactic and immunomodulatory activities[9].

Antimicrobial peptides present generally highly basic and hydrophobic residues, with about 10kDa. They may present different conformations characterized by the presence of hydrophobic and cationic sites spatially organized in distinct regions of the molecule [2].

Among some APs, there are linear molecules whose mechanism of action involve the insertion into the pathogen target membrane and a conformational change to an alpha-helix structure (e.g. Cecropin and magainin, isolated from moth and frog, respectively) [11,12] Differently there is another group of antimicrobial molecules characterized by a rigid antiparallel β-sheet structure, stabilized by disulfide bridges (e.g. defensins) [13–16].

Proteins with linear predominance of one or two amino acids such as indolicidin and PR-39 are frequently found in organisms such as in neutrophils from cow (Bos taurus) and pig (Sus scrofa) respectively (Figure 1). The indolicidin has a large amount of tryptophan residues, while the PR-39 is rich in proline and arginine [17].

The mechanism of action of APs is not fully established and several hypotheses have been suggested, including: a) the pathogen membrane depolarization with the formation of pores and loss of cytoplasmic contents [18]; b) changes in the normal distribution of lipids between the two layers resulting in the collapse of the membrane [12]; c) activation of the cell death process such as hydrolases activation that degrades the cell wall [19]; d) intracellular damage after internalization of these molecules [20]. In general, the primary target of these proteins are components of the lipid bilayer of the pathogens plasma membrane [21].

The composition of the membrane of pathogenic micro-organisms favors the interaction with APs since the outer portion of the lipid bilayer is rich in phospholipids with negatively charged polar head groups. This constitutes an affinity and selective factor that allows this specific interaction between the membrane and these peptides. This is allowed by the plasma membrane of mammals and plant cells that is rich in lipids with polar head groups containing no negatively charged feature [22].

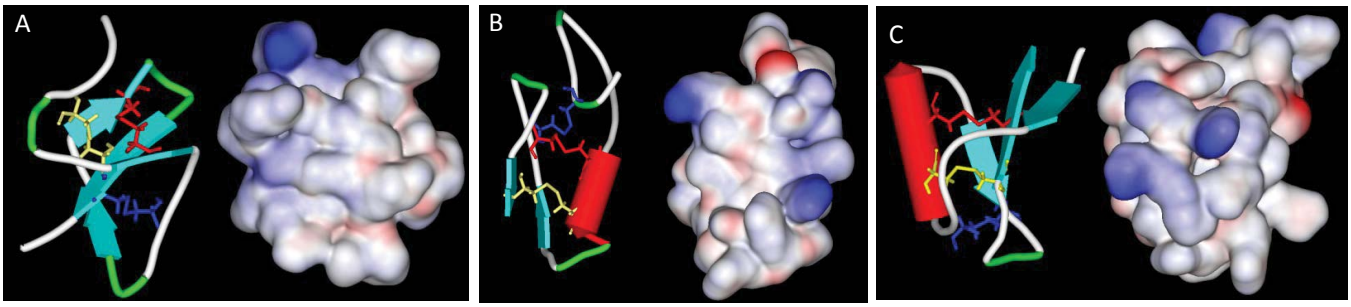

Figure 1: Some defensins from different origins showing the 3-D fold with the secondary structures (a-helix in red tube and b-sheet in light blue arrows), dissulphide bridges (yellow, red and dark blue) (left); and the electrostatic potential surface with the cationic and anionic charged features showed in blue and red respectively (right).(A) Bovine neutrophil beta-defensin-12 (BNBD-12) with the triple-stranded beta-sheet and the beta-bulge preceding the hairpin. (B) The cationic/hydrophobic amphiphilic character is also observed. (PDB ID: 1BNB). (C) Defensin DEF-AAA from the insect *Anopheles gambiae (*PDB ID: 2NY8); and MtDef4, a defensin from the legume *Medicago truncatula*.

