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NEW EDGE OF ANTIBIOTIC DEVELOPMENT: ANTIMICROBIAL PEPTIDES AND CORRESPONDING RESISTANCE

Topic Editors
Nádia S. Parachin and Octavio L. Franco

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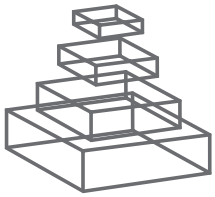

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NEW EDGE OF ANTIBIOTIC DEVELOPMENT: ANTIMICROBIAL PEPTIDES AND CORRESPONDING RESISTANCE

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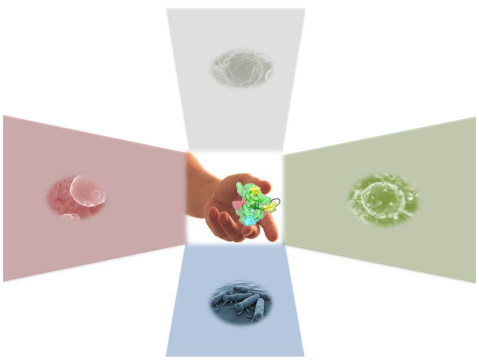

The use of peptides (center) in the control of several pathogens such as yeasts (left), bacteria (bottom) and virus (right) with no deleterious activities against mammalian cells (top).

Antimicrobial peptides, commonly isolated from several organisms, have been considered part of innate immune system and also as potential antimicrobial drugs. Besides its antimicrobial activity, some AMPs also have antifungal activity, inmmunomodulatory and antitumural activities. Lately not only nature has become a source of AMPs. Besides isolation of natural organisms, antimicrobial peptides might be improved or created using computational tools. This opens even more this so amazing field by creating infinite novel and remarkable possibilities.

Overall the current issue highlights the relevance of such Research Topic with perspectives to develop entirely new molecules with vast application within health and agricultural field with higher affinity for its target with concomitant reduction of side effects.

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EDITORIAL
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New edge of antibiotic development: antimicrobial peptides and corresponding resistance

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Keywords: antimicrobial peptide, microbial resistance, antitumoral peptide, host defense peptide

In the last years severe efforts turned into extensive research on development of novel antimicrobial compounds. Among them, antimicrobial peptides, commonly isolated from several organisms, have been considered part of innate immune system and also as potential antimicrobial drugs. AMPs have variable amino acid composition and size (ranging from less than 5–100 amino acid residues), commonly showing cationic and amphipathic properties. Nowadays about 2300 AMPs have been reported in the Antimicrobial Peptide Database (AMP database). Such research endeavor resulted in more than 100 peptide-based drugs available in the market with approximately 500–600 candidates in pre-clinical test development (Craik et al., 2013). Therefore the current issue encloses several aspects when studying within antimicrobial peptides field.

Besides its antimicrobial activity, some AMPs also have antifungal activity, inmmunomodulatory and antitumural activities. For example defensins commonly found in plants, fungi, insect and mammalian cells are small cationic peptides of 45–54 amino acid residues with a conserved signature of cysteines, which can form three to four disulfide bridges. Those have been shown to have inmmunomodulatory activities that can be used as vaccine adjuvants or in the treatment of immune-depressed patients. Therefore, a better understanding of function and mechanism of action of such molecules is a great promise in anti-infective and immunomodulatory therapeutics (Silva et al., 2014).

Defensins isolated from plants also have been considered as a biotechnological tool to improve crop production as recently reviewed (Lacerda et al., 2014). Among their advantages low effective fungicide concentrations and decreased environmental impact have been focused when compared to most common chemicals utilized for fungi control. Therefore many studies have focused in construction of genetic modified plants that over produce such molecules in order to reduce crop losses by fungi infection. Nevertheless it is important to emphasize that although there are many studies occurring at bench scale, indicating that genetic modified plants over producing defensins should appear in the market within the next years (Lacerda et al., not published).

It is also know that antimicrobial peptides have other potential applications since some of them have also antitumoral and imunomodulator activities. Also know as anticancer peptides (ACP) and host defense peptides (HDP) they also represent important candidates as novel drugs and have been carefully addressed in the current issue. For instance, cationic peptides have been considered as anticancer agents for presenting numerous advantages over chemical agents such as higher specific cytotoxicity to tumor cells, lower side effects and easier absorption as recently listed (Mulder et al., 2013). Diverse studies have shown that cancer cells are more anionic than normal cells. Due this property, cationic AMPs seems to bind it faster and selectively resulting in cell death. A better understanding of ACP mechanism of action may result in novel pharmacies with optimized anticancer activity (Gaspar et al., 2013).

Despite the great number of peptides available in databases, isolation of new molecules using classic purification techniques is crucial to identify novel molecules with diverse activities. Indeed a proteinase inhibitor isolated from *J. curcas* seed cake, named JcTI-I, was shown to have a potent activity against the human pathogenic bacteria *S. aureus* and *S. enterica*. Moreover it did present other relevant pharmacologically characteristics such as absence of hemolytic activity against human erythrocytes concomitant to pH and high salt concentration resistance (Costa et al., 2014). Moreover, microorganism have also been focused in this issue were the isolation and characterization of lipopeptide from *Bacillus* sp. were performed. This lipopetide shows strong fungicide activity (specially when this compound was self-assembled) (Roy et al., 2013).

Lately not only nature has become a source of AMPs. Besides isolation of natural organisms, antimicrobial peptides might be improved or created using computational tools. This opens even more this so amazing field by creating infinite novel and remarkable possibilities. Recently a study screened the distribution of known motifs in prokaryotic extracellular and virulence proteins across a range of bacterial species in order to identify novel motifs in virulence proteins (Ruhanen et al., 2014). Such methodologies are able to generate thousands of novel molecules that require high-throughput *in vitro* and *in vivo* validation. In this sense it is also necessary to develop rapid assays that can be performed concomitant to million candidates. A recent method enabled the selection of AMPs directly on peptide microarrays allowing identification of AMPs that bind and are bactericide among those that bind but do not kill bacteria (Wimley, 2010). In another example, an affinity support might be applied for the isolation of AMPs that interact with lipopolysaccharides, the major cause of septic shock (Lopez-Abarrategui et al., 2013). Thus advances in peptide array discovery assays could provide a system to develop

pathogen-specific antibiotics resulting in the discovery of target antibiotics (Diehnelt, 2013).

In alignment with *in silico* models, several systemic strategies such as transcriptome and proteome have been utilized for understanding peptide interaction with its target, which may allow improvement or design new molecule properties (Tavares et al., 2013). These novel approaches could lead us to understand several compounds at the same time which may result in a rapid increased in AMP peptides available in the market for treating several diseases. Finally it is fundamental to understand mechanism of action of AMPs in order to improve its activity and predict possible resistance mechanisms. Although many AMP alters membrane permeability there are other action mechanisms such as synthesis inhibition of cell wall, protein synthesis or nucleic acids as reviewed in this issue (Guilhelmelli et al., 2013). Moreover it is also essential to clear elucidate host-pathogen relationship. A recent study hypothesized that Helmint defense peptides played a critical role in parasite interaction with its host (Robinson et al., 2013). This may be applied as immunomodulators agents since those molecules are able to interact with the host without cytotoxic or cytolytic effects.

Overall the current issue highlights the relevance of such research topic with perspectives to develop entirely new molecules with vast application within health and agricultural field with c higher affinity for its target with concomitant reduction of side effects.

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Strategies and molecular tools to fight antimicrobial resistance: resistome, transcriptome, and antimicrobial peptides

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The increasing number of antibiotic resistant bacteria motivates prospective research toward discovery of new antimicrobial active substances. There are, however, controversies concerning the cost-effectiveness of such research with regards to the description of new substances with novel cellular interactions, or description of new uses of existing substances to overcome resistance. Although examination of bacteria isolated from remote locations with limited exposure to humans has revealed an absence of antibiotic resistance genes, it is accepted that these genes were both abundant and diverse in ancient living organisms, as detected in DNA recovered from Pleistocene deposits (30,000 years ago). Indeed, even before the first clinical use of antibiotics more than 60 years ago, resistant organisms had been isolated. Bacteria can exhibit different strategies for resistance against antibiotics. New genetic information may lead to the modification of protein structure affecting the antibiotic carriage into the cell, enzymatic inactivation of drugs, or even modification of cellular structure interfering in the drug-bacteria interaction. There are still plenty of new genes out there in the environment that can be appropriated by putative pathogenic bacteria to resist antimicrobial agents. On the other hand, there are several natural compounds with antibiotic activity that may be used to oppose them. Antimicrobial peptides (AMPs) are molecules which are wide-spread in all forms of life, from multi-cellular organisms to bacterial cells used to interfere with microbial growth. Several AMPs have been shown to be effective against multi-drug resistant bacteria and have low propensity to resistance development, probably due to their unique mode of action, different from well-known antimicrobial drugs. These substances may interact in different ways with bacterial cell membrane, protein synthesis, protein modulation, and protein folding. The analysis of bacterial transcriptome may contribute to the understanding of microbial strategies under different environmental stresses and allows the understanding of their interaction with novel AMPs.

Keywords: resistome, transcription, genetic, molecular modeling, antimicrobial peptides, NGS applications

INTRODUCTION

According to our recent history, human activity has markedly enhanced the evolution and distribution of resistant bacteria worldwide both in hospitals, human, and animal communities, and in the open environment, although this human activity is not necessarily the only, or even the proximate, cause for antimicrobial resistance phenomenon (Josephson, 2006; Wright, 2010). In this regard, most of the scientific research in antibiotic resistance over the past six to seven decades has been focused on association of drug-resistance with pathogenic bacteria. Given what we now know about the dispersal of resistance genes in nonpathogenic bacteria, this focus on pathogens actually neglects the majority of genes associated with resistance (D'Costa et al., 2006).

Since its use as a therapeutic tool to fight infectious diseases was proposed, antimicrobial drugs have reduced the mortality, but not the persistency of infectious diseases. Due to their use and misuse, these drugs have stimulated bacterial evolution toward the development of resistance, as an adaptive mechanism to the environment. While the selective pressure is maintained, adaptive mechanisms are transmitted to new generations, through the genetic flow. The phenomenon has acquired considerable importance in public health (Levy, 1998). The resistance may be associated with chromosomal mutations or imported genes through genetic recombination. In antimicrobial resistant microorganisms, resistance genes such as plasmids, transposons, and integrons can be inserted into the chromosome or extrachromosomal genome. Resistance may also be associated with a general impermeability of the bacterial cell envelope (El-Halfawy and Valvano, 2012).

The development of microbial resistance to antimicrobials had been going on in nature long before antibiotics were made available to chemotherapy. It is recognized that bacteria, including

human pathogens, may acquire resistance genes in natural environments, particularly in soils (Josephson, 2006; Wright, 2007). Taking the recent methodological approaches, the concept of the antibiotic resistome has been advanced to serve as a framework for understanding the ecology of resistance on a global scale (Wright, 2007).

The resistome consists of a collection of all antibiotic resistance genes including those circulating in pathogenic bacteria, antibiotic producers, and benign non-pathogenic organisms found either free living in the environment or as commensals of other organisms (D'Costa et al., 2006). Most of the so called antibiotic producers live in soils, and as an ecological consequence, most of the susceptible bacteria in their vicinity, including human pathogens, die off, but some develop resistance to these natural products thought of control the microbial population (Wright, 2010; Cox and Wright, 2013).

The limited number of antibacterial classes and the common occurrence of cross-resistance within and between classes have also reinforced the urgent need to discover new compounds targeting novel cellular functions not yet targeted by currently used drugs (Chung et al., 2013). Bacteria are known to employ different strategies for antibiotic resistance. Resistance may be acquired by spontaneous mutation in the coding gene of the target protein resulting in no or reduced affinity to the antibiotic or by horizontal transfer of antibiotic resistance genes from other bacteria (Hassan et al., 2012).

An antibiotic-resistance gene product may act by enzymatic degradation of the antibiotic, by altering the antibiotic target site or by pumping the incoming antibiotic out of the cell by a transport mechanism. Such processes make infection treatment very difficult as we face sophisticated, highly resistant and often multi-resistant pathogens such as *Pseudomonas aeruginosa* (Paterson, 2006), *Escherichia coli* (Overbye and Barrett, 2005), methicillin-resistant *Staphylococcus aureus* (MRSA) (Reynolds et al., 2004) and penicillin-resistant *Streptococcus pneumonia* (Karchmer, 2004).

The antimicrobial peptides (AMPs) is a class of molecules that may be used to overcome the bacterial resistance challenge. Their occurrence is a wide-spread phenomenon in all forms of life, from multi-cellular organisms to bacterial cells. In higher organisms, AMPs contribute to innate immunity and are part of the first defense line against harmful micro-organisms. In bacteria, production of AMPs provides a competitive advantage for the producer in certain ecological niches because the peptide mediates the killing of other bacteria (Hassan et al., 2012). They are constitutively expressed or induced by endogenous or exogenous elicitors, such as developmental stage or pathogen predation (Sachetto-Martins et al., 2000). AMPs are small proteins 20–50 amino acid residues long, often having common properties such as the small number of amino acid residues, cationicity, and amphipathicity (Tavares et al., 2008). The AMPs interact with membranes in different ways, but in general three different models have been used to define their mode of actions in model membrane systems. In the barrel-stave mechanism, peptides integrate into the membrane and form membrane-spanning pores. In the toroidal-pore mechanism, AMPs form membrane-spanning pores together with intercalated lipids. And in the carpet mechanism, peptides accumulate on the membrane surface in a carpet-like manner and at a threshold density so that they dissolve the membrane without forming transmembrane channels (Pietiäinen et al., 2009; Brogden, 2011). However, membrane damage is not the single mechanism whereby AMPs cause cell death. They may also affect functions of several other cell components and act as metabolic inhibitors of cellular processes including biosynthesis of the cell wall, nucleic-acids and proteins. In these cases, the cell death can be the result of multiple inhibitory effects (Brogden, 2005).

AMPs show broad-spectrum antimicrobial activities against various microorganisms, including Gram-positive and Gram-negative bacteria, fungi, and viruses. Many AMPs are effective against multi-drug resistant (MDR) bacteria and possess low propensity for developing resistance probably due to their distinguished mode of action (Seo et al., 2012). AMPs could be very diverse in sequence and structure but most of them are positively charged, allowing their interaction with the bacterial envelope. These peptides are active at very low concentrations (micromolar to nanomolar range) and most of them kill their target microorganism via a non-receptor mediated mechanism involving permeation of the target membrane (Guralp et al., 2013).

AMPs can be classified into four groups based on their structures: α-helical peptides, β-sheet peptides, extended peptides, and loop peptides (Nguyen et al., 2011; Fjell et al., 2012). Understanding the structure-activity relationships (SAR) of AMPs is essential for the design and development of novel antimicrobial agents with improved properties. In particular, the atomic level structures of AMPs can provide versatile information for all stages of drug development, including the peptide design and modification for pharmaceutical application (Seo et al., 2012).

Microbial pathogens have evolved different systems to resist the effect of antimicrobial peptides. These mechanisms can involve the destruction of antimicrobial peptides (by proteolytic digestion), change of antimicrobial peptide target (i.e., the microbial membrane), and removal of antimicrobial peptides from their site of action (through efflux pumps or by alteration of the cell surface composition) (Rio-Alvarez et al., 2012). The modifications of lipopolysaccharide (LPS) to mask the negative charges that allow interaction with AMP are one of the main responses to these compounds in many Gram-negative bacteria (Costechareyre et al., 2013).

Some bacteria such as *Staphylococcus enterica* serovar typhimurium exhibit a regulatory system controls virulence that is involved in the regulation of Mg^{2+} uptake systems, survival in macrophages and resistance to antimicrobial peptides (AMP). Several enzymes, encoded by *pagP, pagO, pmrC, pmrG, lpxO, pmrHFIJKLM*, modify LPS, mostly by adding or modifying palmitate, phosphoethanolamine or 4-aminoarabinose to mask negative charges that allow interaction with cationic AMPs (Costechareyre et al., 2013). Costechareyre et al. (2013) using *Dickeya dadantii,* which is an insect and plant pathogen, to understand the regulation of genes involved in response to AMPs, observed that through transcriptome different genes are involved in response to AMPs

when the bacteria infect the aphid (*Acyrthosiphon pisum)* and plant.

Antimicrobial peptides (AMPs), particularly the so-called bacteriocins produced by bacteria, may be an important contributor in this context as they often have a relatively narrow killing spectrum which comprises mostly bacteria closely related to the producers (Hassan et al., 2012).

Many hundreds of different peptides, differing in size, charge, hydrophobicity, conformation, primary structure, as well as in post-translational modifications, have been demonstrated in frog defensive skin secretions (Evaristo et al., 2013).