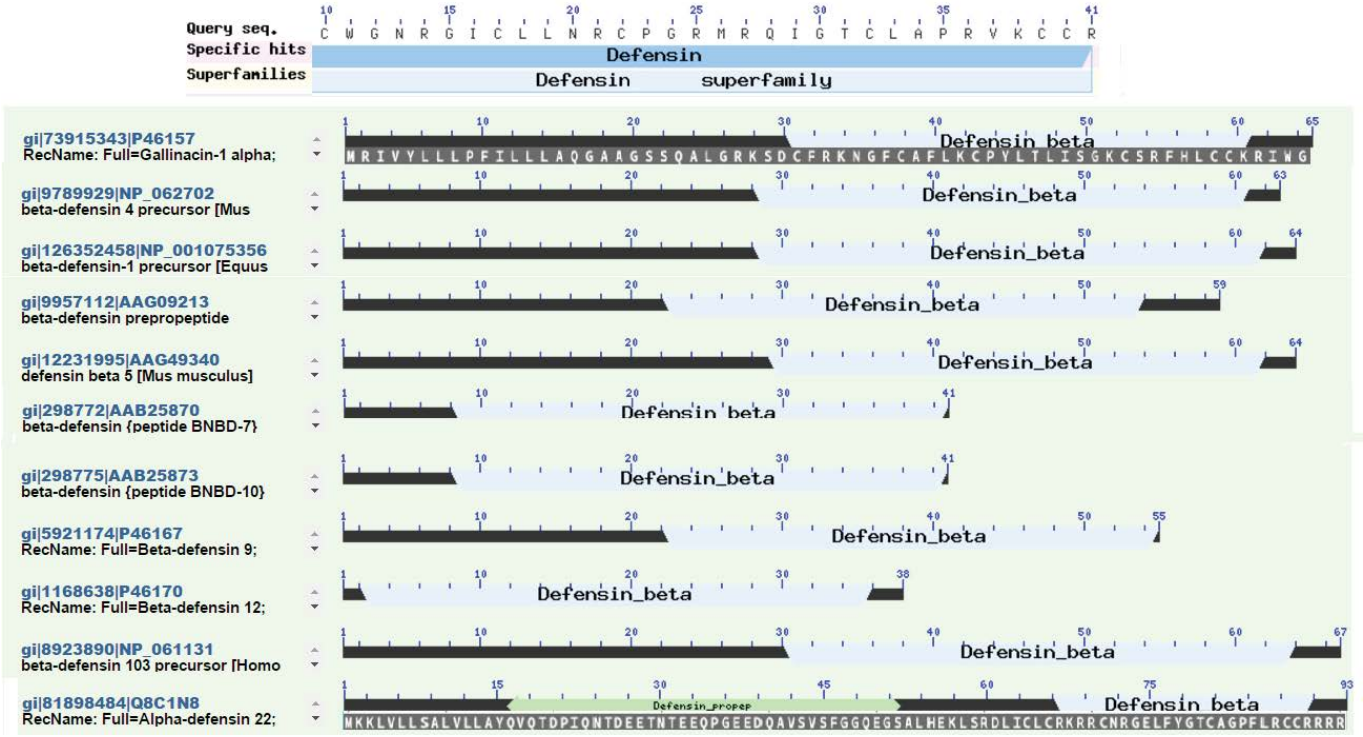

Figure 2: Comparison of some sequences features including size and localization of the defensin domain position by using NCBI site programs.

Defensins structure

The structural variation of APs allows classifying into different families, according to their similar characteristics. Among them is the defensin family (Figure 2), which includes proteins having a compact three-dimensional structure provided with at least one sheet-β. For insects and plant defensins, the structure includes the presence of a α-helix stabilized by disulfide bonds. This profile is observed for isolated mammalian, plants and insect defensins that have two or three antiparallel β sheets [23] (Figure 1).

Defensins are small cationic cysteine rich peptides, which usually contain 18-45 amino acids with a molecular weight of about 5kDa. These molecules display amphiphilic properties and are from different origins [16,24–27].

In general the mammalian defensins are widely found in the animal body, acting as antiviral, antifungal and antibacterial agents. The mammalian defensins are classified into α-, β- and θ- defensins based on their disulfide bonds. Only α- and β-defensins of about 29-45 amino acids are expressed in humans [28,29] Among these amino acids, six cysteines are of importance as they are distributed throughout the molecule, forming three disulfide bonds that are different in α and β - defensins. β-sheets are arranged anti-parallel in both α and β-defensins, but only in β-defensins there is a small α-helix in the N-terminus. The α-defensins are present in blood cells, whereas β-defensins can be found in epithelial tissue and fluids. [28–32].

θ-Defensins are much smaller (18 amino acid residues) than α - and β-defensins. θ-defensin (found in rhesus monkey) displays the same connectivity associated with that of the α-defensins, but is not functionally present in humans. [28,30–32].

The insect defensins are about 40 amino acids, containing six to eight cysteines that are involved in the formation of three to four disulfide bridges. They also present antifungal and antibacterial activities [2,3] Differently plant defensins have around 45 to 54

amino acids with four intramolecular disulfide bonds[33] The three dimensional structure of these defensins have been experimentally determined, revealing highly homologous structures. A segment-type Cys-xxx-Cys in the α-helix is connected via two disulfide bonds to Cys-x-Cys segment of the third β-sheet. This structural pattern called α-helix stabilized by cysteine can also be observed in defensins and the insect neurotoxin from scorpion venom [34] Molecules such as Ta-g1PT from Triticum aestivum [14] , Rs-AFP1 from Raphanus sativus [35] , Ah-AMP1 from Aesculus hippocastanum [15] Psd1 from pea [16] and PhD1 from Petunia hybrida [36] are examples of some defensins structurally described. Comparative structural analysis revealed that the first loop is connected through a disulfide bridge to the end of the first and second β-sheet and connected with the last β-sheet by a third disulfide bridge. An exception to this rule all was seen in PhD1 defense, which has an additional disulfide bridge connecting the first handle of the α-helix [36].

Defensins Biological activity

Overall the plant defensins have few highly conserved amino acids. However a much higher identity can be noticed among some members of this family. Therefore, Land and collaborators proposed the classification of plant defensins in at least three separate groups (I, II and III) based on the conservation of some amino acids and on the homology among their primary chains. [25,37,38] There defensins such as Psd1 that has no sufficient primary sequence identity with members of the groups I, II or III, which raises the possibility that other distinct groups may exist [39].

A great number of the isolated defensins plants have antifungal activity. Among these are those from group I and II. [24,25,37,38] Some defensins from group III has the ability to inhibit α-amylase with no antifungal activity [25] Other defensins, such as Spinacia seed oleracea [26] present antifungal activity and antibacterial.

Because the growth of hyphae is a calcium channel dependent process via second messenger, defensins, particularly those from group II, may interact with specific receptors from fungi membrane or cell wall [38] Importantly, defensins with antibacterial and antifungal profiles and derived from mammals, insects or plants change in the presence of cations. They are dependent on the characteristics of the target organism.

Currently, according to the literature, the main effect of defensins on fungal growth is the microorganisms membrane permeabilization (Figure 3). Studies involving the fungi *N. crassa* incubated with defensins such as Dm-AMP1, Hs-AFP1 and Rs-AFP2 at a concentration 100 times greater than the IC_{50} revealed the induction of formation of nonspecific pores throughout the hyphae of the fungus. These studies revealed that the same concentration of defensins Dm-AMP1 and Rs-AFP2 required to inhibit the growth of the fungus, induced an increased ion flow in the membrane (Ca^{2+} influx, K^{+} efflux) with alkalinization of the environment. [40–42] The

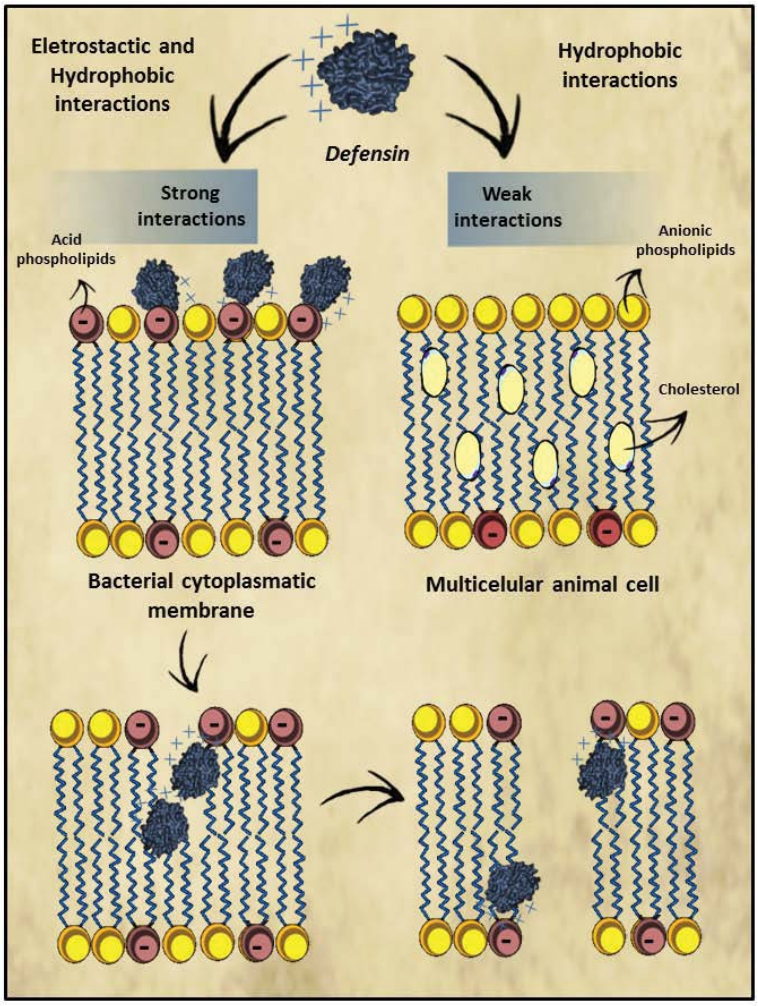

Figure 3: The biological mechanism of APs. Initially the electrostatic attraction and the transmembrane bioelectric field pull the defensin towards and into the pathogen membrane. Then the pathogen membrane is depolarized and pores are formed with subsequent loss of cytoplasmic contents.

Ca^{2+} gradient within the hyphae is of great importance for the fungus growth, leading to the cytoplasm release of intracellular vesicles mediated by inositol 1,4,5-triphosphate (IP_3). The Ca^{2+} channels present in the plasma membrane play a key role in maintaining the calcium concentration inside the cell. In the hyphal growth process, K^+ is also of great importance, and its influx and sometimes efflux are essential to the viability and growth of these cells. Therefore, affecting the channels conductivity causes variation in intracellular pH, which, among other factors, inhibits protein synthesis, growth, and cause the cell death [43–45].

Some defensins can form dependent-ion channels in the cytoplasmic membrane as well as in artificial phospholipid vesicles[46] In these cases, the microbicidal effect is attributed to a disturbance in the electrochemical cell due to the production of these ion channels. This lead to damage to the whole cellular machinery, resulting in death (e.g. Mammalian defensins)[47].

The hypotheses involving the plant defensins mechanisms of action are: a) binding of defensin to a sphingolipid of the fungus membrane and subsequent association with receptors or ion channels of the pathogen membrane; b) binding of defensin to the membrane receptors whose effect is mediated by second messengers include sphingolipids such as M (IP) 2C, c) binding to a membrane receptor or ion channel whose transit from the endoplasmic reticulum to the plasma membrane and that depends on the presence of M (IP) 2C. These factors would promote a change in ionic concentration in the fungus cytoplasm, resulting in inhibition of the growth of hyphae [48].

Nuclear Magnetic Resonance (NMR) and molecular dynamics have allowed identifying interaction sites for molecules such as defensins. Mutations that generate variation in Root Mean Square values and/ or the electrostatic potential of defensins are interesting for determination of important amino acids in the biological characteristics of active proteins. These mutations are likely to enhance or compromise the action of these peptides, which may guide the design of new molecules with great biotechnological potential. Mutations that promote change in RMS, the electrostatic potential and volume of the molecular structure of defensins may be interesting for future experiments that will study the dynamics of mechanisms of action of these defensins, especially in their interaction with the fungal or bacterial membrane [16].

Evidence suggests that naturally occurring or synthetic antimicrobial peptides (AMPs) could be a model for the design and creation of new antibiotics as the Brilacidin a defensin-mimetic compounds. This molecule was shown to have an activity against S. aureus, Enterococcus faecium as well as Gram-negative species (e.g. *E. coli*). This molecule was described as bactericidal and selective against bacterial cells by specifically disrupting the microbial membrane. A phase II study enrolled 215 patients with acute skin and skin structure infections who were randomized into four different groups, with three different doses of brilacidin and daptomycin. Patients were evaluated at days 3, 7, 10 and 28 monitoring for evaluating the clinical response and sustained response [49].

In order to understand the potential of the in silico environment for designing new antimicrobial based on defensins we need to understand more about their effects and on that matter table 1 list some references that can help on increasing this knowledge about them (Table 1).

In order to approach defensins knowledge in a more applied and in silico perspective, we should also understand and know more about the Bioinformatic area and its tools. This knowledge may help on designing new molecules based on the studies involving defensins, their analogues structure and targets.

Bioinformatics a brief view

In the last century, scientists had questioned about the chemical nature of the genetic material. On that time, the researchers came to the conclusion that the DNA was the molecule that stored genetic information and thus in 1953 its chemical structure was unveiled on the classic work of Watson and Crick [50,51].

The discovery of the genetic code and the flow of biological information (nucleic acids encoding proteins) allowed the emergence of new scientific areas such as molecular biology. Soon sequencing methods arose, especially for DNA, and since then, many these sequences have been reported and are available in public databases [52].

In the second half of the 90s, after the creation of automated DNA sequencing machines, an exponential number of decoded sequences needed to be stored. Thus more efficient computing resources were required as well as the analysis of such data demanding efficient computing platforms for interpretation of these results [53].

In this scenario emerged Bioinformatics, a scientific area that includes several different knowledge, including software engineering, mathematics, statistics, computer science and molecular biology among others [54] It is an inter and multidisciplinary area resulting from the initial interaction between biology and computer science [54–56].