The knowledge about AMP action mode and resistance mechanisms shared by different microorganisms may point the direction for discovery and design of new drugs.

NEW APPROACHES TO AMPs RESISTANCE

The knowledge acquired in the last two decades concerning the evolution of antimicrobial resistance to widely prescribed drugs, and the search for new antimicrobial candidates such as AMPs, thought to be natural barriers against bacteria, eukaryotic parasites, viruses, and fungi, has resulted in a better understanding of how microorganisms have become resistant to these proteins (Marshall and Arenas, 2003; Wilcox, 2004; Hancock and Sahl, 2006; Perron et al., 2006).

The variety of already described antimicrobial peptides related to the different sequences, shows that the same peptide sequence is rarely associated with two different species, even closely related. Several multicellular organisms express a collection of peptides of different chemical structures, as a local defensin (Zasloff, 2002). However, despite the structural diversity, most of the already sequenced antimicrobial peptides show at least 50% hydrophobic amino acid residues and a low proportion of both neutral polar and negatively charged amino acids (Hancock and Chapple, 1999). It is accepted that this structural skeleton may explain why the majority of AMPs persists at water-lipid interfaces and then disturb microbial membrane components (Ruissen et al., 2001). Membrane damage is considered the primary antimicrobial mechanism of the so called cationic antimicrobial peptides (CAMPs) or ribosomally synthesized antimicrobial peptides (RAMPs) (Perron et al., 2006), and requires interaction with microbial membrane lipids and hydrophobic properties to enable integration of the peptide into the hydrophobic core of the membrane (Peschel and Sahl, 2006).

Studies with CAMPs thrombocidins, defensins, and cathelicidins show a potential use as skin and epithelia protectors against invading microorganisms, such as *Staphylococcus aureus* and *Salmonella enterica,* by reducing the net negative charge of the bacterial cell envelope through covalent modification of anionic molecules (e.g., teichoic acids, phospholipids, and lipid A) resulting in repulsion of CAMPs. Other mechanisms have also been reported such as expelling CAMPs through energy-dependent pumps, altering membrane fluidity and CAMPs cleavage with proteases (Peschel, 2002; Marshall and Arenas, 2003).

Although nonspecific targets led researchers to suggest that it would be difficult for the bacteria to develop resistance to some peptides (Ge et al., 1999a,b; Schroder, 1999; Zasloff, 2002; Boman, 2003; Jenssen et al., 2006), molecular mechanisms of resistance to CAMPs have been suggested in several groups (Zasloff, 2002). In *S. aureus,* changes in the cell wall appear to involve the operon *dltABCD*, which results in carriage of positively charged D-alanine from the cytoplasm to anionic teichoic acids (Peschel et al., 1999; Kristian et al., 2003; Nizet, 2006). Perron et al. (2006) have studied the effects of resistance to pexiganan, CAMP analog of magainin, in different bacterial strains (mutants for *mutS* and *mutL* genes—*Pseudomonas fluorescens* and *Escherichia coli*) and observed MIC50 increased in both mutant strains. They also observed a reduction in the lag phase after subsequent growth in pexiganan presence. The contribution of these resistance mechanisms in bacterial pathogenesis may be confirmed by studies with mutants. It is accepted that such prospective investigations are of extreme relevance, since these potential AMPs are thought to be an alternative to well established antibiotics used in chemotherapy against multiresistant bacteria (Nizet, 2006; Brogden and Brodgen, 2011; Maróti et al., 2011). Mechanisms such as peptidases production, down regulation of host AMP production, and cellular filamentation have also been related (Nizet, 2006; Maróti et al., 2011).

AMPs may interact with intracellular targets, binding to DNA, RNA and protein, or even interfering with the characterized *FtsZ* gene, responsible for bacterial cell division septum or with protein synthesis such as DNA gyrase and DnaK (Brogden, 2005; Chauhan et al., 2006; Handler et al., 2008; Maróti et al., 2011). Genetic markers related to the defensins and cathelicidin mediated AMPs resistance include *kasB* in *Mycobacterium marinum* (Gao et al., 2003), *sak* in *S. aureus* (Jin et al., 2004)—for defensins; and *emm1* in Group A *Streptococcus* (Lauth et al., 2009).

Additionally, some AMPs have non-protein targets such as the peptidoglycan precursor lipid II and ATP (Hilpert et al., 2010; Sass et al., 2010). Modifications on cell surface have also been correlated with the AMPs resistance and several genetic markers have already been described, such as *mprF/lysS* in *S. aureus* (Peschel et al., 2001; Nishi et al., 2004), *dlt* operon in Group B *Streptococcus* and *Listeria monocytogenes* (Abachin et al., 2002; Poyart et al., 2003), *htrP* in *Haemophilus influenzae* (Starner et al., 2002), *pmr* in *Pseudomonas aeruginosa* (Moskowitz et al., 2004).

The active efflux of AMPs has already been observed and might be related to different genetic markers in various bacteria species, such as *mtr* in *Neisseria gonorrhoeae* (Jerse et al., 2003), *sap/sapA* operon in *S. enterica* and *H. influenzae* (Parra-Lopez et al., 1994; Mason et al., 2005) and *qacA* in *S. aureus* (Kupferwasser et al., 1999).

Moreover, the degradation of AMPs has being correlated to several genetic markers: *lasB* in *P. aeruginosa* (Schmidtchen et al., 2002), *gelE* in *Enterococcus faecalis* (Schmidtchen et al., 2002), *zapA* in *Proteus mirabilis* (Schmidtchen et al., 2002), *speB*/*ideS* in Group A *Streptococcus* (Schmidtchen et al., 2002), *aur* gene in *S. aureus* (Sieprawska-Lupa et al., 2004), *degP* in *Escherichia coli* (Ulvatne et al., 2002), and *rgpA/B* in *Porphyromonas gingivalis* (Devine et al., 1999).

The use of AMPs as pharmaceuticals will promote selective pressure for bacterial strains that are resistant also to the repertoire of host-defense peptides in the human body (Bell and Gouyon, 2003; Nizet, 2006). In this context, the bacterial resistome must also consider endogenous housekeeping genes which

may interact with the AMPs. To select genetic markers related to the bacterial resistome in this holistic point of view remains as an important challenge (Islam et al., 2001; Taggart et al., 2003; Wright, 2007). To illustrate the role of the housekeeping genes in the AMPs resistance, several authors have reported the importance of regulatory genes such as *phoP/phoQ* in *S. enterica* and *P. aeruginosa, pmrB* in *P. aeruginosa,* and *rpoE* in *S. enterica* (MacFarlane et al., 2000; Ernst et al., 2001; McPhee et al., 2003; Crouch et al., 2005).

Considering different organisms, such as fungi, a lot is known about the mechanism of resistance to antimicrobial drugs, but there are few reports on AMPs resistance. So far, AMPs in these organisms include modification of *erg11/mdr1* gene and *pdr5* locus, over expression of specific drug efflux pumps, alteration in sterol biosynthesis and alteration in AMP target, AMP inactivation and reduction in the intracellular concentration of target enzymes (Ghannoum and Rice, 1999; Balkis et al., 2002; Gulshan and Moye-Rowley, 2007). As observed for bacteria, antifungal drug resistance is quickly becoming a major problem, especially considering the expanding population of immunocompromised patients who have contributed to an increased incidence of opportunistic and systemic fungal infections.

With regards to the antifungal drug resistance mechanisms, the genetic markers codifying for multidrug efflux pumps and their upregulation have been highlighted (Balkis et al., 2002; Gulshan and Moye-Rowley, 2007). Jabra-Rizk et al. (2004) described two different types of efflux pumps in *C. albicans* and *C. dubliniensis*: adenosine triphosphate–binding cassette (ABC) transporters encoded by the *cdr* genes (*CDR1* and *CDR2*) and major facilitators encoded by the *mdr* genes.

Overall, it is accepted that further prospective studies on antimicrobial resistance are needed to enable a better understanding of the microbial genetic diversity that underlies resistance. Such knowledge will help and guide our efforts to develop new potential drugs to overcome the resistance phenomenon (Wright, 2007).

NEXT GENERATION SEQUENCING AND AMP PREDICTION

The next generation sequencing technologies have opened the opportunity to access genomes and transcriptomes at high throughput level allowing the researchers to understand a wide variety of physiological response of various types of organism. As a consequence new tools are available for antimicrobial discovery and design (**Figure 1**). The knowledge of host resistance mechanisms vs. susceptibility is important to the development of new approaches to prevent and/or treat human infectious diseases (Teles et al., 2013). The innate immune response in different organisms has the potential to reveal new and/or novel molecules for antimicrobial purpose. During transcriptome analysis of the oral chicken *Salmonella* infection four steps were observed and none of the genes was directly involved in bacterial infection, but associated with inflammatory response (Matulova et al., 2013). On the other hand persistence of *Salmonella* in several other niches is observed by resistance to AMPs and its sensibility is increased by adrenaline, down regulating the promoter of the *pmr operon* that controls resistance genes to AMPs (Karavolos et al., 2008). The combination of transcriptome and proteomic

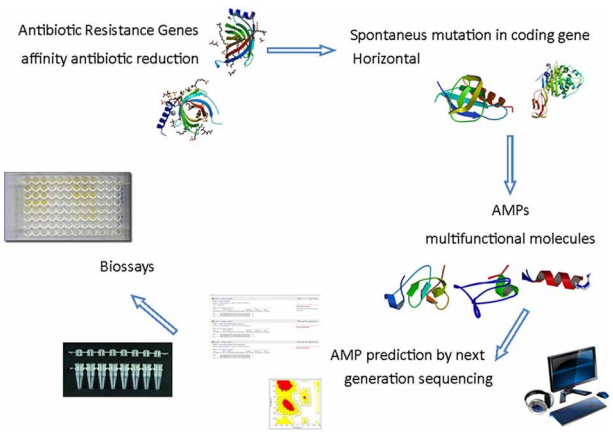

FIGURE 1 | Schematic antimicrobial peptides prospecting from the *in silico* analysis of sequences obtained by next generation sequencing. The demand for new antimicrobials to prevent microbial resistance from multifunctional action encourages the search for AMPs through prediction for next-generation sequencing followed by analysis of bioinformatics and computational modeling in order to produce effective peptides after antimicrobial trials. PDB IDs 3GP6; 1THQ; 2JSO; 3HUM; 2L24; 2KUS; 2LB7; 2NY8.

strategies were used to study the Australian scorpion, revealing that the molecular weight found for proteomics analysis was not completely adjusted to amino acid sequence deduced from cDNA cloned genes. Some reasons are pointed out by the authors: the level of gene expression is not necessarily the same information obtained from the cDNA, posttranslational modifications, or sample preparation (Luna-Ramírez et al., 2013). Regardless of problems encountered, some potential therapeutically peptides were identified in those samples. In ladybird *Harmonia axyridis* the successful invasive behavior was revealed by 454 sequencing. The two layer innate immune system is composed of a chemical weapon, mediated by the secondary metabolite harmonine, associated with a wide range of AMPs resulting from multiple gene duplication and divergence events (Vilcinskas et al., 2013). In the scorpion *Hetermetrus petersii* its venon showed four families of antimicrobial and cytolyc peptides identified by 454 sequencing platform (Ma et al., 2010). In *Spodoptera exigua* larvae upon AcMNPV infection the 454 analysis demonstrated that some genes, including genes encoding for AMPs, are down regulated (Choi et al., 2012). Summarizing, the association of transcriptome and proteomics technologies offers new points of view for AMP mode of action in different organisms, showing different potential and different strategies for prospection. In the bivalve mollusk *Ruditapes philippinarum,* for example the use of 454 platforms allowed the identification of 36 AMP sequences (Moreira et al., 2012). The analysis of transcriptome of the American dog tick infected with different microorganisms allowed the researcher to identify a novel elicited defensin in the Arachnids immune system response transcripts (Jaworski et al., 2010). Facing up to the high diversity of organisms, various tissues and physiological approaches, the number of novel and new AMPs derived from biodiversity is a vast field for research.

PREDICTION OF ANTIMICROBIAL PEPTIDE FROM DNA/RNA LIBRARY

ANTIMICROBIAL PEPTIDES SEARCH TOOLS

The antimicrobial peptides are directly related to the innate and acquired immune response of organisms, and their potential to kill microorganisms resistant to many antibiotics has attracted the interest of the pharmaceutical industry. In this aspect tools to find and produce antimicrobial peptides have created a revolution in research for new drugs. According to Belarmino et al. (2010), the development of bioinformatics tools for predicting patterns in biological sequences has already allowed a routine search in databases of ESTs (Expressed Sequence Tags) of plants by defensins and a subsequent validation by antimicrobial testing.

The APD2 (Antimicrobial Peptide Database Second Version) is one of the main databases of antimicrobial peptides, allowing users to search for families of peptides, post translational modified peptides, among other options (Wang et al., 2009). APD provides an option to calculate and to predict AMPs in order to extract important information about peptides such as total charge, hydrophobic rate, in addition to providing an alignment with deposited sequences (http://aps.unmc.edu/AP/main.php). The information provided can be linked to the data on the hydrophobic moment calculated by the web-program HydroMcalc (Tossi et al., 2002) available at http://www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html, allowing the prediction of antimicrobial peptides.

In addition to the APD, another database of AMPs is the CAMP (Collection of Antimicrobial Peptides) available at http://www.bicnirrh.res.in/antimicrobial. Such tools provide information related to the sequence, definition of protein biological activity, taxonomy of the source organism, target organisms—indicating the MIC (minimum inhibitory concentration), hemolytic activity of the peptide and links to external databases such as SwissProt, PDB, PubMed and the NCBI Taxonomy (Thomas et al., 2010). The iAMP-2L, available at http://www.jci-bioinfo.cn/iAMP-2L, is a web-server used for the prediction of uncharacterized sequences as antimicrobial. Once the subject sequence is identified as an antimicrobial, the server indicates to which class (antibacterial, anticancer, antifungal, anti-HIV and anti-viral) the peptide belongs. Peptides are promiscuous molecules (Franco, 2011) and are invariably classified in more than one class and family (Xiao et al., 2013). All of these characteristics reinforce how these new technologies can make available an unlimited source for new drugs and biologically active molecules. Tools of bioinformatics for modeling are fundamental for development of this research area.

TYPES OF MODELING AND PREDICTING STRUCTURES APPLIED TO ANTIMICROBIAL PEPTIDE

In the following steps we present a summary of a method to develop such peptides that can predict the structure of peptides and proteins in two ways: experimentally, through methods such as nuclear magnetic resonance (NMR), X-ray diffraction and crystallography, and theoretically, by computational modeling methods, which involve *comparative modeling*, *threading (folding) modeling* and *ab initio* (*de novo*). Experimental methods of peptide prediction and modeling have typical difficulties. The lack of structural conformation of plant bactericidal peptides prevents more detailed classification of AMPs (Porto and Franco, 2013). The use of computational tools and methods has become an important strategy in the search for bioactive peptides. However, there are still some limitations in this prediction method, such as the difficulty of developing a general method for predicting the nature and activity of antimicrobial peptides, due to low homology sequences that can occur (Lata et al., 2007; Torrent et al., 2012).

Comparative modeling

Comparative homology modeling is a method based on the structures similarity, i.e., similar amino acid sequences tend to have a very similar secondary structure. Thus, it is possible to use as a template structures solved by experimental means, in order to predict the 3D conformation of peptides and proteins using computational algorithms. In comparative modeling, the alignment of the sequence to be predicted and the template must present an identity of at least 30% (Baker and Sali, 2001), a large number of cases with alignments with low identity between target and template can lead to better models of 3D structure (Rayan, 2009). In fact the identity of the alignment can be put aside when we are in a situation of functionality. For example, imagine a protein A having 90% identity to another protein B, but a different function, and also a protein C that has 70% identity and the same function as B. In this case protein C would be the best template and B is not the shape desired. The alignment score should also be considered when working with whole proteins, since in certain programs, such as BLAST, alignments may appear above 30%, but still low coverage.

The *in silico* prediction method by homology modeling is divided into four main steps: (1) identification of structures and selection of templates, (2) alignment of the target sequence with the chosen model structure, (3) generation of models for the target structure, using information about the structure of the template; (4) validation of the models generated for the target (Martí-Renom et al., 2000). It can also be interesting to implement a fifth step which is the model of refinement by energy minimization, which is important in the context that during the production of the geometric errors can occur in regions of the main chain (Vyas et al., 2012).