Bioinformatics came up with the initial purpose to organize and analyze complex data from modern techniques of molecular biology, biochemistry and genetics. It works through the database maintenance and creation of programs to simulate biological phenomena observed in living organisms. This new area of knowledge is based on the existence of a hierarchical relationship among the structures of genes, their arrangement relative to the genome, the protein and peptide structure and their functions and interactions among them and/or in a particular organism, resulting in diverse processes. Thus, Bioinformatics can be considered an important "tool" to understand how the information contained in genes is reflected in physiological and pathological processes [57,58] In general, the first research on proteins and peptides such as defensins is initially guided by getting data obtained by two-dimensional electrophoresis and mass spectrometry techniques together with a functional analysis.

One of the great challenges of Bioinformatics comes in the post-genomic era. Proteomics becomes a major target of study to understand the relationship between the proteins structural and functional profiles [59].

After collecting the information regarding the isolation process and protein/peptide identification, the next steps aimed at the 3-D structural characterization of the molecule [60] Through Bioinformatics it is possible to search and evaluate a complex set of crystallographic data from X-rays and nuclear magnetic resonance (NMR) to construct three dimensional models of protein and peptides by comparative molecular modeling [61,62].

The rapid increase in the number of 3D structures available in databases such as the Protein Data Bank (PDB) has provided the creation of the molecular modeling subarea and its tools. This allowed the emergence of guidelines, structural analysis, visualization and storage of structural information at atomic levels with prediction of protein/peptides, receptors and complex structures, becoming an area of great interest. The comparative molecular modeling approach based on the homology of protein primary sequences has emerged as a major category [63].

Description from / of the Reference	Keywords	Reference about Defensin Related Subject
"This review introduces opportunities for insect-derived AMP production, like the choice of the expression system…All of these aspects are discussed with regard to large-scale processes and costs."	antimicrobial peptides, large-scale purification, large-scale production, elastinlike, polypeptides, intein	Müller et al., 2015. Biotechnol Prog. 31(1):1-11
"It highlights 3D structure-based design of peptide antimicrobials and vaccines, surface coating, delivery systems, and microbial detection devices involving antimicrobial peptides."	antimicrobial peptide; bacterial detection; biofilms; mechanism of action; nanoparticle; peptide discovery; sensors; structure-based design; surface coating	Wang et al., 2015. Pharmaceuticals (Basel). Mar 23;8(1):123-50.
"This article provides a critical review of the empirical data to shed light on the wider role of Antimicrobial peptides in the robust and resource-effective defense responses of plants."	PTI/ETI; antimicrobial peptides; hormonal cross-talk; plant immunity; sugar signalling	Bolouri et al., 2015. Mol Plant Pathol. 29. doi: 10.1111/mpp.12299.
"The purpose of this paper is to introduce and highlight a few classes of traditional antimicrobial peptides with a focus on structure-activity relationship studies…"	antimicrobial peptides; mechanism of action; peptidomimetics	Mojsoska 2015 Pharmaceuticals (Basel). 13;8(3):366-415.
"Overview focuses on natural and synthetic antimicrobial peptides in human and experimental sepsis and their potential to provide significant improvements in the treatment of critically ill with severe infections."	Sepsis, Antimicrobial Peptides, Synthetic, Naturally Occurring Peptides, Therapy .	Martin 2015 Front Immunol. 20;6:404.
Editorial briefly approaching some articles about Antimicrobial Peptides as therapeutic agents.	Antimicrobial peptides, potential applications, therapies.	López-Meza et al., 2015 Res Int.; 367243.
"It considers the anti-infective properties of short AMPs lacking disulfide bonds, which are active against dermatologically important microflora… and consider the challenges that need to be addressed to facilitate the prophylactic application of AMPs in personal care products."	Anti-infective, Antimicrobial peptides, Cosmetic industry, Dermal pathogens, Prophylaxis, Skin diseases	Mohammad et al., 2015 Appl Microbiol Biotechnol 99(21): 8847–8855.
"It shows the potentiating functional interaction of co-occurring insect Antimicrobial peptides (the bumblebee linear peptides hymenoptaecin and abaecin) resulting in more potent antimicrobial effects at low concentrations.'	antimicrobial peptides, innate immunity, insects, abaecin, hymenoptaecin, bumble bees	Rahnamaeian M et al. 2015 Proc. R. Soc. B 282: 20150293
"This review describes modern research into bioengineering honey and venom from bees, silk, cantharidin, antimicrobial peptides, and maggot secretions and anticoagulants from blood-sucking insects into medicines. Problems and solutions encountered in these endeavours are described …	Insect; natural products; medicines	Ratcliffe et al., 2014 Evid Based Complement Alternat Med. 904958
"The unique activities and features of AMPs are discussed, with a focus toward the clinical importance of priming the antibiotic pipeline and the role AMPs can fulfill in the future of fighting drugresistant bacteria."	antimicrobial peptides; drug-resistant bacteria; peptide design	Steckbeck et al., 2014 Expert Opin Biol Ther. 14(1):11-4
"It summarizes the newest studies of drug development using cationic antimicrobial peptides as lead molecules for novel antimicrobial drugs."	Cationic antimicrobial peptides, therapeutic agents, neonates, children	Ashby et al., 2014 Curr Opin Infect Dis. 27(3):258-67.
It discusses current knowledge and recent progress in several classes of insect AMPs, including ins"ect defensins, cecropins, attacins, lebocins and other proline-rich peptides, gloverins, and moricins, with a focus on structural-functional relationships and their potential applications	alpha-helical peptide, cysteine-rich peptide, glycine-rich peptide, proline-rich peptide, lipopolysaccharide, conformational changes	Yi HY et al,. 2014 Appl Microbiol Biotechnol. 98(13):5807-22.
"In this mini-review, we will discuss current knowledge and recent progress in several classes of insect AMPs, including insect defensins, cecropins, attacins, lebocins and other proline-rich peptides, gloverins, and moricins, with a focus on structuralfunctional relationships and their potential applications."	Alpha-helical peptide . Cysteine-rich peptide .Glycine-rich peptide . Proline-rich peptide .Lipopolysaccharide . Conformational changes	Yi et al., 2014 Appl Microbiol Biotechnol, 98(13):5807-22.
"This article reviews an area of increasing interest, namely self-assembled peptides and their potential therapeutic applications as innovative hydrogels and biomaterials in the prevention of biofilm-related infection "	antimicrobial; bacteria; biofilm; biomaterial; infection; peptide; self-assembly	McCloskey et al., 2014 Pathogens. 2014, 3(4):791-821.
"..this review makes a few considerations about those molecules as a potential new class of antimalarial ..."	AMP, amphipathic, antimalarial, antimicrobial, cationic, membranolytic, peptide, Plasmodium spp.	Vale et al., 2014 Front Pharmacol.5:275.