The first two steps to create and predict a three-dimensional model of a protein or peptide involve query of database structures experimentally determined by crystallography techniques, X-ray diffraction or nuclear magnetic resonance (Kiefer, 2012). First, research tools in the databases such as BLAST (Basic Local Alignments Search Tool) that allow local alignment. Searching sequence of regions similar to other regions of sequence is an essential step to find sequence templates. The BLAST minimizes the time spent on research, discarding alignments in which regions between the query and subject sequences have few chances to exceed a pre-determined score (Altschul et al., 1990). During the search for similar sequences in databases, attention should be given to the best method, i.e., one that is both sensitive (able to identify sequences related bit) and selective (relations between the query and subject sequences are true). At this point it is worth mentioning one of the most used tools in the search for

similar sequences, PSI-BLAST (Position-Specific Iterated Basic Local Alignment Tool) from NCBI, which differs from conventional BLAST due to its higher accuracy and greater statistical sensitivity (Li et al., 2011).

After choosing sequences of templates that will be used in the second stage, the optimal alignment between this sequence and the target is required to build three-dimensional models (Centeno et al., 2005). The main strategies used are: progressive alignment between the sequences using the software Clustal W (Larkin et al., 2007), the sequence-profile alignment, HMM-based method (HIDDEN MARKOV MODEL) between query and profile of families of templates, using up the database profiles Pfam (Finn et al., 2010) and HMMER web server (Finn et al., 2011) one can still perform profile-profile alignment, from building a profile for the target and matching with the profile templates in a database of profiles (Centeno et al., 2005; Ramachandran and Dokholyan, 2012; Venclovas, 2012).

The models are generated based on the structural information provided by the template and the sequence alignment between them and predicted (Kiefer, 2012). Currently there are several programs and web servers that can be used to build models of proteins and peptides, the main one is Modeler, developed by Sali and Blundell (1993). The Modeler is used to compare the target structure by satisfaction of spatial constraints involving restrictions on atomic distances, angles dihedral, and stereochemistry. The information modeler generated is also combined with the statistical calculation preferences of constraints derived from the sequence template homology (Eswar et al., 2003; Vyas et al., 2012). Another tool also used in model building by homology is SwissModel, which unlike the modeler searches in a homologous target database with the BLAST protein and then determines a three dimensional model, finding the core backbone and modeling loops and chain laterals (Schwede et al., 2003).

In homology modeling each step is directly linked to the previous, so in the event of accidental errors, these can be propagated. Thus, it becomes necessary to validate the final model and interpretation of the target. The generated model can be validated as a whole or for individual regions (Martí-Renom et al., 2000), the basic need is for a good model built on good stereochemistry (Hillisch et al., 2004). The main tools for analysis and validation of models generated by comparative modeling are: Procheck (Laskowski, 1993) and Molprobity (Chen et al., 2009), both for quality analysis stereochemistry; Whatchek (Hooft et al., 1996) and Qmean (Benkert et al., 2009) used to evaluate the quality of the model, and the ProsaWeb (Wiederstein and Sippl, 2007) used in the analysis of interaction energy between the model residuals. According to Martí-Renom et al. (2000), the most common errors that may occur during modeling are positioning errors of side chain distortions in regions aligned, regions with an inefficient mold alignment and wrong choice of template.

The refinement of the model in general uses methods of molecular dynamics calculations of force fields, the most common being the CHARMM (Brooks et al., 1983) and GROMOS (Schuler et al., 2001). A refinement process can be defined as walking on the surface of covalent and hydrogen bonds in the model, the search for a better minimum energy than the energy of departure, therefore, a difficult task (Gront et al., 2012). The energy minimization can promote excessive deviation of the model structure, compared to the original, which actually is not ideal; therefore, you should keep the number of cycles of minimization to a minimum, which is sufficient for improved stereochemistry of the model (Peitsch, 2002).

b-Threading modeling

The modeling threading or by folding pattern recognition is a method of predicting three dimensional structures by looking for folding patterns, applying the combination-linear alignments profile and adjusting the profile structure of the target reference frames (obtained from folding profile libraries).

The LOMENTS is a meta-server which includes nine major servers threading (PPA-I, SP3, PPA-II, sparks2, PROSPECT2, FUGUE, HHSEARCH, PAINT, SAM-T02), allowing the selection of models through research for 30 models for each of the individual servers, excluding short alignments, and defining models of greater structural similarity (Wu and Zhang, 2007). It is an important tool for the study of structures in modeling template folding. Among commonly used tools in modeling protein folding are the ROSETTA (Kaufmann et al., 2010) and I-TASSER (Wu et al., 2007; Roy et al., 2010). Such tools have their operation based on either amino acid sequence of the target and information about structures in the template experimentally resolved, or using predictors of secondary structure and folding as mentioned above, with libraries of fragments.

c-Ab initio modeling

This method of predicting three-dimensional structures ignores in principle the use of reference structures solved experimentally. Prediction *ab initio* (*de novo*) makes use of the energy minimized functions and research of spatial conformations that the target can take, and this is important for the use of force fields and methods of molecular dynamics and Monte Carlo simulations (Lee et al., 2009). According to Helles (2008), the three factors that make *ab initio* interesting for homology modeling, are that this does not provide accurate information about how a given protein or peptide acquires structure, many proteins and peptides do not have sufficient (> 30%) experimentally solved homology molecules, and even if the target presents high similarity with templates, it does not mean they will present the same structural profile.

ab initio Software such as ROSETTA and I-Tasser, cited above, have been used as *de novo* prediction programs (Wu et al., 2007; Kaufmann et al., 2010). However, by considering information frames of reference they are not actually *ab initio* techniques. A tool that is completely is LINUS (Local Independently Nucleated Units of Structure), which does not make use of structures or reference sequences, initiating the construction of the target from the extended chain as a result. The simulation performed by the software promotes the disruption of conformations of three randomly chosen residues, and evaluates the energy, using Monte Carlo procedure to validate the favorable conformation predicted (Srinivasan and Rose, 2002). Another tool also used currently is the QUARK (http://zhanglab.ccmb.med.umich.edu/QUARK/), a tool that builds models from small fragments (residues 1–20) using Monte Carlo simulations (Xu and Zhang, 2012).

The intensive growth of research of AMPs and development of robust databases the discovering of novel and new biological active peptides (Amaral et al., 2012). The development of antimicrobial peptides from genomic and transcriptome databases can be an alternative strategy to the studies with research and development of AMPs.

MODIFICATION MEDICINES

As long as antimicrobials were made available in the 1940s, there were no concerns related to the antimicrobial resistance mechanisms. However, the discovery of other antimicrobial agents and even the modification of those already described were not able to stop microbial evolution, such as the rapid emergence of β-lactamase-producing *Staphylococcus aureus* strains (Spellberg, 2009; Theuretzbacher, 2009; Choffnes et al., 2010).

The accelerated increase and global expansion of bacterial resistance made it necessary the search for new fighting agents (Spellberg, 2009; Choffnes et al., 2010). One of the main factors associated with this increasing antimicrobial resistance was the misuse of antimicrobials (Gwynn et al., 2010).

Driven by high profitability, the pharmaceutical industry has focused its production on blockbuster drugs (or global FMCG)–such as those used in the treatment of chronic diseases such as cancer or sexual dysfunction, for example—rather than the development of antimicrobial drugs used for short term treatment of acute infectious diseases (Theuretzbacher, 2009).

The economic advantages offered by blockbuster drugs coupled with the high cost of production and the low economical income related to the antimicrobial production, if compared to the profitability of other drug production, led to a lack of investment in the development of new antimicrobial agents in the 1990s (Spellberg, 2009; Theuretzbacher, 2009). In this regard, the production of new antibiotics becomes, now, very expensive due to the rationale and steps of manufacturing and preclinical testing and clinical trials, up to their insertion in the market. The searches for new agents has to overcome the mechanisms of bacterial resistance, and therefore, are based on the search for new routes of administration, new targets or mechanisms of action toward the same target, which ends up limiting the production of effective potential agents (Gwynn et al., 2010). Add to that the availability of generic formulations and the development of drugs kept to treat only severe diseases to avoid quick bacteria resistance development, further also contributed to the economical failure related to new antimicrobial releases (Spellberg, 2009). In this point of view the *in silico* prediction of antimicrobial peptides becomes an advantage for industry due to low cost and time consumption.

Furthermore, the wide use of broad-spectrum antimicrobials has contributed to the need for new drugs given the emergence of the so called multi-drug resistant bacteria (MDR) (Choffnes et al., 2010; Gwynn et al., 2010). The decline in production of new agents was compounded by the loss of effectiveness of existing antimicrobials without a concomitant replacement by new therapeutic options. In a study by Shlaes and Moellering (2002), the medical community was alerted to the lack in new drug discovery, and the authors concluded that the development of new antibacterial agents was even lower than that related to hyperactivity disorder and male erectile dysfunction (Spellberg, 2009).

Within the current scenario of increasing bacterial resistance, however, it becomes necessary to resume production of new antimicrobial agents, or discussion of new strategies for the use of the available drugs. This discussion has motivated and encouraged scientific research on the subject, in order to decrease the cost of production within large pharmaceutical companies (Spellberg, 2009). For example, while other drugs require 15 candidates to yield one FDA-approved product, antibiotics require 72 candidates to yield an FDA-approved product, which currently costs 400–$ 800 million per approved agent (Spellberg et al., 2008; Forsyth, 2013; IDSA, 2013). The production of antimicrobials is not profitable also because: the drugs are used for a short period of time (7–14 days), sold for low price and prescription controlled market (Forsyth, 2013). As a result, it is estimated that about two million Americans per year develop hospital infections, mostly caused by multidrug-resistant bacteria pathogens, which increases treatment costs in about U$ 21 million to U$ 34 billion, compared to antibiotic-susceptible pathogens (Roberts et al., 2009; Spellberg et al., 2011). Nosocomial infections such as pneumonia and sepsis, killed about 50,000 Americans in 2006 and cost to the US health care system more than U$ 8 billion (Eber et al., 2010).

To encourage the production of new antimicrobials, Government policies have been issued, such as the GAIN (Generating Antibiotics Incentives Now) Act, which states: (i) warranty for new approved drugs protection from competition in the marketplace by limiting FDA approval of similar drugs during the a certain exclusivity period; (ii) review and fast-rack approval priority for qualified antimicrobial drugs, antibiotic applications will be eligible for both priority review and fast-track approval through the FDA new drug application process; and (iii) study of incentives for Qualified Infectious Diseases Biological Products, to encourage research, development, and marketing for qualified infectious disease biological products (Forsyth, 2013). Besides this, proposals have been discussed for new ways of using drugs already known and established for the microorganisms which have been made resistant (Spellberg, 2009).

In this regard, considering the evolution of bacterial pathogens associated with infectious diseases today, the need to develop new agents to control multiresistant bacteria is presumed, or to prospect new ways of using the inefficient well-established antimicrobial arsenal, aiming to overlap the existing limitation in antibacterial chemotherapy (Rai et al., 2009; Spellberg, 2009; Choffnes et al., 2010).

With regards to the AMPs and their eventual modifications as an alternative strategy to overcome the need of new drugs, it is important to undergo a retrospective analysis of the co-evolution of antimicrobial peptides and bacterial resistance. Initially several peptides had been reported in the scientific literature and among them, cationic peptides called attention by their mechanisms of action: using positively charged molecules, amphiphilic, with affinity to bacterial membranes. However, during initial *in vitro* and in preclinical trials, resistant strains have been noticed. Overall, variations in the peptide sequences are proposed leading to conjugate molecules (Peschel and Sahl, 2006).

Obtaining AMPs can be performed in three different ways: direct isolation of the producer, by chemical synthesis or

recombinant expression (Li et al., 2010; Parachin et al., 2012). Modifications in AMP composition, structure and function are being used to create more stable molecules. Six distinctive new classes of AMPs have already been reported (Brogden, 2011; Brogden and Brodgen, 2011; Tossi, 2011). The first class includes mimetic peptides, which are non-peptidic, synthetic molecules, which mimic the natural properties of AMPs. Its structure requires a different composition such as peptoids, arylamides oligomers, β-peptides, or phenylene ethynylenes (Rotem and Mor, 2009). The second class includes hybrid peptides, AMPs constructed of the active regions of two to three peptides, such as cecropinA-melittin (CEME/ CEMA/ CP26/ CP29) (Piers and Hancock, 1994). The potential benefits of each individual fragment are combined to increase antimicrobial activity, reduce antimicrobial spectrum of activity or reduce cytotoxicity for host cells. The third class includes peptide congeners, a chemical compound closely related to another in composition, such as congeners of CAP18, LL-37, SMAP28, ovispirin, and Q25. They may contain changes in tertiary structure, change of specific amino acids in the sequence to load change, among other characteristics. The fourth class includes cyclotides and stabilized AMPs. Cylotides are cyclopeptides with a head-to-tail cyclic backbone, containing 30 amino acid residues with three conserved disulfide bonds (i. e., cyclized angiotensin and cyclic diastereomeric lysine ring) (Ireland et al., 2010). The fifth class includes peptide conjugates which is connected to micelles, liposomes, antibodies, steroids or fatty acids, such as lactoferrin—lauric acid (Chu-Kung et al., 2010), and the sixth class includes immobilized peptides via incorporation into distinct materials or absorbed to a variety of surfaces where they still retain their ability to bind and kill bacteria. These groups of new peptides have a variety of potential medical and industrial applications in many different areas (medicine, veterinary, agriculture, pharmaceutical, food) (Costa et al., 2011).

In conclusion, the misuse of anticrobials lasting recent decades has increased the spread of mutations allowing the development of multidrug resistant microbes. The antimicrobials were neglected due to economic interest. Thus, for infectious diseases the development of new antimicrobial with low cost and broad spectrum of action becomes of great importance, because the lifetime of such molecules is very short and a wide range of molecules is important to overcome the novel resistant pathogens. The molecular modeling of AMPs from transcriptome has arisen in current times as an important alternative for drug development.

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LPS inmobilization on porous and non-porous supports as an approach for the isolation of anti-LPS host-defense peptides

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Lipopolysaccharides (LPSs) are the major molecular component of the outer membrane of Gram-negative bacteria. This molecule is recognized as a sign of bacterial infection, responsible for the development of local inflammatory response and, in extreme cases, septic shock. Unfortunately, despite substantial advances in the pathophysiology of sepsis, there is no efficacious therapy against this syndrome yet. As a consequence, septic shock syndrome continues to increase, reaching mortality rates over 50% in some cases. Even though many preclinical studies and clinical trials have been conducted, there is no Food and Drug Administration-approved drug yet that interacts directly against LPS. Cationic host-defense peptides (HDPs) could be an alternative solution since they possess both antimicrobial and antiseptic properties. HDPs are small, positively charged peptides which are evolutionarily conserved components of the innate immune response. In fact, binding to diverse chemotypes of LPS and inhibition of LPS-induced pro-inflammatory cytokines from macrophages have been demonstrated for different HDPs. Curiously, none of them have been isolated by their affinity to LPS. A diversity of supports could be useful for such biological interaction and suitable for isolating HDPs that recognize LPS. This approach could expand the rational search for anti-LPS HDPs.

Keywords: LPS, antiendotoxic, antimicrobial peptides, affinity chromatography, LPS immobilization

INTRODUCTION

Sepsis is characterized by an uncontrolled inflammatory as well as anti-inflammatory process driven by the host immune system in response to bacteria (Adib-Conquy and Cavaillon, 2012). This syndrome is one of the leading causes of death in intensive care units worldwide and its incidence is progressively increasing (Kotsaki and Giamarellos-Bourboulis, 2012). Although major wall components of Gram-positive bacteria (peptidoglycan and lipoteichoc acid) can induce sepsis, the highest incidence of this syndrome is caused by lipopolysaccharides (LPSs) from Gram-negative bacteria (De Kimpe et al., 1995). Consequently, research in this field has been focused on LPS. LPSs are the major molecular component of the outer membrane of Gram-negative bacteria. This molecule represents a pathogen-associated molecular pattern (PAMP), responsible for the development of local inflammatory response through Toll-like receptor-4 (TLR-4) signaling (Miller et al., 2005). The inflammatory response is essential for bacterial clearence, but in extreme cases an exacerbated reaction may lead to septic shock (Salomao et al., 2012). Unfortunately, despite substantial advances in the pathophysiology of sepsis, there is no efficacious therapy against this syndrome yet (Schulte et al., 2013). As a consequence, septic shock syndrome continues to increase, reaching mortality rates over 50% in some cases (Buttenschoen et al., 2010).