Table 1: Some recent references from the last 2 years that may be taken into account when looking defensins as potential therapeutic and/or biotechnological agents yet to be explored.

The molecular modeling enables the analysis of genomic and proteomic data, providing the acquisition of large amounts of information on different molecules that are sometimes important as therapeutic targets not only for the treatment of different pathologies, but also for understanding the intricate process of establishing and maintaining them. This information is stored in public databases and can be crossed, analyzed and compared to get information relevant to the advance of biological and technological researches [64].

Molecular Modeling tools for working with defensins

Molecular modeling is the use of computational chemistry and graphical visualization techniques in order to provide a three-dimensional representation of molecular structures as well as their molecular properties [65] In a broader view, molecular modeling comprises different theoretical or computational methods used to describe and predict molecular structures; transition state and thermodynamic properties and reactions; among others. The main purpose is to predict and understand the behavior of real systems. Such methods include studies of minimization energy of the molecules, conformational analysis and molecular dynamics simulations, being applicable from single atoms to biomacromolecules [62].

The molecular modeling involves calculations of selected parameters for a particular molecule, providing information about

their properties. The methods of estimation or calculation of molecular properties can be divided into:

- Interpolation: where the correlation is found between the desired property and other properties and molecular characteristics;
- Extrapolation: wherein the correlation does not include the molecule of interest, but by extrapolating the correlation, it is possible to estimate the desired property.
- Computational calculations: calculations of molecular properties held on the in silico environment of the computer, varying several parameters that can replace and/or be compared with experimental measurements [66]

Some properties such as HOMO and LUMO distribution and energy, dipole moment, hydrophobic character, molar refractivity (MR), the partition coefficient (LogP), among other atomic and electronic characteristics can be obtained by theoretical methods for calculation computing properties. The three main theoretical methods for these calculations are classified into: ab initio calculations, semi-empirical and empirical methods or molecular mechanics [67].

- Ab initio: derivative methods of quantum-mechanical (e.g. Gaussian) and include sets of STO-3G bases 6-31 + G *, G ** ++ 6-311, among others. These methods are purely theoretical and demand more computational time than the others. It can be used for molecules with up to 100 atoms.
- Semi-Empirical: These methods are also derived from quantum mechanics, but are not entirely theoretical. Several molecular modeling programs such as Spartan and MOPAC may include the Hamiltonian - mathematical operator functions used in molecular orbital calculations as AM1 (Austin model 1) and PM3 (Parametric method 3), among others. These methods may be somehow inaccurate depending on the approach, such as conformational analysis, and are used for molecules of less than 1000 atoms.
- Molecular Mechanics: empirical methods based on experimental values such as CHARMM that MMFF force fields. They are used for molecules with up to 100,000 atoms and require less computational time than other methods [68].

The molecular mechanics and semi-empirical methods involve calculations through parameterization, while ab initio calculations reproduce an experiment without using parameters. The best results in the first two methods are applied in interpolation with experimental data. In general, these methods differ depending on the nature of the force field. [69] In order to obtain more reliable data on energy, new semi-empirical methods were introduced as MINDO-3 (Modified Intermediate Neglect of Differential Overlap) and MNDO (Modified Neglect of diatomic Overlap). The latest methods used parameterizations derived from ab initio calculations based on extended Huckel theory and CNDO program (Complete Neglet of Differential Overlap), which help the orbital calculations and loads [67] It is important to notice that all methods have limitations and that each one works differently from others, having its own application within the molecular modeling.

Homology or Comparative molecular modeling for studying defensins

Gene mutation during the replication process is associated with evolutionary mechanisms that eventually form families of related proteins [70] These protein families may have some differences in the amino acid sequence; however they maintain a high degree of conformational similarity. Proteins that evolve from a common ancestor can be substantial conformational identical, or similar to in many aspects or even quite different due to various mutations [71] An important concept in the homology modeling approach is that the sequence similarity may be smaller and less conserved than the 3D final structure [72].

The three-dimensional structures of homologous proteins can be preserved during the process of evolution. This is even more important regarding to functional residues, as the conservation of the structure is critical for the maintenance and performance of specific functions [73] The differences among homologous proteins occur more frequently in “loops”.

Physicochemical properties of the mutated residues are sometimes very different from the original structure [73] In general, residues located within the proteins vary less frequently, and when they do occur, usually with less distinction of physicochemical properties. The amino acids which simulate the core of the protein and the main elements of the secondary structure preserved in a family of homologous proteins [72].

The homology modeling is a method that considers the structural conformation of a protein often more conserved than its amino acid sequence during the evolutionary process. Small changes in the sequence, in general, result in subtle changes [74] Thus, for predicting a protein three-dimensional structure by comparative modeling is necessary to find at least one sequence homologous protein with 3D structure experimentally defined and available [75].

The process of constructing a virtual protein model such as for new defensins through molecular modeling by homology involves four main steps: a) search for homologous proteins, b) alignment of the sequences, c) the construction and optimization of models, d) validation of the final structure (Figure 4) [76].

a) Search of homologous proteins

This first step should identify proteins with three-dimensional structures experimentally defined, by crystallographic methods or Nuclear Magnetic Resonance (NMR), and that present sequence similarity to the desired defensin (Protein Target). These proteins will act as templates for creating defensin 3D model [76] This identification can be performed taking into account several aspects, such as: similarity function, expression by the same group of genes, sequence similarity and/or evolutionary correlation [77].