In this context, the search for new therapeutics that can inhibit the activation of the innate immune system by LPS is of major importance (Pulido et al., 2012). Even though many studies in animal models and clinical trials have been conducted, there is no effective drug yet that interacts directly against LPS (Buttenschoen et al., 2010). Host-defense peptides (HDPs) could be a possible alternative solution since they possess antimicrobial, antiseptic, and immunomodulatory properties (Giuliani et al., 2010). These molecules have been identified as a defense strategy across many forms of life from prokaryotic organisms to vertebrates (Zasloff, 2002). HDPs are generally small, commonly having around 12–50 amino acid residues, cationic (net charge of +2 to +7), and are frequently quite hydrophobic and amphipathic (Jenssen et al., 2006). Furthermore, binding to diverse chemotypes of LPS and inhibition of LPS-induced pro-inflammatory cytokines from macrophages have been demonstrated for different HDPs (Scott et al., 2000; Lee et al., 2010). Interestingly, none of them have been isolated taking advantage of their affinity to LPS. As the search for new LPS-binding peptides is imperative for the development of more effective therapies, the use of LPS immobilized on different supports could be useful and suitable for isolating them. This approach could expand the rational search for anti-LPS HDPs.

LIPOPOLYSACCHARIDE ENDOTOXIN

Lipopolysaccharides are the major molecular component of the outer membrane of Gram-negative bacteria. This molecule is essential for the survival of Gram-negative bacteria, contributing to the correct assembly of the outer membrane. In this context, LPS provides a permeability barrier to many different classes of molecules such as detergents, antibiotics, and metals. Due to their localization, LPS molecules participate in host-bacterium interactions like adhesion, colonization, virulence, and symbiosis (Silipo and Molinaro, 2011).

Lipopolysaccharide is an amphiphilic molecule composed of three domains: lipid A, core oligosaccharide, and O-antigen repeats. Lipid A represents the hydrophobic component of LPS, which is located in the outer leaflet of the outer membrane and carries the endotoxic properties of LPS. This domain is the most conserved region of the lipopolysaccharide molecule. The hydrophilic portion of the molecule is composed of the glycan, O-antigen. The core oligosaccharide joins the lipid A and O-antigen domains. The core oligosaccharide domain can be divided into two regions: the inner core (proximal to lipid A) and the outer core (proximal to O-antigen). In contrast to lipid A, core oligosaccharide and O-antigen domains are displayed on the surface of bacteria (Brandenburg et al., 2010).

The activation of the immune system by LPS occurs through the transmembrane protein TLR-4, a pattern recognition receptor (PRR) found on the surface of many cells from the innate immune system. MD-2, a small membrane-bound glycoprotein, associates with TLR-4 for the recognition of LPS. Other proteins such as CD14 and LPS-binding protein (LBP) enable the interaction of LPS with MD-2. After LPS recognition, TLR4 homodimerises, initiating the recruitment of intracellular adapter molecules such as MyD88, Mal, Trif, and Tram and leading to the expression of diverse inflammatory genes (Miller et al., 2005; Bryant et al., 2010; Mcgettrick and O'Neill, 2010).

ANTI-LIPOPOLYSACCHARIDE HOST-DEFENSE PEPTIDES

The efficacy of HDPs against Gram-negative bacteria has been widely documented (Vaara, 2009; Cao et al., 2010; Park et al., 2011). The antimicrobial activity of these molecules is not only a consequence of their direct action against bacteria (at the cellular membrane and/or intracellular targets) but also of their anti-infective (modulation of the immune system) capacity (Hale and Hancock, 2007; Wieczorek et al., 2010). Although the outer membrane of Gram-negative bacteria constitutes an excellent permeability barrier to antibacterial agents, the interaction of HDPs with LPS permits this resistance mechanism to be bypassed. The process by HDPs across the outer membrane has been termed self-promoted uptake (Sawyer et al., 1988). In this mechanism, the peptides firstly interact with the negative surface of LPS and competitively displace the divalent cations that bridge the LPS barrier. This causes disturbance of the outer membrane, promoting peptide movement through it.

Host-defense peptides are very attractive molecules for use as therapeutics against septic syndrome due to their affinity for LPS and their antibacterial activity (Pulido et al., 2012). In fact, a number of natural HDPs from various sources bind to diverse chemotypes of LPS and reduce LPS-induced release of pro-inflammatory cytokines (Bowdish and Hancock, 2005; Bhattacharjya, 2010). For example, *in vitro* and *in vivo* LPS neutralization by the human cathelicidin peptide LL-37 has been demonstrated (Scott et al., 2002). The pretreatment of monocytes with this peptide inhibited *Pseudomonas aeruginosa* LPS-induced IL-8 production.

Interestingly, pro-inflammatory cytokine inhibition was abolished upon removal of LL-37 from the media before LPS stimulation, suggesting that the capacity of LL-37 to inhibit LPS signalling is dependent on extracellular LPS neutralization (Scott et al., 2011). Nevertheless, LL-37 may also have direct effects on macrophage function. Scott et al. (2002) demonstrated the upregulation of 29 genes and downregulation of another 20 genes in macrophages treated with the peptide using gene expression profiling experiments. Among the genes predicted to be up-regulated by LL-37 were those encoding chemokines and chemokine receptors, without stimulating the pro-inflammatory cytokine, TNF-α. Furthermore, an intracellular receptor for this peptide in monocytes has been discovered (Mookherjee et al., 2009). In order to increase the antiendotoxic activity of LL-37, various cathelicidin-derived peptides have been studied (Nagaoka et al., 2002; Nell et al., 2005). The antiendotoxic activity can be improved by increasing the hydrophobicity and cationicity of the parental peptide (Nagaoka et al., 2002). The LPS neutralization capacity of cathelicidins from another species has also been proved (Tossi et al., 1994; Tack et al., 2002; Bhunia et al., 2009).

Although the LPS neutralization properties of α-defensins are low (Scott et al., 2000), a potent antiendotoxic activity for some β-defensins has been established (Motzkus et al., 2006). Indeed, it has recently been demonstrated that LPS-binding activity and TNF-α release inhibition in RAW264.7 cultures for the human β-defensin DEFB114. Additionally, protection against LPS-induced reduction of human sperm motility *in vitro* and LPS-induced lethality of D-galactosamine-sensitized C57BL/6 mice were also demonstrated for DEFB114 (Yu et al., 2013). The antiendotoxic activity of DEFB114 was dependent on disulfide bond. On the other hand, fluorescence experiments demonstrated that DEFB126, another human β-defensin with anti-sepsis activity, can penetrate RAW 264.7 cells and diminish the production of LPS-stimulated inflammatory factors. In the same way, DEFB126 might also participate in intracellular immune regulation beyond its direct LPS neutralization (Liu et al., 2013). Perhaps DEFB126 uses a similar intracellular pathway to that of LL-37. Moreover, the differences in the antiendotoxic activity between α-defensins and β-defensins suggested that antibacterial activities do not necessarily correlate with anti-LPS properties (Bhattacharjya, 2010). Finally, the anti-LPS properties of invertebrate defensins have also been demonstrated (Saido-Sakanaka et al., 2004; Koyama et al., 2006). Other HDPs also have the capacity to inhibit LPS effects (Jacks et al., 1996; Giacometti et al., 2006; Lin et al., 2013; Schadich et al., 2013). These examples evidence the natural role of HDPs in the defense against LPS-induced septic shock.

Different mechanisms of LPS inhibition have been described for HDPs (Pulido et al., 2012). In general, the direct interaction of HDPs can disaggregate, or induce a change in the unilamellar/cubic structure of LPS to multilamellar, inhibiting the recognition of this molecule by the immune receptor complex

(Andra et al., 2005; Kaconis et al., 2011; Singh et al., 2013). Besides, HDPs also can inhibit LPS-induced sepsis by their modulation of immune cells. In this context, the inhibition of pro-inflammatory mediators (Liu et al., 2013), inhibition of surface expression of TLR-4 by interacting with microtubules (Li et al., 2013), and the normalization of the coagulation (Kalle et al., 2012) have been demonstrated.

Otherwise, the structural knowledge of LPS-high affinity binders such as Limulus anti-LPS factor (Hoess et al., 1993), Bactericidal/permeability-increasing protein (Beamer et al., 1997), Factor C (Tan et al., 2000) and Polymyxin B (Pristovsek and Kidric, 1999) among others has allowed the development of synthetic antiendotoxic peptides (Pristovsek and Kidric, 2001; Andra et al., 2006; Ding et al., 2008). Although preclinical data are very encouraging, only one of the synthetic variants, the recombinant fragment of protein BPI (rBPI21), has been tested successfully in clinical studies (Domingues et al., 2012). The situation is similar for natural compounds where there is no Food and Drug Administration (FDA)-approved drug yet that interacts directly against LPS. Only the apheresis procedures for endotoxin adsorption with Polymyxin B (lipopeptide produced by the bacterium *Bacillus polymyxa*) immobilized in the fiber column have been used for the treatment of septic shock patients in Japan since 1994 (Ruberto et al., 2013). Due to the failure to obtain antiendotoxic molecules with clinical efficacy, searching for new LPS-binding peptides is imperative (Giuliani et al., 2010).

LIPOPOLYSACCHARIDE IMMOBILIZATION

Affinity chromatography is one of the most efficient protein purification strategies. This technique is a method for selective purification of molecules from complex mixtures based on highly specific biological interaction between the immobilized ligand and the molecule of interest. The highly selective interactions that guide this procedure allow for a fast, often single-step process, with potential for purification in the order of several hundred to 1000-fold (Urh et al., 2009). Successful affinity purification is determined by the selection of a suitable support and immobilized ligand. The affinity support (the matrix onto which the ligand is immobilized) should selectively capture the molecule of interest while at the same time exhibiting low non-specific adsorption (Gustavsson and Larsson, 2006).

ISOLATION OF LIPOPOLYSACCHARIDE-BINDING PROTEINS

Affinity supports based on LPS immobilization could be a powerful tool for the isolation of anti-LPS HDPs. Indeed, the isolation of LBPs using these supports has been described (Minetti et al., 1991; Chiou et al., 2000; Shahriar et al., 2006). For instance, the Limulus endotoxin-binding protein-protease inhibitor (LEBP-PI), a 12 kDa protein from Limulus amebocytes, was purified using an LPS affinity column. In this study, the authors immobilized LPSs onto Affi-Gel Hz support (Bio-Rad). This support is based on hydrazide coupling chemistry. Affi-Gel Hz hydrazide gel is an agarose support which reacts with the aldehydes of oxidized carbohydrates (periodate oxidation) to form stable, covalent hydrazone bonds (O'Shannessy and Wilchek, 1990). A high yield of active LEBP-PI was achieved after elution with LPS or sodium citrate (Minetti et al., 1991). On the other hand, Chiou et al. (2000) isolated an LBP by affinity chromatography based on LPS from *Escherichia coli* O55:B5 coupled to cyanogen bromide (CNBr) activated Sepharose CL-4B. The isolated glycoprotein showed an apparent molecular mass of about 40 kDa and 72.2% identity to tachylectin-3, a lectin isolated from the amebocyte of T. tridentatus, previously characterized by its affinity to the O-antigen of LPS. Shahriar et al. (2006) also used LPS-affinity chromatography for isolation of LBPs in porcine milk. The affinity support was prepared by coupling 100 mg of *E. coli* F4 LPS – 3 g of CNBr-activated Sepharose 4B. Low affinity LBPs were eluted using mild conditions (Tris 10 mM, 1 M NaCl, pH 7.2) whereas high affinity binders were eluted using 0.1 M glycine-HCl, pH 2.5. The LBPs lactoferrin, soluble CD14, serum amyloid A, alpha-S1 casein, beta-casein, and kappa-casein were isolated by this approach. The coupling reagent used in the last two examples for synthesizing the affinity support was CNBr, which is very efficient for immobilizing proteins. It activates hydroxyl groups on the resin to create reactive cyanate esters, which then can be coupled to amine-containing ligands forming an isourea bond (Urh et al., 2009). The LPS molecule is thus immobilized on these resins by their hexosamines located at the core outer region and in lipid A.

ISOLATION OF LIPOPOLYSACCHARIDE-NEUTRALIZING PEPTIDES

Despite the success in purifying LBPs by affinity chromatography, this approach has not yet been used for isolating anti-LPS HDPs. The idea that interaction of LPS and anti-LPS peptides could be affected when one of them is immobilized on a matrix is possible, and therefore isolating anti-LPS peptides with immobilized LPS could be less efficient. Nevertheless, an interesting attempt to study LPS interaction with anti-LPS peptides when the latter were immobilized (Gustafsson et al., 2010) showed that immobilization of HDPs does not inhibit their capacity to neutralize LPS, although there are differences between the peptides assayed. The interaction of LPS with immobilized peptides was efficient both in LPS binding and inhibiting cytokine production induced by LPS.

Otherwise, the binding of HDPs to LPS occurs through the lipid A moiety: specifically, basic aminoacids interact with phosphates and hydrophobic aminoacids with the acyl chains (Bhattacharjya et al., 2007). For this reason, the immobilization of LPS using hexosamines located in the lipid A region may affect recognition by HDPs. Therefore, a conjugation method keeping free the lipid A moiety in the lipopolysaccharide molecule could be more efficient to immobilize LPS for the isolation of anti-LPS HDPs. In fact, Pallarola and Battaglini (2008, 2009) performed the coupling of LPS with probes bearing hydrazine or primary amino groups. LPS was modified through the activation of the hydroxyl groups present in its O-antigen moiety. Conjugates with good labeling ratios were obtained with the preservation of its endotoxic activity. A similar strategy was discussed above for the purification of LEBP-PI (Minetti et al., 1991). Otherwise, LPS molecules are capable of form micelles and aggregates even at very low concentrations (Yu et al., 2006). For this reason, the efficiency of LPS immobilization could be extremely low. Unfortunately, there is no data reported about the efficiency of LPS immobilization. Moreover, the coupling of LPS through the O-antigen domain may render a

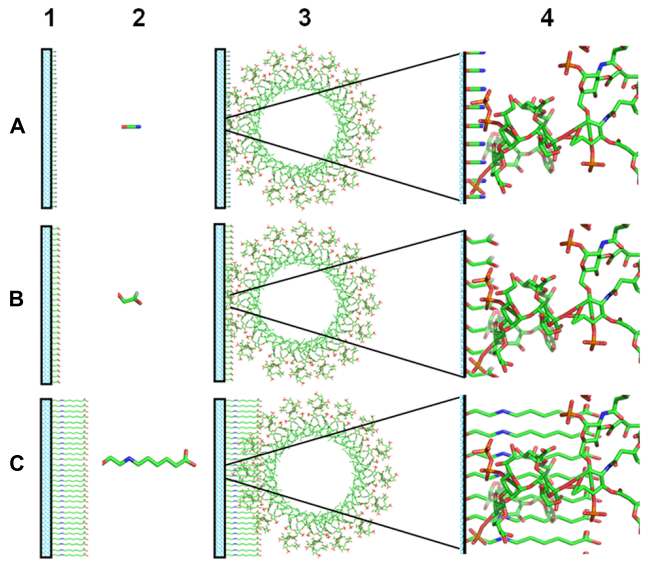

FIGURE 1 | Scheme of LPS immobilization on affinity supports. (1) Activated supports. (2) Coupling reagents. (3) Miscellar lipid A immobilized on affinity supports. (4) Zoom of the interaction of lipid A with the activated supports. **(A)** Cyanogen bromide activated support. **(B)** Glyoxyl activated support. **(C)** Ethyloxy-6-aminocaproic acid activated support.

higher immobilization efficiency than coupling LPS through hexosamines due to the poor reactivity of the latter (Eller et al., 2000). Nevertheless, the LPS immobilization through hexosamines could not be discarded due the large numbers of commercial resins that could be activated to react with amine groups (Urh et al., 2009). In this sense, the accurate use of coupling reagents may influences on the LPS affinity support synthesis. For example, coupling reagents with a higher length seems to better interact with LPS (**Figure 1**).

On the other hand, magnetic field-based separations using magnetic nanoparticles have received considerable attention in the last two decades (Horak et al., 2007). This methodology can be used on viscous materials, simplifying the purification process by removing sample pretreatment. Furthermore, the highly specific surface area of the nanoparticles enables the immobilization of a larger amount of molecule. Therefore, the immobilization of LPS on magnetic nanoparticles could be a very attractive procedure for isolating anti-LPS HDPs by magnetic separation. Although this approach has not been used yet, there are different examples of LPS immobilization on magnetic nanoparticles (Fornara et al., 2008; Piazza et al., 2011). For example, LPS was reversibly immobilized in a magnetic nanoparticle system consisting of oleylamine-coated iron oxide nanocrystals by hydrophobic interactions. LPS-magnetic nanoparticles were stable enough to mimic natural LPS aggregates for investigating the interaction of the LPS with TLR4 receptor (Piazza et al., 2011). In another approach, Fornara et al. (2008) synthesized magnetic nanoparticles for detection of *Brucella* antibodies in biological samples. Thermally blocked nanoparticles obtained by thermal hydrolysis were functionalized with LPS from *Brucella abortus*. LPS was attached to magnetic nanoparticles by adsorption through hydrophobic interactions, and the variation in magnetic relaxation due to surface binding of antibodies to LPS-functionalized nanoparticles was used to detect the disease. This method showed high sensitivity, with detection limit of 0.05 μg/mL of antibody in the biological samples without any pretreatment. Interestingly, the same approach could be used for detecting anti-LBPs from different sources. The same principle is not feasible for HDPs due to their small molecular weight. Furthermore, both systems described above could be used as affinity purification procedures, but the stability of hydrophobic LPS-functionalized nanoparticles in drastic elution conditions (0.1 M glycine-HCl, pH 2.5) would have to be evaluated.