There are some programs freely available that search for homologous sequences in databanks, for example, “Basic Local Alignment Search Tool” (BLAST) [78] This tool uses statistical formulas to evaluate the degree of similarity between fragments of the sequences (local alignment) as well as to distinguish important similarities of biological or structural standpoint, and random coincidences that may not show any significant importance. The search may also be conducted in a database of 3D structures, such as the PDB. The introduction of the sequence of the target protein allows determining sequence similarity and identity. The secondary structure prediction for the target sequence based on this search becomes one main aspect in the construction of the molecular model of the target protein [79].

b) Alignment of sequences

After checking the structural correlation between the target protein and the template selected in the first step, it is necessary to perform a sequence alignment. The main purpose is to identify the correlation between the amino acid residues of each sequence

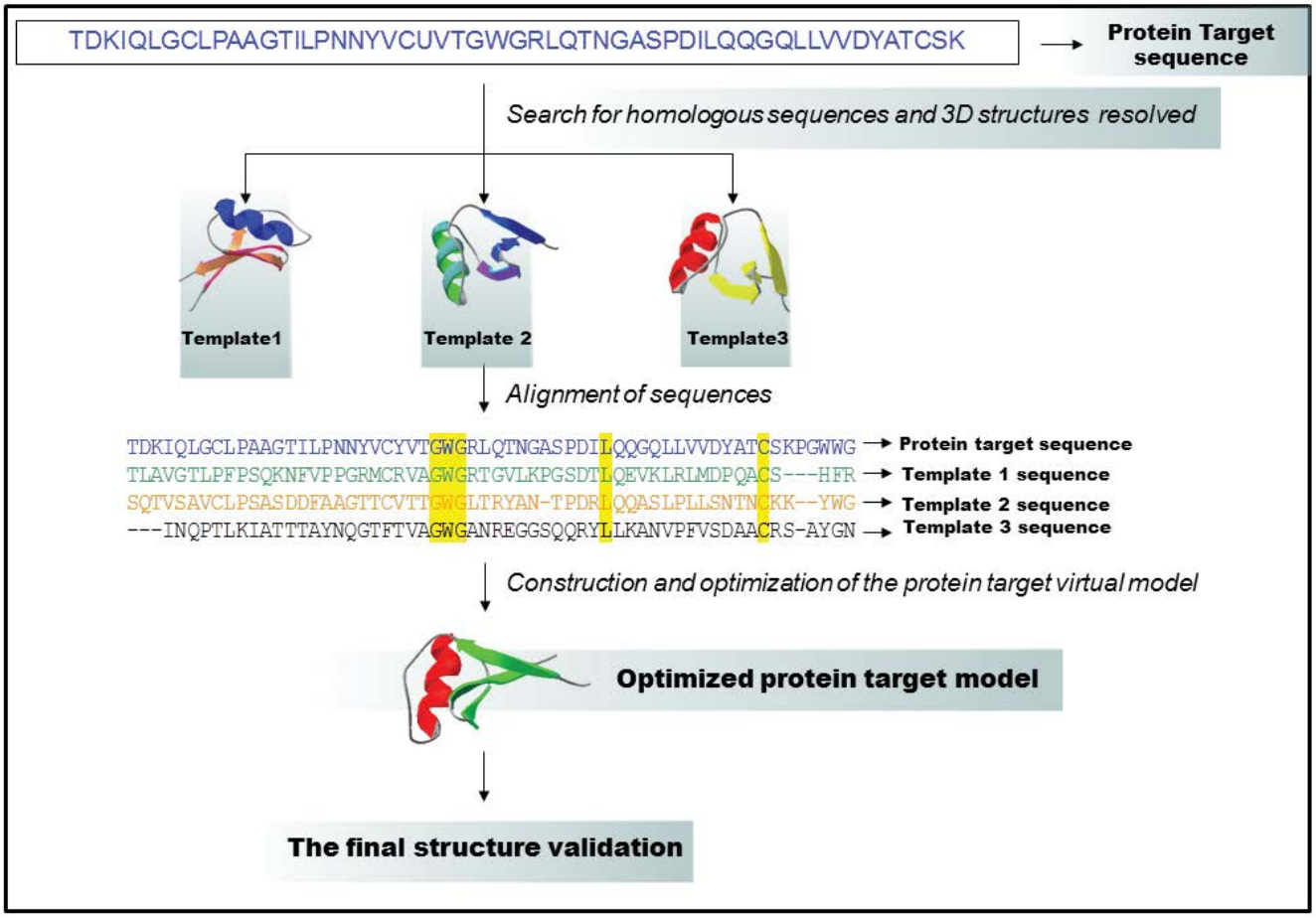

Figure 4: Main steps of molecular modeling by homology to construct a protein model.

[76] When only one protein is identified, it is held only an alignment "pairwise" with the target sequence. When multiple templates are identified, it becomes necessary to obtain a multiple alignment, in which all sequences are aligned together with the target sequence. To generate a final alignment it should be used a more specific software such as the computer package "Alignment of Multiple Protein Sequences "(AMPS) and CLUSTAL [80] Programs in this category originate an alignment that spans the entire polypeptide sequence.

The acceptable range of sequence identity for using homology/ comparative molecular modeling includes values above 25-30% sequence identity between the template and the target protein. This value is dependent on the number of residues of the protein to be modeled, being less critical the larger the sequence length [81].

c) Defensins model construction and optimization

The methods used in the automated construction of structural homology models employ optimization techniques of geometric distances to satisfy space constraints. This automated approach for homology modeling was inserted in programs such as Modeller and Swiss Model [82].

In these programs, the alignment between the template and the target sequence serves as the "input data" to the structure prediction of the molecule. The "output data" generated is a set of atomic coordinates for the number of 3D models chosen by the user for the target protein. From the alignment of the sequences, the program calculates various restrictions distances and torsion angles in the target sequence, formulating extra parameters that are added to the force field to orient the calculations in a certain direction [83].

Primarily a newly generated virtual model does not present an ideal geometry. Thus, a geometry optimization process involving energy minimization should be applied to the model. In this context, the molecular structure will be subjected to a relaxation process so that the minimum energy state is achieved.

The model virtual optimization occurs primarily through force fields based on molecular mechanics. They have the advantage of being quick and allow results with a good accuracy level. Through molecular mechanics calculations of the total energy of the atomic coordinates, it is minimized according to pre-defined force field parameters. AMBER and CHARMM are examples of force fields often used for macromolecules. Because of the complexity and the high computational demand, mechanical-quantum methods are reserved only for some specific cases [72].

Another method is molecular dynamics related to molecular mechanics, which can be applied to the optimization of geometry models generated by comparative modeling. The molecular dynamics simulations comprise the Newton's equations of motion to perform a systematic conformational search to determine the lowest energy conformation of a flexible molecule. Theoretically, it generates all possible conformations of a given model [84].

d) The final Structure Validation

An important step in the homology modeling process is the evaluation of the quality and reliability of the models [85] From the moment in which a model is generated, the quality and reliability levels should be determined. This depends on several properties present in different degrees of structural organization such as

stereochemistry accuracy and folding reliability according to the chemical environment of the residues [72].

There are several softwares available, including Prochek, What If and Verify3D. To check the stereochemical quality of the models, the accuracy of parameters such as bonds length and angles, torsion angles and chirality of amino acids needs to be evaluated [72] The Procheck program evaluates various stereochemical parameters such as torsion angles of the main chain (Φ and Ψ), torsional angles of the side chains, forbidden contacts or steric hindrance, energy of hydrogen bonds, planarity of the peptide bonds, α-carbons deviations from the tetrahedral geometry, among others [86].

Several methods that employ information from protein structures experimentally determined (e.g. Globular proteins) may be used to estimate the quality and stability of the model structure [72] Assuming that the atom-atom interactions are crucial in the formation of a protein, Nabuurs and Vriend developed the program What If, which checks the quality of protein models of packaging through calculations of the so-called contact quality content. This index is a measure of correlation between the distribution of the atoms around the side chain of an amino acid and equivalent distributions observed in 3D-Known proteins. Therefore, currently there is a database that contains the distribution of atomic contact probability for all amino acid side chains. This database describes the probability of an atom being in a particular region around the side chain. The probability values are used for evaluating the quality of atomic contacts in the model. The greater the correlation between the model and the crystallized structures, the higher will be the quality index [87].

In order to determine the reliability level of a model folding, the quality of chemical environments can also be assessed. The Verify3D program [88] can be used for this purpose. It determines the chemical environments of each model residue and assigns a score with reference to a matrix constructed from a statistical analysis involving protein structures from PDB. The program detects low quality regions and the sum of these points is compared

Defensins and Analogues	Species Targeted	Biological Activity
Indolicidin Bovine neutrophils	*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium*	Antibacterial
Cecropin A	*Acinetobacter bauannii, P. aeruginosa*	Antibacterial
Gageostatins 1-3	*P. aeruginosa*	Antibacterial
DefMT3, DefMT5, DefMT6 (Ixodes ricinus)	*E. coli; P. aeruginosa*	Antibacterial
Omiganan Indolicidin analogue	*E. coli. P. aeruginosa, E. faecalis, Klebsiella* spp., *A. bauannii*	Antibacterial
Tritrpticin Porcine	*E. coli, P. aeruginosa, Klebsiella pneumoniae, Proteus mirabilis*	Antibacterial
Bovine lactoferricin analogues LFB17-27 e Octa1	*E. coli, P. aeruginosa*	Antibacterial
Bovine lactoferricin analogues LfcinB20-25, *LFB 4-14 e Undeca 9*	*E. coli*	Antibacterial
Copsin	*Enterococcus faecium and Listeria monocytogenes*	Antibacterial
Bovine Indolicidin	*Staphylococcus aureus, S. epidermidis*	Antibacterial
Omiganan Indolicidin analogue	*S. aureus, S. epidermidis, Streptococci* spp., *Corynebacterium* spp., *E. faecium, Bacillus ssp.*	Antibacterial
Bovine Lactoferricin (LFB17-41)	*S. aureus*	Antibacterial
Gageostatins 1-3	*B. subtilis, S. aureus, S. typhimurium)*	Antibacterial
Def1, Def2 and Def3 from *Tribolium casteneum*	*Micrococcus luteus, B. thuringiensis serovar tolworthi, S. epidermidis*	Antibacterial
Bovine lactoferricin analogues LFB17-31 and LfcinB17-31	*S. aureus, Bacillus subtilis*	Antibacterial
Bovine lactoferricin analogues LFB17-27, LFB 4-14, Undeca 9, Octa 1	*S. aureus*	Antibacterial
Tritrpticin Porcine	*S. aureus, P. aeruginosa, S. epidermidis, Streptococcus* group D	Antibacterial
Ixodes ricinus defensins	*Fusarium spp*	Antifungal
Gageostatins 1-3	*Rhizoctonia solani, Botrytis cinerea, and Colletotrichum acutatum*	Antifungal
Indolicidin Bovine neutrophils	*Trichosporon beigelii, Candida albicans, Saccharomyces cerevisiae*	Antifungal
Tritrpticin Porcine	*Aspergillus fumigatus*	Antifungal
Bovine Lactoferricin (LFB17-41)	*C.albicans, C.tropicalis, C. neoformans*	Antifungal
LFB17-31 or LfcinB17-31 Synthetic, bovine lactoferricin analogue	(*Penicillium digitatum, P.italicum, P. expansum, Alternaria sp., Aspergillus nidulans, Botrytis cinerea,F.oxysporum, Magnaporthe grisea)*	Antifungal
LfcinB20-25 Synthetic, bovine lactoferricin analogue	*S. cerevisiae, P. digitatum, P. italicum, P. expansum, Alternaria sp., A. nidulans, B. cinerea, F. oxysporum, M. grisea)*	Antifungal
Indolicidin Bovine neutrophils	Herpes simplex virus - HSV	Antiviral
Indolicidin analogue	Human immunodeficiency virus - HIV	Antiviral
10R, 11R Indolicidin analogues	Tobacco mosaic virus - TMV	Antiviral
Bovine Lactoferricin (LFB17-41)	Human Cytomegalovirus (HCMV), HSV	Antiviral
Indolicidin Bovine neutrophils	*Giardia lamblia*	Antiparasite
α-defensin-5 (DEFA-5)	Toxoplasma gondii	Antiparasite
Prophenin 2	Trichomonas vaginalis	Antiparasite
Bovine Lactoferricin analogue LFB17-41	anticancer, apoptosis inducer, antiangiogenic	Anticancer
Bovine lactoferricin analogue LTX-302	Anticancer activity	Anticancer
Cecropin A	Anti-inflamatory	Antiinflamatory

Table 2: Some defensins and analogues and their current targets that can be explored in the context of *in silico* studies.

to the ideal expected value for a protein of equal length. The sum should be greater than 45% of the expected value (ideally), which is the range found in good quality and resolution structures [89].