As was exemplified above, the synthesis of affinity supports is no longer used only for purification of specific biomolecules. It is also rapidly becoming a method of choice to study biological interactions. In fact, Genfa et al. (2005) identified LPS-binding molecules in herb fractions by coating affinity optical biosensor cuvettes with lipid A via hydrophobic interactions after pre-incubating extracts with LPS. Such a concept demonstrated that LPS can be immobilized, keeping its ability to efficiently bind LPS binding molecules, and it could represent a high-throughput approach for the identification of LPS-neutralizing peptides (Zheng et al., 2010).

CONCLUDING REMARKS

Despite substantial advances in the pathophysiology of sepsis, there is no effective therapy against this syndrome yet. A therapeutic alternative could be the use of HDPs due to the capacity of some of them to neutralize LPS. As it is vital to find new LPS-binding peptides for the development of more effective therapies, the use of LPS immobilized on different supports could be useful and suitable for isolating them. This approach could expand the rational search for anti-LPS HDPs.

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From antimicrobial to anticancer peptides. A review

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Antimicrobial peptides (AMPs) are part of the innate immune defense mechanism of many organisms. Although AMPs have been essentially studied and developed as potential alternatives for fighting infectious diseases, their use as anticancer peptides (ACPs) in cancer therapy either alone or in combination with other conventional drugs has been regarded as a therapeutic strategy to explore. As human cancer remains a cause of high morbidity and mortality worldwide, an urgent need of new, selective, and more efficient drugs is evident. Even though ACPs are expected to be selective toward tumor cells without impairing the normal body physiological functions, the development of a selective ACP has been a challenge. It is not yet possible to predict antitumor activity based on ACPs structures. ACPs are unique molecules when compared to the actual chemotherapeutic arsenal available for cancer treatment and display a variety of modes of action which in some types of cancer seem to co-exist. Regardless the debate surrounding the definition of structure-activity relationships for ACPs, great effort has been invested in ACP design and the challenge of improving effective killing of tumor cells remains. As detailed studies on ACPs mechanisms of action are crucial for optimizing drug development, in this review we provide an overview of the literature concerning peptides' structure, modes of action, selectivity, and efficacy and also summarize some of the many ACPs studied and/or developed for targeting different solid and hematologic malignancies with special emphasis on the first group. Strategies described for drug development and for increasing peptide selectivity toward specific cells while reducing toxicity are also discussed.

Keywords: anticancer peptides, tumor selectivity, electrostatic interactions, membrane disruption, apoptosis induction, necrosis, drug development

INTRODUCTION: LEADING ANTIMICROBIAL PEPTIDES INTO ANTICANCER THERAPY

Inappropriate and irrational use of antibiotics has induced the worldwide emergence and spreading of resistant microorganisms. Nowadays, understanding the biological and biomedical importance of antimicrobial peptides might be regarded as an advance toward new and resistance-free therapies for infectious diseases. Antimicrobial peptides constitute a mechanism of immune defense with low antigenicity (Iwasaki et al., 2009) that can be found in innumerous eukaryotic organisms of different species (Reddy et al., 2004). These are small and generally amphipathic molecules, most of them containing cationic and hydrophobic residues in elevated proportion, thus capable of interacting with microbial membranes (Brandenburg et al., 2012; Seo et al., 2012) through non-specific interactions with the membrane lipids (Arouri et al., 2009). The short time-frame of interaction promotes the microbe rapid death and decreases the probability of resistance development (Fernebro, 2011). There is immense structural diversity in the several hundred AMPs that have been studied until today (Maroti et al., 2011). There are α-helical (such as cecropins), cysteine-rich and β-sheet AMPs (such as defensins). It is also common to find AMPs rich in His, Arg, Pro, and Trp (like indolicidin for instance) (Reddy et al., 2004).

Although Gram-positive and Gram-negative bacteria are the most studied targets for AMPs, many other different targets have been described, like fungi, protozoa (Giuliani et al., 2007) and enveloped viruses, such as HIV and herpes virus (Hancock and Diamond, 2000). The scientific literature is also rich in studies providing information on the mechanisms of action of AMPs (Friedrich et al., 2000; Mika et al., 2011). It is well established that the ability of these small cationic molecules in disrupting and permeating cell membranes is dependent on several biophysical properties, such as peptides' secondary structure, overall net charge, amphipathicity, hydrophobicity, size and balance between hydrophobic and polar regions (Reddy et al., 2004; Teixeira et al., 2012). This ability in permeating the cellular membrane is correlated with the antibiotic effect of several AMPs, such as defensins and cecropins (Steiner et al., 1988; Cociancich et al., 1993). Membrane disruption by AMPs can occur through different modes. It can either consist in pore formation in the lipid membrane (barrel stave and toroidal pore models), thinning of the membrane bilayer, membrane dissolution (carpet model), or lipid-peptide domain formation. In other cases AMPs are capable of intracellular targeting of the pathogen (Yeaman and Yount, 2003; Brogden, 2005; Papo and Shai, 2005; Bechinger and Lohner, 2006; Chan et al., 2006) since AMPs can bind to nucleic acids and proteins (Hancock and Sahl, 2006). There are also reports describing immunomodulatory activities for AMPs (Jerala and Porro, 2004; McPhee et al., 2005) such as the stimulation of chemokine and cytokines production as well as chemotaxis for leukocytes (Bowdish et al., 2005). For further detail on AMPs

structures, mechanisms and potential pharmaceutical applications, the reader is referred to the reviews by Li et al. and Brogden et al. (Brogden, 2005; Li et al., 2012). In addition to these well-known and described activities and targets, a growing number of studies report a broad spectrum of cytotoxic activity against cancer cells by these peptides (Moore et al., 1994; Mader and Hoskin, 2006; Hoskin and Ramamoorthy, 2008; Berge et al., 2010).

Cancer remains a major cause of death affecting millions of people and is caused by the growth and spreading of abnormal cells in an uncontrolled manner. Estimates from the international Agency for Research on Cancer (IARC) indicate that 12.7 million of new cancer cases and 7.6 million cancer deaths occurred worldwide during 2008 (Ferlay et al., 2010). Also, the worldwide statistics reveal that the most commonly diagnosed cancers are lung, breast and colorectal (Parkin et al., 2005; Ferlay et al., 2010). In the last decades many effort has been devoted in creating new therapies that are at the same time more selective and less harmful for the patients. Despite this, the methods today available such as surgery and chemotherapy have a relatively low success rate as well as they present a risk of reoccurrence (Harris et al., 2011). Indeed, chemotherapy treatment of prostate, bladder, kidney and pancreatic cancer as well as metastatic melanoma is being inefficient (Riedl et al., 2011a). For these cases where reoccurrence and/or metastasis occur, chemotherapy is the first line of defense (Riedl et al., 2011b). The therapeutic arsenal includes natural products, DNA-alkylating agents, hormone agonists/antagonists and antimetabolites but all of them presenting an insufficient selectivity and consequently an unspecific targeting of healthy mammalian cells with many deleterious effects (Kalyanaraman et al., 2002; Al-Benna et al., 2011). In fact, as chemical agents that are designed to attack the rapidly cancer dividing cells, they are expected to induce side-effects on normal cells that divide at the same rate. Consequently, it is very frequent the occurrence of myelossupression and thrombocytopenia (decreased production of blood cells), mucositis (inflammatory event on the digestive tract) and alopecia (hair loss) due to the non-selective targeting of cells from bone marrow, gastrointestinal tract and hair follicles (Riedl et al., 2011b). Moreover, once many of these compounds pass through the cell membrane and enter the cytosol they are transported back to the outside of the cell as a part of a mechanism of resistance from the cancerous cells (Perez-Tomas, 2006). Besides the increase of the drug transporters that carry the anticancer agent out of the cell, other mechanisms of multiple drug resistance (MDR) may be described. These include the ability of the cell to repair suffered DNA damage, tolerance to stress conditions and abnormal expression of drug detoxifying enzymes (Gatti and Zunino, 2005).

In a time where the number of people suffering from a cancer-related disease increases each day and where conventional therapies gather a worrying number of deficits and drawbacks, new treatment options are a demand for symptoms relieving and ultimately the eradication of the disease. In this context, anticancer peptides have been proved to be a resourceful strategy for the molecularly targeted cancer drug discovery and development process. Small molecules with an efficient tissue penetration and uptake by the heterogeneous cancer cells, endowed with intrinsic activity or synergizing with existing therapeutics, are expected to result in improved anticancer drugs with higher selectivity for neoplastic cells and reduced harmful effects over healthy tissues.

ANTICANCER PEPTIDES—CLASSIFICATION, SELECTIVITY, AND MODES OF ACTION

In a structural point of view, most ACPs have either α-helical or β-sheet conformation but some extended structures have already been reported (Hoskin and Ramamoorthy, 2008; Rodrigues et al., 2009; Wang et al., 2009a; Hammami and Fliss, 2010). Concerning cell targets, they can be classified into two major groups. The first one includes peptides active against microbial and cancer cells while not being active against healthy mammalian cells, such as cecropins and magainins. The second group contains ACPs that act against all three types of cells: microbial, normal and cancerous (Papo and Shai, 2005; Hoskin and Ramamoorthy, 2008), such as human neutrophil defensins HNP-1 to 3 (Papo and Shai, 2005; Droin et al., 2009). For a complete list of ACPs the reader is referred to the database available on http://aps.unmc.edu/AP/database/antiC.php.

The mechanism and selectivity criteria by which ACPs kill cancerous cells is still a controversial theme although some major conclusions can be outlined. ACPs oncolytic effects may generally occur either by membranolytic or non-membranolytic mechanisms (Harris et al., 2013). The mechanism underlying each membranolytic peptide activity is dependent on the ACP characteristics as well as on the target membrane features, which in turn modulate peptides' selectivity and toxicity (Schweizer, 2009). In fact, cancer and normal mammalian cells have a number of differences that are accounted responsible for the selectivity of some of the ACPs. These differences rely firstly in the membrane net negative charge that characterizes malignant cells (Schweizer, 2009). Anionic molecules such as the phospholipid phosphatidylserine (PS), O-glycosylated mucins, sialylated gangliosides and heparin sulfate are present in the membrane of cancer cells, conferring them a net negative charge which contrasts with the normal mammalian cell membrane, typically zwitterionic in nature (Hoskin and Ramamoorthy, 2008; Schweizer, 2009). Increased sialic acid content on the membrane affects membrane charge by stimulating surface concentration of acid groups (Dobrzynska et al., 2005). In addition to the modified glycosilation profile typical of cancer tissues and which is directly associated with the tumor phenotype (Dube and Bertozzi, 2005), during cell transformation PS molecules will present themselves on the outer membrane leaflet, accumulating on site and counteracting the typical phospholipid asymmetry of the membrane (Utsugi et al., 1991; Hoskin and Ramamoorthy, 2008; Schweizer, 2009). Along with the zwitterionic lipids, normal cell membranes have high contents of cholesterol which has been proposed as a protective molecule of the membrane by modulating the cell fluidity and blocking the entry/passage of cationic peptides (Schweizer, 2009). On the opposite, most cancer cell membranes are described to be more fluid than normal cells (Kozlowska et al., 1999; Sok et al., 1999) allowing membrane destabilization by ACPs. Nevertheless, there has been also shown that certain tumors, like breast and prostate, present a higher content of cholesterol in the cell membranes (Li et al., 2006) posing an obstacle to the lysis by ACPs. The cell surface area is also a factor controlling ACPs activity since the

elevated number and distorted features of microvili present on the malignant cells confer them higher surface area and higher contact with ACPs molecules (Domagala and Koss, 1980; Chaudhary and Munshi, 1995; Chan et al., 1998).

The negative surface charge of the cancer cell membrane is a characteristic also shared by the bacterial cells (Mader and Hoskin, 2006; Hoskin and Ramamoorthy, 2008). This fact lead to the hypothesis that AMPs and ACPs share similar molecular principles for selectivity and activity (van Zoggel et al., 2012). However, not all AMPs are ACPs (Hoskin and Ramamoorthy, 2008) and so it is of crucial importance the comprehension of all factors that allow ACPs to recognize and lyse neoplastic cells for understanding efficacy and selectivity phenomena. Unraveling the specific targets that are expressed and presented within a certain tumor type will be a valuable source of information in the process of drug design.

ACPs' membranolytic and selective mode of action on tumor cells can be due to the increased anionicity of the cytoplasmic membrane of these cells. The same "carpet" and "barrel-stave" models, for instance, used for describing AMPs interaction with bacterial membranes are also applied in this case (Pouny and Shai, 1992; Oren and Shai, 1998; Schweizer, 2009). Further membranolytic events involve the permeation and swelling of mitochondria with release of cytochrome c and apoptosis events (Mai et al., 2001). Although the rapid killing associated to ACPs might imply the prevalence of a non-receptor mediated mode of action, some non-membranolytic activities for ACPs have also been described (Sharma, 1992; Wachinger et al., 1998; Winder et al., 1998). Different attempts in controlling tumorigenesis involve the targeting of the angiogenesis process. Peptides that block the function of receptors expressed on angiogenic endothelial cells and that by this way perturb the formation of the vasculature associated to a tumor have been described (Arap et al., 1998; Mader and Hoskin, 2006; Schweizer, 2009; Lee et al., 2011; Rosca et al., 2011). The main goal nowadays when using an anti-angiogenic therapy is to normalize the tumor vasculature instead of reducing the density of tumor blood vessels (Shang et al., 2012). The development of therapeutic molecules which by their own or in a combination with other chemotherapy agents target several aspects of the angiogenic events might prove fruitful in cancer treatment (Rosca et al., 2011).

ANTICANCER PEPTIDES FOR SOLID AND HEMATOLOGICAL TUMORS

Regardless the many scientific studies published in which peptides are shown to successfully eliminate tumor cells both *in vitro* and *in vivo* and also prevent metastases formation (Cruciani et al., 1991; Ellerby et al., 2003; Papo et al., 2003, 2004, 2006), there has always been difficulties in establishing a clear structure-activity relationship for ACPs that might facilitate drug development. Targeted peptides which recognize tumors and metastases in a specific manner are difficult to obtain. In this section, information concerning ACPs that have been designed, synthesized or isolated and studied for targeting specific tumor cells is provided. Due to the vast broad spectrum of cancer cells tested for each ACP in study, the different tumors have been divided into two main groups, hematological and solid, and some of the ACPs that have been described to target cells from each group are reviewed with special emphasis on the solid tumors. Selectivity, efficacy and major requirements for anticancer activity are discussed. As the literature is vast concerning this matter, this review is focused in a period covering nearly 20 years of AMP cancer cell treatment. **Table 1** shows the primary sequence of some of the peptides with anticancer activity described in this review.

SOLID TUMORS

Solid tumors are characterized by a mass of tissue without cysts or liquid areas. In these tumors it is possible to distinguish malignant cells and the stroma where these cells are maintained (Dvorak et al., 1986). The tumor masses are represented by phenotypically heterogeneous populations of cancer cells, each having its own ability in proliferating and forming a new tumor (Reya et al., 2001; Al-Hajj and Clarke, 2004). The physiology and morphology of these tumors is largely deviated from the normal tissues (Brown and Giaccia, 1998) and these differences are currently being explored for selective cancer treatments in an attempt of circumvent the low specificity of the actual chemical and radiation therapy. Regarding the use of peptides in cancer therapy, many ACPs have been developed for targeting different types of solid tumors. Conclusions about structure requirements for the selective targeting of this type of tumors remain elusive. Available results show that ACPs target solid tumors by a variety of mechanisms.