Defensins: why exploring through theoretical tools

At various stages of a rational planning process of drugs based on the structure, there are several applications for the structural information of defensins and those models generated by homology. The contribution of structural information of defensins can significantly help in the identification and optimization of compounds or mutants that become candidates for clinical trials [76] This should not be ignored due to the different targets of the defensins known by far (Table 2). These tools may also help on identifying structural origins of defensins, such as described by Zhu in 2007 [100].

Since it is important to consider the ligand-target interactions, current computer simulations seek dynamic aspects of the drug-receptor complexes to provide the best description of the biological processes, which is key for drug development. The homology models can be applied to identify the interactions with the active site of the therapeutic target. Studies of point mutations may be combined with in vivo and / or in vitro data as well as computer simulations involving modeling also the target. This strategy enables the identification of amino acid residues which are functionally or structurally important in both structure (e.g. defensins and receptors) [90].

The structural information available from a homology model may allow the determination of the influence of mutations for the protein structure and activity profiles [91] In the literature several examples of using the comparative modeling may be described [74] Ring and collaborators in 1993 identified protease inhibitors based on the use of molecular modeling by homology. In 2004, Park and Lee described the optimization of histone diacetilase inhibitors activity through computer simulations involving models generated by homology.

Vangrevelinghe and collaborators generated homology models that were applied to identify potent inhibitors of protein kinase CK2 in a Novartis collection of 400,000 compounds [92–94] In case of defensins, different parameters of the model could be correlate with these biological activities. These parameters could enhance the affinity of drug to microorganisms or the membrane destructive capability and because of these ilimited possibilities that thes proteins and their analogues should be explored in silico.

Several molecular targets are described in the literature by using molecular modeling to create new drugs [74,95] It is possible to develop compounds based on models by homology and then use them as tools in the study of the physiological role of the target protein. These simulations help in identifying new drugs that come from databases, with great potential for interaction with the target protein [96].

The defensins mechanism of action is based on the interaction of its amino acids with several targets in the microorganism involved. Through the comparative molecular modeling is possible to study all residues involved in this inhibitory process, allowing mutations and evaluation of their stereoelectronic profile, simulating their main interactions. Recently, Huang and coworkers (2015) reported the use of the comparative molecular modeling techniques to study the site-directed mutagenesis effect on antimicrobial profile of Porcine Beta Defensin 2. This defensin has broad antimicrobial activities against bacteria and plays an important role in host defense.

In order to enhance its antimicrobial activity and better understand the effect of positively charged residues on its activity, this study has proposed the replacement of eight amino acid residues with arginine or lysine respectively using mutagenesis tests and computational techniques. All mutants were cloned and expressed in BL21 (DE3) plysS and the mutant proteins were then purified. These mutants had higher positive charges, but similar structural configurations to the wild-type pBD2. Moreover, these mutants showed different antimicrobial activities against *E. coli* and *S. aureus*. Interestingly mutant I4R of pBD2 had the highest antimicrobial activity. In addition, all the mutants showed low hemolytic activities.

The Huang and coworkers (2015) data indicated that the positively charged residues were not the only factor that influenced antimicrobial activity, but other factors such as distribution of these residues on the surface of defensins might also contribute to their antimicrobial potency. This analysis was possible only by using Bioinformatics tools [97].

On molecular modeling there are databanks to be used when constructing or testing molecules. Some of these databases are made up of molecules that have the properties "drug-like" [98]. Moreover, such models may be used in simulations of "docking" together with the structure of active molecules identified in biological assays. This may predict the binding mode of these compounds in the target binding site, and extrapolate this prediction for the success of other molecules which have not undergone experimental biological assays. This may include simulations of "docking" and molecular dynamics and free energy perturbation calculations. In these studies, the models serve as the structural basis for the support of hypotheses associated with the activity of the prototypes [92].

One challenge with the prototype optimization step is not only identifying potent compounds, but also to develop those with: a) suitable pharmacokinetic properties to achieve the systemic circulation, b) resist metabolic inactivation and c) present no or lower side effects. The structural knowledge of defensins can aid in the development of molecules that specifically interact with micro-organism proteins, with the aim of reducing toxicity and/or improve the pharmacotherapeutic profile [76].

Literature described antimicrobial peptides of about 2,000–5,000 Da that may guide the design of new highly active molecules with 600–1,000 Da (e.g. arylamides) [101]. Theoretical tests for these and other new molecules such as regarding cardiovascular or hepatic toxicities may be helpful and may receive special attention in the drug development process [99].

Given the potential of molecular modeling by homology and theoretical toxicologic tests, they may enlighten the structure-activity relationship of defensins of animal or plant origin. The role as a pre-analysis of experimentally site-directed mutation methodology is obvious and also permits to observe significant changes that may influence the biological action of defensins.

Conclusion

Defensins are widely distributed in nature with different mechanisms of action and a common goal, which is the system integrity maintenance. Due to their diversity and biotechnological potential use, understanding defensins are necessary to exploit them as a novel therapeutic alternative.

In that perspective, molecular modeling can be used as a tool for obtaining information about parameters of fundamental importance for designing new and potent antimicrobial and antifungal molecules. By using molecular modeling tools it may be possible to understand defensins antimicrobial activity relationship with their structure and how their mechanism of action works.

The structural analysis of the mutant defensins may help us not only to understand deeply their mechanism of action, but also to build more powerful and specific antimicrobial molecules to propose new alternative therapies.

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