Breast and prostate cancers are the most frequently diagnosed cancer in women and men aside from skin cancer (Jemal et al., 2006; Gross and Andra, 2012). Estimates indicate that breast and prostate cancers accounted for 23 and 14% of the total new cancer cases only in 2008 (Ferlay et al., 2010). Moreover, prostate cancer does not respond adequately to single or multiple drug regimens (Papo et al., 2004). Breast, prostate, uterine cervix, liver and lung are some of the targeted tumors for the development of ACPs. Some of these peptides defy the malignant cells by apoptotic or necrotic mechanisms after damaging cellular membranes, others by intracellular targets and it is also possible that one single peptide presents more than one mode of anticancer activity. **Table 2** summarizes some of the ACPs studied in the targeting of solid tumors.

Electrostatic interactions are key activity modulators

In a pioneer study, Iwasaki et al. evaluated by flow cytometry the PS density on the surface of several cancer cell lines and established a correlation with cell sensitivity to AMPs using four enantiomeric AMP analogs derived from beetle defensins (D-peptides A, B, C, and D) (Iwasaki et al., 2009). The results showed a selective cytotoxic activity dependent on the negative charge of the cancer cell membrane and PS, providing direct proof of PS role in the anticancer activity (Iwasaki et al., 2009). Papo et al. had also reported on a short host defense-like peptide that inhibits the growth of primary human prostate and breast carcinomas after being injected intratumorally (Papo et al., 2004, 2006). The D-K_6L_9 peptide induced a two-step cytolytic effect via membrane disruption and necrosis of tumor cells alongside with a decrease in the tumor vessel density, formation of new capillary tubes and in the secretion of prostate-specific antigen secretion. It was also

Table 1 | Peptides with anticancer activity toward solid and hematological tumors.

Peptide name	Amino acid sequence	References
D-peptide A	RLYLRIGRR	Iwasaki et al., 2009
D-peptide B	RLRLRIGRR	
D-peptide C	ALYLAIRRR	
D-peptide D	RLLLRIGRR	
D-K6L9	LKLLKKLLKKLLKLL	Papo et al., 2006
NRC-03	GRRKRKWLRRIGKGVKIIGGAALDHL	Hilchie et al., 2011
NRC-07	RWGKWFKKATHVGKHVGKAALTAYL	
Gomesin	ZCRRLCYKQRCVTYCRGR	Rodrigues et al., 2008
Hepcidin TH2-3	QSHLSLCRWCCNCCRSNKGC	Chen et al., 2009
Dermaseptin B2	GLWSKIKEVGKEAAKAAAKAAGKAALGAVSEAV	van Zoggel et al., 2012
PTP7	FLGALFKALSKLL	Kim et al., 2003
MGA2	GIGKFLHSAKKFGKAFVGEIMNSGGKKWKMRRNQF–WVKVQRG	Liu et al., 2013
HNP-1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	Wang et al., 2009c
Tachyplesin	KWCFRVCYRGICYRRCR	Chen et al., 2005
Temporin-1CEa	FVDLKKIANIINSIF	Wang et al., 2012
NK-2	KILRGVCKKIMRTFLRRISKDILTGKK	Schroder-Borm et al., 2005
Bovine lactoferricin B6 (Lbcin B6)	RRWQWR	Richardson et al., 2009
Cecropin CB1	KWKVFKKIEKMGRNIRNGIVKAGPKWKVFKKIEK	Srisailam et al., 2000

observed a decrease in the spontaneous and experimental metastases formation (Papo et al., 2006). PS was identified as the target for this peptide which colocalizes with the negatively charged phospholipid and exerts a membrane-depolarizing lytic activity on the neoplastic cells interacting with them in a selective way (Papo et al., 2006). Further than PS molecules, many other membrane components which contribute to the negative membrane of the malignant cell have been selected as anticancer activity triggers. Binding to negatively charged gangliosides expressed on the cell surface can be a potential alternative for directing ACPs activity and a source of information to elucidate the mechanisms by which AMPs bind to normal and cancerous cells (Hanai et al., 2000; Bitton et al., 2002; Guo and Wang, 2009; Miyazaki et al., 2012). NRC-03 and NRC-07 are two peptides from the AMP pleurocidin family with activity against human breast cancer cells including drug-resistant variants and with decreased affinity toward human healthy cells even by intratumoral administration (Hilchie et al., 2011). Peptides are able to bind the cancer cells and cause membrane effects through negatively-charged molecules that are exposed on the cells' membrane, specifically heparan and chondroitin sulfate. Cell death also involved mitochondrial damage and reactive oxygen species (ROS) production (Hilchie et al., 2011). Many other peptides were described, such as MPI-1 (Zhang et al., 2010), Gomesin (Rodrigues et al., 2008), tilapia hepcidin TH2-3 (Chen et al., 2009) and SVS-1 (Gaspar et al., 2012; Sinthuvanich et al., 2012), that seem to target cancer cells on the basis of charge rather than cell growth. MPI-1 is an analog of the AMP polybia-MPI, a peptide isolated from the venom of the wasp *Polybia paulista* (Wang et al., 2009b). MPI-1 peptide has a thioamide bond substitution that selectively binds to human prostate and liver cancer cells causing injury, swelling, bursting, and final cell death by necrosis (Zhang et al., 2010). Scanning electron microscopy (SEM) studies show the disruption of the cell membrane and the authors point the peptide amphipatic α-helical structure as crucial for its activity as well as the surface charge of the cell (Zhang et al., 2010). It is also shown that the thioamide bond substitution can be a valid strategy for designing ACPs representing a conservative modification of the peptide backbone structure (Zhang et al., 2010). The same authors have previously demonstrated that the original peptide, polybia-MPI selectively inhibited the proliferation of prostate and bladder cancer cells with low cytotoxicity for normal murine fibroblasts and that the α-helical conformation was an important feature for achieving an anticancer effect (Wang et al., 2008). The exposure of PS on the cells' membranes was suggested as a possible trigger for the peptides selective killing ability (Wang et al., 2008).

Gomesin is a cationic AMP with a hairpin-like two-stranded antiparallel β-sheet structure isolated from hemocytes of *Acanthoscurria gomesiana* (Rodrigues et al., 2008). Rodrigues et al. showed that this AMP possesses anticancer activity *in vivo* after topical treatment for subcutaneous murine melanoma and *in vitro* for melanoma, breast and colon carcinomas (Rodrigues et al., 2008). Although the precise mode of action is not described and may include pore formation, the cytotoxic activity was dependent on the β-hairpin structure and electrostatic forces as well as hydrophobic interactions which were already proved to be important factors for the AMP activity (Fazio et al., 2007). Most of the AMPs active against tumor cells adopt either a bioactive helical conformation at the cell surface or a β-sheet structure prior to engaging the membrane. SVS-1 peptide, a small designed anticancer peptide, folds only at the surface of cancer cells and acquires a β-sheet structure that disrupts the cell membrane via pore formation (**Figure 1**) (Sinthuvanich et al., 2012). The published studies show that this small 18-residue ACP folding is electrostatically induced and cell death occurs before the peptide neutralizes the cells' negative membrane charge (Gaspar et al., 2012). SVS-1 showed cytotoxic activity against lung, epidermal and breast carcinoma cells and low toxicity against healthy cells

Table 2 | Peptides and their respective oncolytic properties against solid tumors.

Peptide	Cancer cell	Experimental test	Selectivity	Anticancer activity	References
D-peptides A, B, C and D	Human cervix, glioma, lung, mouse myeloma, african green monkey kidney	ICL	Yes	Cell membrane disruption	Iwasaki et al., 2009
D-K_6L_9	Human prostate	ICL/GEM	Yes	Necrosis via membrane depolarization	Papo et al., 2006
NRC-03, NRC-07	Human breast	ICL/GEM	No	Cell membrane lysis with possible pore formation in mitochondria and ROS production	Hilchie et al., 2011
MPI-1	Human cervix, prostate and hepatocellular adenocarcinoma,	GEM	Yes	Necrosis after cell membrane targeting	Zhang et al., 2010
Polybia-MPI	Human bladder and prostate	ICL	Yes	Cell membrane disruption with probable pore formation	Wang et al., 2008
Gomesin	Murine melanoma, human breast, colon and cervix adenocarcinoma	ICL	Nd	Unclear; possible pore formation	Rodrigues et al., 2008
Hepcidin TH2-3	Human cervix, hepatocellular carcinoma, fibrosarcoma	ICL	Yes	Cell membrane lysis	Chen et al., 2009
SVS-1	Human lung, epidermis and breast	ICL	Yes	Cell membrane disruption via pore formation	Gaspar et al., 2012; Sinthuvanich et al., 2012
Epinecidin-1	Human lung, cervix, hepatocellular carcinoma, fibrosarcoma, histiocytic lymphoma	ICL	Yes	Cell membrane lysis mediated by necrosis inhibitory activity	Lin et al., 2009
Dermaseptin B2	Human prostate and breast	ICL/GEM	Yes	Necrosis	van Zoggel et al., 2012
PTP7	Human lung, prostate, breast and hepatocellular carcinoma	ICL	Yes	Apoptosis induction	Kim et al., 2003
BEPT II and BEPT II-1	Human prostate	ICL	Nd	Apoptosis induction	Ma et al., 2013
TfR-lytic peptide	Human breast and prostate, gliobastoma, pancreas and bile-duct	ICL/GEM	Yes	Apoptosis induction	Kawamoto et al., 2011
BPC96	Human cervix	ICL	Yes	Apoptosis induction	Feliu et al., 2010
RGD-Tachyplesin	Human prostate, melanoma	ICL/GEM	Some selectivity	Apoptosis induction	Chen et al., 2001
MG2A	Human cervix and lung, melanoma, rat glioma	ICL/GEM	Yes	Both necrosis and apoptosis	Liu et al., 2013
A_9K	Human cervix, kidney	ICL	Yes	Both necrosis and apoptosis	Xu et al., 2013

(Continued)

Table 2 | Continued

Peptide	Cancer cell	Experimental test	Selectivity	Anticancer activity	References
HNP-1	Mouse colon and breast	GEM	Nd	Mediation of antitumor immunity	Wang et al., 2009c
Hecate, Phor14 and Phor21 -BCG	Human prostate, breast, ovarian and testicular cells	ICL/GEM	Yes	Necrosis	Leuschner et al., 2003; Leuschner and Hansel, 2005; Hansel et al., 2007
Myristoyl-Cys-Ala-Val-Ala-Tyr-(1,3 dimethyl)His-OMe	Several human cell lines (lung, colon, breast, ovarian, renal, . . .)	ICL	Nd	DNA synthesis/replication inhibition	Ourth, 2011
9 somatostatin peptide analogues	Human colon	ICL	Yes	DNA polymerase β nhibition	Kuriyama et al., 2013
Pentastatin-1, chemokinostatin-1, properdistatin	Human breast	ICL/GEM	Nd	Tumor growth and angiogenesis inhibition	Koskimaki et al., 2009
ERα17p	Human breast	ICL/GEM	Nd	Apoptosis induction and massive necrosis	Pelekanou et al., 2011; Byrne et al., 2012
A-8R	Human prostate	ICL/GEM		ROS generation and DNA damage	Gao et al., 2013
CR1166	Human breast and pancreas	ICL	Yes	Apoptosis induction	Patra et al., 2012
Peptide aptamers	Human cervix, mouse melanoma, rat colon	ICL/GEM	Nd	Apoptosis induction by inhibition of HSP-70	Rerole et al., 2011
Tachyplesin	Human prostate	ICL	Nd	Activation of the classic complement pathway	Chen et al., 2005
Temporin-1CEa	Human breast	ICL	Yes	Membrane disruption, calcium release, ROS production	Wang et al., 2012, 2013a,b

Nd, not determined; ICL, immortal cancer-cell lineage; GEM, grafts experimental model (xenografts).

(HUVEC and erythrocytes) (Gaspar et al., 2012; Sinthuvanich et al., 2012). Recent studies point to a neutralization of the bacterial membrane charge that coincides or is closely related to minimal inhibitory concentration (MIC) values (Alves et al., 2010) contrary to what might happen with ACPs (**Figure 2**). Therefore, SVS-1 studies together with others which have been conducted with different types of peptides (Kim et al., 2003) clearly show that antitumor cell activities may actually not parallel AMPs mode of action and that differences should be expected. Furthermore, the different expression patterns of negatively charged molecules on cancer cell membranes will be a limiting factor conditioning the binding and engagement of peptides in the membrane and consequently dictating the ability of each peptide in killing specific cells. This points to the possibility of the same peptide to act by different mechanisms depending on the cell type in question (Yoo et al., 1997; Eliassen et al., 2006) and to be selective against determined types of cancer.

Interfering with necrosis and apoptosis mechanisms

After engagement of ACPs in the cell membrane, the peptides may penetrate into intracellular spaces. This entrance might lead to the disruption of the cell membrane accompanied by pore formation and/or changes on the cell membrane charge (Janek et al., 2013) and finally the interference with necrotic (Maher and McClean, 2008; Huang et al., 2011; Ausbacher et al., 2012; Wang et al., 2013a) and apoptotic pathways (Ausbacher et al., 2012). Pore formation is a mechanism associated to some AMPs and has been reported for ACPs as well (Rodrigues et al., 2009). The insertion of bulky hydrophobic amino acids on the cell membrane hydrophobic core with the acquisition of a stable structure can be the driving events for pore formation (Hilchie et al., 2011). Cell death might be a result of apoptosis and/or necrosis, which are characterized by different cellular morphological changes (Elmore, 2007). Indeed, apoptosis of cancer cells is a recognized strategy in cancer therapy (Qiao and Wong, 2009). While analyzing the

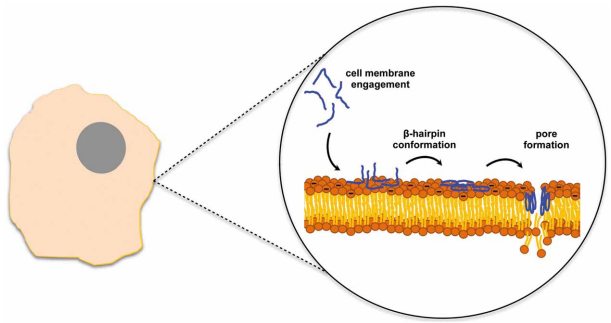

FIGURE 1 | SVS-1 anticancer peptide disrupts the cell membrane after engaging the membrane surface and folding into a β-hairpin conformation (Sinthuvanich et al., 2012).

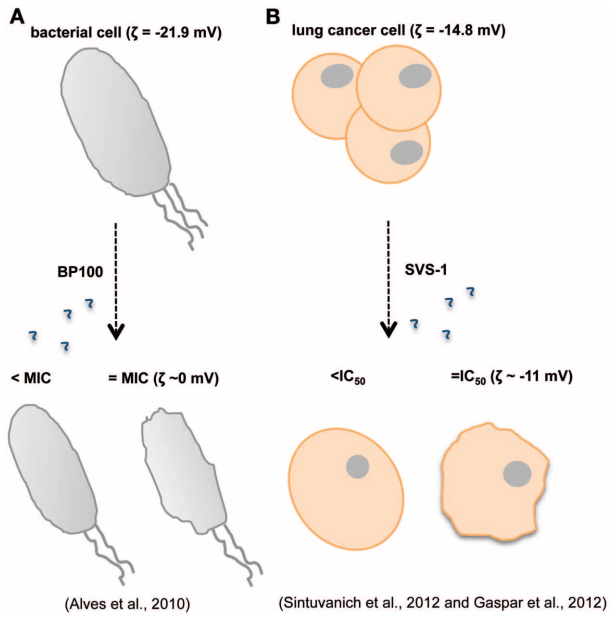

FIGURE 2 | Antimicrobial and anticancer peptides (AMPs and ACPs) are expected to show different modes of action. While for the BP100 AMP is possible to establish a correlation between the minimal inhibitory concentration (MIC) and membrane charge (Alves et al., 2010) **(A)**, for the ACP SVS-1 membrane neutralization does not occur even after cell death **(B)** (Gaspar et al., 2012; Sinthuvanich et al., 2012).

effects that ACPs exert upon cancer and healthy cells using microscopy and fluorescence tools, it is common to search for cell shrinkage or swelling, chromatin condensation, cytoplasmic vacuoles or even membrane blebbing (Elmore, 2007). There are many peptides capable of inducing these cellular changes. For instance, the synthesized AMP epinecidin-1 selectively kills cancer cells at low concentration and studies of necrosis inhibition test and real-time PCR indicate a membrane disruptive activity as well as an anti-necrosis effect by inhibition of necrosis gene expression (Lin et al., 2009). Dermaseptin B2 is one other example of a necrosis-inducing peptide. Increased lactate dehydrogenase (LDH) release, the positive staining with propidium iodide (PI) as well as confocal microscopy studies points to a necrotic mechanism which in turn might be induced after binding and disruption of the plasma membrane (van Zoggel et al., 2012). Also, induced apoptosis in several human cancer cell lines from breast, uterine cervix, liver and prostate has been described as the mode of action for different potential ACPs (Kim et al., 2003; Feliu et al., 2010; Kawamoto et al., 2011; Ma et al., 2013). It is also possible to attack metastatic tumor cells with peptide-induced apoptosis (Yang et al., 2008) as well as induce apoptosis in the tumor associated endothelial cells (Chen et al., 2001). As metastases are responsible for increased therapy failure, peptides that can specifically intervene in the process of metastases formation stimulating neoplastic cell death are valuable resources in cancer treatment. Chen et al. studied the effect of RGD-tachyplesin on human prostate cancer and melanoma cells (Chen et al., 2001). In this study, the natural tachyplesin was linked to a homing domain which facilitates the AMP internalization into the cells by the binding to integrins on tumor and endothelial cells (RGD sequence). Results showed that the peptide inhibited not only the growth of cancer cells both *in vivo* and *in vitro* with some degree of selectivity, but also affected membrane function triggering apoptosis (Chen et al., 2001).

Peptides that share both necrotic and apoptotic modes of action have been also described. The conjugation of magainin II (MG2) with the N-terminus of the cell penetrating peptide penetratin (Antp) resulted in a fusion peptide, MG2A, active against tumor cells with an IC_{50} in the micromolar range that target chondroitin sulfate on the surface of tumor cells (Liu et al., 2013). Tests involving apoptosis assessment by annexin V and PI staining, fluorescence microscopy and FACS analysis suggest necrotic cell death by membrane lysis while observing apoptotic cells. A different peptide also with dual mode of action was described by Xu et al. (2013). A_9K is a short designed amphiphilic AMP which combines a short length with other properties such as inherent surfactant-like and AMP activities, protease stability and absence of immunological responses. Presents high selectivity for leukemia, uterine cervix and kidney cancer cells killing cells by membrane disruption and apoptosis (Xu et al., 2013).

Diversified modes of action and molecular targets

The modes of action for ACPs are not limited only to the disruption of the plasma and mitochondrial membranes with the subsequent damages above-mentioned. Other mechanisms do exist and have been described, while it is not unusual to find peptides that combine more than one mechanism. These may involve alternative pathways such as, mediated immunity (Wang et al., 2009c), hormonal receptors (Leuschner and Hansel, 2005), DNA synthesis inhibition (Ourth, 2011; Kuriyama et al., 2013) and anti-angiogenic effects (Koskimaki et al., 2009) (**Figure 3**). Indeed, one study showed that human neutrophil peptides HNP-1 to 3 can exert several antitumor effects and that these might occur by a variety of mechanisms (Wang et al., 2009c). HNP-1 to 3 belong to the α-defensin group and are potent AMP with ∼30 amino acid residues (Droin et al., 2009). Different studies revealed that these peptides have potential as cancer prognostic markers (Albrethsen et al., 2005, 2006; Droin et al., 2009), are

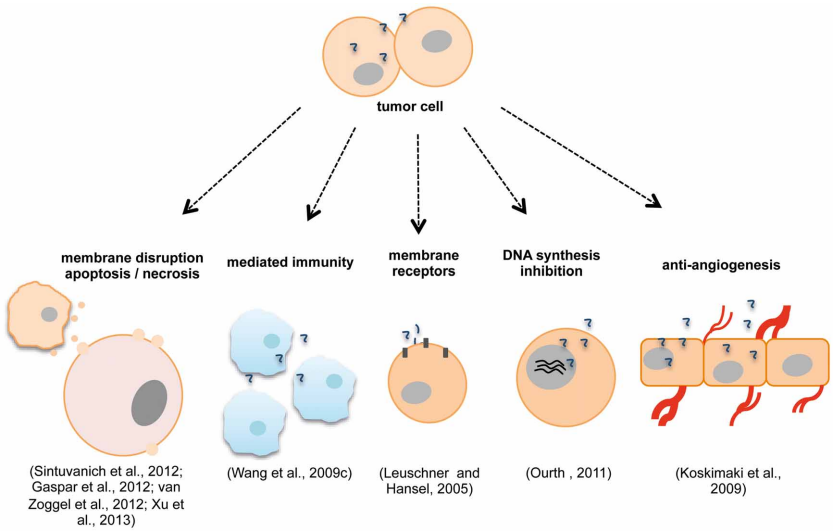

FIGURE 3 | Anticancer peptides (ACPs) modes of action may include disruption of plasma/ mitochondrial membranes (Sinthuvanich et al., 2012), necrosis, apoptosis (van Zoggel et al., 2012; Xu et al., 2013), mechanisms of mediated immunity (Wang et al., 2009c), membrane receptors involvement (Leuschner and Hansel, 2005), inhibition of DNA synthesis (Ourth, 2011) and anti-angiogenic effects (Koskimaki et al., 2009). Different ACPs can act by more than one mechanism simultaneously (Wang et al., 2009c; Xu et al., 2013).

active against a variety of healthy and malignant mammalian cells (Nishimura et al., 2004; McKeown et al., 2006) and that they are found in the tissue of epithelial tumors as well as they are associated with tumor necrosis when expressed intratumorally (Bateman et al., 1992; Muller et al., 2002). In this particular study, Wang et al. showed that the expression of mature HNP-1 in models of breast and colon tumors induced the recruitment and activation of dendritic cells which led to an immune response to the tumor from the host. HNP-1 intratumoral expression in its mature form may inhibit and eradicate established tumors (Wang et al., 2009c). Increased apoptosis and decreased angiogenesis events are also reported with the antitumor effects. Other studies reveal the potential in using peptides that target or mimic hormonal receptors and hormonal-regulated genes for treating cancer (Leuschner et al., 2003; Leuschner and Hansel, 2005; Hansel et al., 2007; Kampa et al., 2011; Pelekanou et al., 2011; Byrne et al., 2012; Gao et al., 2013). Leuschner et al. studied the ability of a series of compounds formed by synthetic membrane-disrupting peptides and a 15-amino acid residues segment of the beta chain of chorionic gonadotropin in targeting cells expressing luteinizing hormone/chorionic gonadotropin (LH/CG) receptors (Leuschner and Hansel, 2005; Hansel et al., 2007). These formed conjugates were able to destroy metastases and disseminated cells derived from human prostate cancer xenografts in nude mice and cells died by necrosis as revealed by histological examinations (Leuschner and Hansel, 2005). These studies prove that the lytic peptide conjugates might be useful for the inhibition of the development of metastases after surgical removal of the primary tumor (Hansel et al., 2007). In a different study, the ERα17p peptide originated from part of the sequence of the estrogen receptor α (ERα) was shown to interact with the polar part of the plasma cell membrane, to penetrate it and induce cell membrane damage at high concentration (Byrne et al., 2012).

Many other targets can be found for developing specific ACPs and many proteins have been highlighted for this effect. Patra et al. reported on the cell-permeable lipopeptide CR1166 that inhibits the PDZ domain of the GIPC (GAIP-interacting protein, C terminus) protein which is over expressed in breast and pancreas tumors (Patra et al., 2012). This inhibition interferes with the protein-protein interactions disturbing the events involved with GIPC activity and which in turn include tumor progression (Patra et al., 2012). Effects were observed *in vivo* and *in vitro* and account for decreased proliferation, cytotoxic effects and apoptosis on breast and pancreas cancer cells (Patra et al., 2012). The heat shock protein 70 (HSP70) has been also described as a potential protein target for treating neoplastic diseases (Rerole et al., 2011), since its upregulation induces an increased tumorigenicity of cancer cells in rodent models (Jaattela, 1995) and its downregulation kills cancer cells or renders them susceptible to apoptosis (Nylandsted et al., 2000). Molecules that inhibit matrix metalloproteinases (MMPs) activity are also interesting potential drugs (Destouches et al., 2012). MMPs are a family of membrane-bound zinc endopeptidases that display an important activity in what concerns remodeling of the extracellular matrix (ECM) in processes as tumor development, angiogenesis and metastatic progression (Coussens and Werb, 2002; Coussens et al., 2002; Egeblad and Werb, 2002; Visse and Nagase, 2003; Jang et al., 2011; Destouches et al., 2012). Many cancers express aberrant MMPs quantities (Derrico et al., 1991; Davies et al., 1993) and this fact can be used for creating strategies to block metastasis process. For instance, buforin IIb, that displays activity against 60 human tumor cell lines (Lee et al., 2008), was fused with a modified magainin sequence, a negative charge that would equilibrate the overall positive charge of buforin II, generating the MMIS:buforin IIb fusion peptide. Both peptide sequences were in turn linked by an octapeptide, cleavable by the MMP-2 (gelatinase

A) and MMP-9 (gelatinase B) enzymes which are over expressed in tumor tissues, allowing the release of buforin IIb (Jang et al., 2011). Cells expressing high amounts of MMPs such as mouse melanoma, human fibrosarcoma and gliobastoma were sensitive to this peptide. On the other hand, human uterine cervix cells which deficiently express these MMPs were resistant, and the fusion peptide anticancer activity was shown to be dependent on enzymatic activity (Jang et al., 2011). Two final different examples concerns to tachyplesin and temporin-1CEa. Tachyplesin is a peptide with 17 amino acid residues isolated from the horseshoe crab, which revealed an antitumor activity connected to a binding to hyaluronan or related glycosaminoglicans on the surface of cells and activation of the classic complement pathway leading to the disruption of the plasma membrane (Chen et al., 2005). The AMP temporin-1CEa causes breast cancer cell death by significant membrane disruption, intracellular calcium release and ROS over production (Wang et al., 2012, 2013a,b).

HEMATOLOGICAL TUMORS: LEUKEMIAS, MYELOMAS AND LYMPHOMAS

Hematological malignancies consist in a broad spectrum of diseases which comprise blood, bone marrow and lymph nodes cancer and are classified as leukemia, myeloma and lymphoma, respectively (Alvarez-Calderon et al., 2013). Estimates indicate a 3.4% of deaths caused by leukemia in 2008 (Ferlay et al., 2010). At the present, hematologic cancer is treated with cytotoxic drugs, radiation therapy or with bone marrow transplantation, which is known to cause severe long-term effects on patients (Alvarez-Calderon et al., 2013). As a complex group of diseases affecting multiple cell types, the literature provides numerous examples of peptides developed to target blood and bone marrow cells, many of them designed also to be active against solid tumors (**Table 3**).

The multiple roles of the negatively charged cancer cell membrane

As above-mentioned, the existence of negatively charged molecules on the cancer cell membrane might render cells susceptible to ACPs. Many peptides targeting non-solid tumors take advantage from electrostatic attraction, such as the NK-2 peptide derived from the cationic core region of NK-lysin from porcine and T-cells. NK-2 has a positive net charge and selectively kills cancer cells by a necrotic mechanism (Schroder-Borm et al., 2005). This killing ability correlates with the exposure of negatively charged PS on the surface of the cancer cell and the intercalation of the peptide into PS-containing membranes, being the leukemia cells with lower PS exposure the least sensitive (Schroder-Borm et al., 2005). This study involved also cells from solid tumors and Schroder-Borm et al. equally demonstrated the importance of PS presence on the cell membrane for NK-2 activity toward neuroblastoma cells (Schroder-Borm et al., 2005). The permeabilization of the plasma membrane due to pore formation by peptides was also demonstrated to occur by an increase in the electrostatics interactions and the transmembrane potential (Lemeshko, 2013). In this same study, the designed polycationic peptides revealed a selective anticancer activity against cancer human acute T cell leukemia which the authors attribute to higher values of surface and membrane potential of tumor cells when compared with normal cells (Lemeshko, 2013). Other different examples regarding the importance of electrostatics interactions in ACP—membrane interplay are patent on many studies such as the ones with the already described AMP polybia-MPI (Wang et al., 2009b) and with bovine lactoferricin 6 (LfcinB6) (Richardson et al., 2009). The short α-helical peptide polybia-MPI is selective toward leukemia cells probably due to the different amount of PS exposed in the cancer cell membrane (Wang et al., 2009b). Cell proliferation, viability and cytotoxicity assays revealed that polybia-MPI impaired the proliferation of sensitive and MDR cells while inducing LDH activity. On the contrary, the peptide showed lower effect on normal fibroblasts (Wang et al., 2009b). The mechanism of action relied on the disruption of the plasma membrane by pore formation which was shown by microscopy analyses. Upon contact with the negative cell membrane, the peptide acquires helical conformation capable of destroying the membrane (Wang et al., 2009b). Electrostatics is thus the main force attraction and the hydrophobic interactions allow the peptide insertion into the membrane. Leukemia cells died by a necrosis effect, with cells swelling and bursting (Wang et al., 2009b). Bovine lactoferricin (LfcinB) is a 25 amino acid residues cationic AMP isolated from cows' milk (Hoskin and Ramamoorthy, 2008). This peptide presents an amphipathic β-sheet configuration and displays anticancer activity against leukemia cells and various other carcinomas (Mader et al., 2005) being able to bind to GAGs of the cell membrane (Jenssen et al., 2004). LfcinB is capable of inducing apoptosis by direct disruption of the mitochondrial membrane (Furlong et al., 2008), but is also capable of lysing the membrane depending on the cancer cell type (Eliassen et al., 2006). LfcinB6 is the antimicrobial core of LfcinB peptide with a +3 net charge conferred by the three arginine residues in its sequence (Ueta et al., 2001). This charge is expected to promote bacteria death (Ueta et al., 2001) but it was insufficient for allowing the peptide binding and interaction with T cell leukemia membranes. Indeed, it was shown that a net charge of at least +7 was required for a strong cytotoxic activity toward the tumor cells (Richardson et al., 2009). On the other hand, the peptide showed cytotoxic activity when delivered by fusogenic liposomes into the cytosolic compartment of the same cells involving cathepsin B and caspase activities (Richardson et al., 2009). On a different study, CB1a, a cecropin-derived peptide, showed high cytotoxic activity against leukemia and stomach carcinoma with low hemolysis (Wu et al., 2009). In this case, the net positive charge of the peptide (+12) proved to be important for its activity even though the exact mode of action for this peptide is still poorly understood (Wu et al., 2009).

Even though peptides' net positive charge is shown essentially to have a promoting role in ACPs binding to the membrane negative charge of cancer cells, the negative components of the membrane might function also as inhibitors of the activity. Indeed, LfcinB and KW5, a derived peptide from LfcinB with 21 amino acid residues designed to adopt an idealized amphipathic α-helical structure, were shown to have decreased effect on lymphoma cells expressing heparan sulfate (HS) on the cell surface (Fadnes et al., 2009). It was proposed that the HS at the surface of cells sequester the peptide molecules leading them away from the membrane bilayer and thus poorly differentiated tumors with low expression of cell surface HS are

Table 3 | Peptides and their respective oncolytic properties against hematological tumors.

Peptide	Cancer cell	Experimental test	Selectivity	Anticancer activity	References
NK-2	Human chronic myelogenous leukemia, histiocytic lymphoma, acute T cell leukemia, acute lymphoblastic leukemia, neuroblastoma, colorectal adenocarcinoma	ICL	Yes	Necrotic death after peptide intercalation into PS-containing membranes	Schroder-Borm et al., 2005
Polycationic peptides	Human acute T cell leukemia	ICL	Yes	Plasma membrane permeabilization by pore formation	Lemeshko, 2013
Polybia-MPI	Human chronic myelogenous leukemia, promyelocytic leukemia, mouse lymphocytic leukemia	ICL	Yes	Disruption of the plasma membrane by pore formation	Wang et al., 2009b
Bovine Lactoferricin (LfcinB)	Human acute lymphoblastic T leukemia, acute T cell leukemia	ICL	Yes	Apoptosis by direct disruption of the mitochondrial membrane	Furlong et al., 2008
Bovine Lactoferricin B6 (LfcinB6)	Human acute T cell leukemia, acute lymphoblastic T leukemia	ICL	Yes	Intracellular cytotoxicity by cathepsin B and caspase activation	Richardson et al., 2009
Cecropin CB1a	Human acute lymphoblastic T-leukemia cells, lung carcinoma, stomach carcinoma	ICL	Yes	Unclear mode of action	Wu et al., 2009
SK84	Human leukemia, liver and breast	ICL		Membrane disruption	Lu and Chen, 2010
Magainin analogues	Human acute T and B cell leukemia, human chronic myelogenous leukemia, human histiocytic/Burkitt lymphoma, Ape T cell leukemia, human breast, prostate and neuroepithelioma	ICL	Yes	Membrane Lysis	Cruciani et al., 1991
Cecropin CB1	Human chronic myelogenous leukemia, acute T cell leukemia, acute lymphoblastic T-leukemia	ICL	Nd	Membrane Lysis	Srisailam et al., 2000
Pep 2 and Pep3	Human chronic myelogenous leukemia, acute lymphoblastic T-leukemia cells	ICL	Yes	Apoptosis of cancer cells through activation of caspases -3 and -9	Edison et al., 2012
BIM $SAHB_A$	Human histiocytic lymphoma, chronic myelogenous leukemia, acute myeloid leukemia	CT	Yes	Apoptotic resistance overcoming	Labelle et al., 2012

Nd, not determined; ICL, immortal cancer-cell lineage; CT, clinical trial.

more susceptible to peptides' activity (Fadnes et al., 2009). Apart from this inhibitory effect of the negatively charged molecules expressed on cancer cell surface, it is also possible to find peptides that seem insensitive to differences in the membrane charge, like SK84 (Lu and Chen, 2010). SK84 is a glycine-rich non-cationic AMP isolated from *Drosophila virilis* which membrane disruption activity of leukemia cells was evidenced by SEM (Lu and Chen, 2010). This disruption might be consequence of the perturbation of the membrane not due to electrostatic forces but by the formation of an elastic structure via the peptide flexible

N-terminal glycine-rich domains (Lu and Chen, 2010). The SK84 peptide, with this unusual mode of action, is toxic to leukemia, liver and breast cancer cells, while remains non-toxic to mouse and human erythrocytes (Lu and Chen, 2010).

Peptide activity on non-solid tumors

Magainins are naturally occurring peptides isolated from the skin of *Xenopus laevis* that present antibiotic activity toward different microorganisms (Cruciani et al., 1991). These molecules are α-helical peptides with separate cationic and hydrophobic faces comprising 21–27 amino acid residues (Hoskin and Ramamoorthy, 2008). Cruciani et al. showed that nine synthetic magainin peptide analogs lyse in a rapid and irreversible way several hematopoietic tumors with a cytotoxicity comparable to their antibacterial activity and with relatively non-toxicity to differentiated normal cells, peripheral blood lymphocytes (PBLs), and polymorphonuclear lymphocytes (PMNs) (Cruciani et al., 1991). The study revealed that all the tested hematopoietic cell lines were sensible to the peptides derivatives with varying degrees of cytolytic activity within minutes. It also showed that the magainin derivatives were selective toward the tumor cells at concentrations 5–10 times greater than those required for antibiotic effects but 10–20 times less than those which are toxic to normally differentiated cells (Cruciani et al., 1991). In agreement to magainins described mode of action, the permeability of tumor cell membranes was affected by α-helical channel formation on the cell membrane while non-cytolytic concentrations of these peptides were not sufficient to form selective α-helical ion channels capable of compromising cell viability (Cruciani et al., 1991).

Also α-helical peptides, cecropin derivatives have been studied as potential alternatives for targeting leukemia cells (Srisailam et al., 2000; Wu et al., 2009). In a study conducted using a custom AMP, cecropin B1 (CB1), researchers point out that parallel to the importance given to peptide structure, the orientation of the peptide after approaching the surface of the polar lipid heads conditions peptides' activity (Srisailam et al., 2000). CB1 activity was compared with two amphipatic α-helical segments derived from the natural cecropin B (CB) and no selective activity for CB1 was described while the differences reported for the IC_{50} values for different leukemia cell lines have been attributed to the heterogeneity regarding the different tested cells. Thus, adding to the structure and helix stability and to peptide self-orientation, the peptide flexibility appears to be a key factor for the efficient insertion on the membrane in the first events of membrane lysis (Srisailam et al., 2000).

Apart from LfcinB, other peptides have been recently described as apoptosis inducers in leukemia cells (Edison et al., 2012; Labelle et al., 2012). Pep2 and Pep3 are short synthetic peptides derived from the C-terminus of the proapoptotic mitochondrial protein ARTS and were shown to efficiently kill cells from human leukemia (Edison et al., 2012). Also, a stapled peptide combining the Bcl-2 interacting mediator of cell death (BIM) with stabilized α-helix of Bcl-2 domain (SAHBs) named BIM $SAHB_A$ was recently developed for targeting the Bcl-2 pathway (Labelle et al., 2012). This peptide disables survival proteins and activates the Bcl-2 family proteins resulting in cancer cell death by overcoming the apoptotic resistance expressed in hematological cancers (Labelle et al., 2012). Experiments showed that the peptide was able to suppress the growth of drug-resistant leukemia tumors in mice and also showed a synergistic anticancer effect when administered with other drugs (Labelle et al., 2012).

PERSPECTIVES AND OPEN QUESTIONS ON ANTICANCER PEPTIDES DESIGN AND DEVELOPMENT

The use of peptides in clinical treatments has many advantages as well as drawbacks. The challenge in ACP designing lies on the improvements of their delivery to the tumors while maintaining a low profile of toxic effects. The low selectivity of some of the ACPs molecules, the high cost of production in large scale, and their low resistance to proteolytic cleavage (Hu et al., 2011) are some of the main reasons why peptides have been retained in drug development pipelines. There are also some concerns related to the use of AMPs whose sequences are close to human and natural AMPs due to a possible compromise of the human natural defense and consequently threat to public health (Chen et al., 2012). On a positive view, since ACPs are not directed to a specific extracellular or intracellular receptor, some mechanisms of resistance can be impaired (Giuliani et al., 2007; Torfoss et al., 2012b) and actually some AMPs have shown cytotoxic activity against MDR cancer cells (Johnstone et al., 2000). The success for obtaining an optimal ACP relies then on the manipulation of its sequence, net charge, secondary structure, oligomerization ability, amphipathicity and hydrophobicity while maintaining high serum stability. The result should be a balanced equilibrium between these characteristics. Although there are no defined rules for designing ACPs, some facts established through structure-activity studies might help in elucidating the lack of selectivity for some peptides and potentiate drug development strategies. During the rational drug designing process differences in the pattern expression of surface molecules or in membrane fluidity between malignant cells types which may dictate the preference of the peptides for certain cancer cells in detriment of others (Fadnes et al., 2011) should not be neglected. Peptides' ability in crossing the cell membrane to reach intracellular targets is a major requirement for developing an anticancer agent. Peptides' structure might condition its internalization as well as contribute exponentially to the productions cost. Identifying the amino acid sequence in the peptides full sequence which might be responsible for the anticancer activity will certainly help reducing the high cost production by allowing the synthesis of shorter fragments that retain full biological activity. Many pharmacological parameters will be improved with this process, such as bioavailability and stability, and also immunogenicity is expected to decrease (Fadnes et al., 2011). It might also be expected that the shorter peptides are more efficient in reaching the membrane phospholipid bilayer with a concomitant increase of peptides' cytotoxicity (Fadnes et al., 2011). One clear and recent example reports to FK-16 peptide, a fragment of LL-37 which is the only peptide from the cathelicidin familiy found in humans (Ren et al., 2013). This shorter fragment showed an improved anticancer activity when compared to the original sequence and was described as capable to kill colon cancer cells by autophagic cell death, an additional cell death pathway, while reducing cost production

(Ren et al., 2013). It has been shown that arginine residues in cationic AMPs interact strongly with zwitterionic phospholipids which may result in toxicity events (Yang et al., 2003; Giuliani et al., 2007). It is therefore expected that other cationic residues might be used when constructing an ACP, such as lysine, to direct the peptide binding toward the negatively charged cells and simultaneously avoid hemolytic events. Serum stability might be improved by the incorporation of D-amino acids on the peptide sequence (Riedl et al., 2011b) and by cyclization of the structures (Torfoss et al., 2012b). The incorporation of lipophilic $\beta^{2,2}$ amino acids building blocks into heptapeptides resulted in a potent anticancer activity toward human and murine lymphoma cells, as well as high proteolytic stability and low toxicity against non-tumor cells (Torfoss et al., 2012a,b). The authors of this study state that the incorporation of the disubstituted $\beta^{2,2}$ amino acids in an α-helical peptide added an extra methylene group to the structure which in combination with two bulky lipophilic substituents, increased stability to protein degradation. Indeed, the central $\beta^{2,2}$ amino acid is flanked by two tryptophan residues to increase bulkiness and forming a lipophilic sequence where lysine cationic residues, are located at the N- and C-terminals (Torfoss et al., 2012a). The cyclization of these peptides resulted in structures with increased rigidity, amphipathicity and with changes in the secondary structure conformation which lead to an improvement of the anticancer potency against human lymphoma cells (Torfoss et al., 2012b).

The hydrophobicity of the peptides is also an important property when considered the hydrophobic environment that characterizes the cell membrane and it can be easily modulated in order to increase anticancer activity (Huang et al., 2011). In one recent study, the authors proved that manipulating the hydrophobicity of a 26-amino acid residues amphipathic peptide, V13K, by changing between alanine—leucine residues, it was possible to increase peptide activity against cancer cells, having human cervix cells shown high sensitivity, and thus showed a correlation between hydrophobicity—anticancer activity (Huang et al., 2011). Increasing hydrophobicity on the nonpolar face of the peptides enhanced their helical structure which in combination with the hydrophobicity resulted in stronger self-association and anticancer activity (Huang et al., 2011). In turn, helical structure acquisition can be controlled by D-amino acid substitution which may also modulate peptide specificity (Huang et al., 2012). The same study allowed the simultaneous observation of an increased hemolytic activity with the increase of the hydrophobicity of the peptides revealing a low degree of specificity. In 2013, a different study supported the same correlation between increasing hydrophobicity and anticancer and hemolytic activities (Yang et al., 2013). The authors suggest that high cationicity for enhancing neoplastic cell specificity and controlled hydrophobicity for equilibrating the hemolysis effect might be a suitable strategy for drug design (Yang et al., 2013).

Tumorigenesis is a multistep process where many factors intervene for the tumor growth and progression as well as in the metastatic and angiogenic events. For an effective targeting of each step, new therapeutic agents with the ability to kill slow-growing and drug resistant cancer cells, despite their proliferative capacity, are needed. The design of an oncolytic peptide with optimized properties and with high impact on the area of cancer treatment requires obtaining precise information concerning peptides' activity on the cell membranes at high resolution and detail. Once this goal is achieved, and combined with the advance knowledge on cancer biology, the optimized peptide may prove to be economically and therapeutically viable and a valuable alternative to current chemotherapeutics. These new chemotherapeutic drugs may synergize with the existing agents to restrict tumor activity and it is unlikely that they elicit multidrug resistance mechanisms and increase side-effects on healthy tissues and organs.

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Current scenario of peptide-based drugs: the key roles of cationic antitumor and antiviral peptides

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Cationic antimicrobial peptides (AMPs) and host defense peptides (HDPs) show vast potential as peptide-based drugs. Great effort has been made in order to exploit their mechanisms of action, aiming to identify their targets as well as to enhance their activity and bioavailability. In this review, we will focus on both naturally occurring and designed antiviral and antitumor cationic peptides, including those here called promiscuous, in which multiple targets are associated with a single peptide structure. Emphasis will be given to their biochemical features, selectivity against extra targets, and molecular mechanisms. Peptides which possess antitumor activity against different cancer cell lines will be discussed, as well as peptides which inhibit virus replication, focusing on their applications for human health, animal health and agriculture, and their potential as new therapeutic drugs. Moreover, the current scenario for production and the use of nanotechnology as delivery tool for both classes of cationic peptides, as well as the perspectives on improving them is considered.

Keywords: cationic peptides, antiviral, antitumor, target selectivity, therapeutic drugs

INTRODUCTION

Antimicrobial peptides (AMPs) are natural peptides found in microorganisms, plants, and animals, and are considered the evolutionarily conserved effectors in innate immunity (Hancock and Chapple, 1999; Hancock and Sahl, 2006). These peptides can be classified by physical–chemical properties such as cationic, anionic, hydrophilic, and amphipathic (Peter et al., 2010). Although they are diverse in length and sequence, two physical-chemical features are often the hallmarks of these molecules: they are cationic, often ranging from +2 to +7 at pH 7, and amphipathic, therefore their stereogeometry confers relatively polarized hydrophilic and hydrophobic facets (Nguyen et al., 2011). Despite their structural conservation, they have a broad spectrum of activity such as antibacterial (Okubo et al., 2012; Tavares et al., 2012), antioxidative (Power et al., 2013), antihypertensive (Escudero et al., 2012), antifungal (Mandal et al., 2013), antiviral (Findlay et al., 2013), antitumor as well as modulation of the immune response (Silva et al., 2012). The modulation of the immune response role is specific to a group of peptides named host defense peptides (HDPs). Moreover, multiple functions may be associated with a single peptide according to the concept of promiscuity. They may act over different targets, therefore presenting different functions depending on their physical-chemical (namely pure promiscuity) or on their amino acid modification (namely family promiscuity) (Franco, 2011). These features confer to AMPs and HDPs many physiological advantages over other molecules for application in the field of drug development. As is summarized here, many AMPs and HDPs isolated from a wide variety of organisms including mammals, amphibians, insects, plants, and bacteria have been reported to have antiviral and/or antitumor activities. This review comprises their mechanisms of action and targets, as well as their potential as new therapeutic strategies to combat both viruses and malignant cells.

CATIONIC ANTITUMOR PEPTIDES

Cancer has become a major concern in relation to human morbidity and mortality. All types of cancer are characterized by irregular cell growth originating from a small number of inherited or environmentally-stimulated genetic mutations (Renan, 1993). Many strategies have been adopted to combat the propagation of cancer cells and their elevated growth such as chemotherapy, surgery, and radiation (Wang and Zhang, 2013). These typical procedures have often been revealed to be non-specific for cancer cells (Yang et al., 2013), additionally acting on the cell division of healthy cells, consequently impairing the restoration of normal tissues (Smith et al., 2000). Antitumor drugs are subject to differences in absorption, metabolism, and target tissue, which can be particular to each patient; moreover, tumors can be positioned in places into which drug penetration is impaired or possibly sheltered by restricted environments due to amplified hydrostatic pressure in the tissue or modified tumor vasculatures (Szakacs et al., 2006). Furthermore, the intrinsic or acquired drug resistance is considered the widespread cause for tumor recurrence (Szakacs et al., 2006).

Acknowledging the limitations of these currently available therapies, researchers have been encouraged to seek novel anticancer agents with only one exclusive mechanism of action (Szakacs et al., 2006; Hoskin and Ramamoorthy, 2008). In this context, natural AMPs from different sources and their synthetic analogs have been the basis for a number of studies performed to discover new therapies for treating malignant cells (**Table 1**).

Table 1 | Cationic antitumor peptides from different sources, their application, and their mechanisms of action.

Peptides	Source	Group	Application	Mechanism of action	References
AGAP	*Buthus martensii*	Insect	Lymphoma, leukemia, human malignant glioma, human colon cancer	Cell cycle arrest	Cao et al., 2010; Zhao et al., 2011; Gu et al., 2013
Alloferon	*Calliphora vicina*	Insect	Leukemia	Induces NK and IFN—Immune-modulatory	Chernysh et al., 2002
Aplidine	*Aplidium albicans*	Tunicate	Melanoma, non-small cell lung, prostate, ovarian, colorectal	Multifactorial apoptosis inducer/cell cycle arrest/inhibition of protein synthesis	Faivre et al., 2005
Apratoxin A	*Lyngbya majuscula*	Cyanobacteria	HeLa (human cervical cancer)	Cell cycle arrest	Ma et al., 2006
Arenastatin A	*Dysidia arenaria*	Sponge	Human KB carcinoma	NR	Kobayashi et al., 1994
Aurein 1.2	*Litoria raniformis*	Frog	Leukaemia, lung, colon, CNS, melanoma, ovarian, renal prostate, breast	Barrel stave mechanism	Rozek et al., 2000
BMAP-27 BMAP-28	*Bos taurus*	Mammal	Leukemia, human erythromyeloblastoid leukemia, human leukemic monocyte lymphoma	Influx of Ca^{2+} DNA fragmentation Increase membrane permeabilization	Risso et al., 1998, 2002
Brevinin-2R	*Rana ridibunda*	Frog	Leukemia, lymphoma, colon carcinomas, fibrosarcoma, breast adenocarcinoma, lung carcinoma	Depolarize the transmembrane potential/lysossomal pathway	Ghavami et al., 2008
Buforin IIb	*Bufo bufo gargarizans*	Frog	Leukemia, breast cancer, non-small cell lung cancer, CNS cancer, melanoma, renal, ovarian, prostate and colon cancer	Apoptosis by a mitochondria-dependent pathway/caspase-9 activation/cytochrome c	Lee et al., 2008
Cecropins	*Hyalophora cecropia*	Insect and mammals	Leukemia, bladder	Carpet mechanism/Membrane disruption	Steiner et al., 1981; Chen et al., 1997; Papo and Shai, 2005; Lehmann et al., 2006; Suttmann et al., 2008; Xu et al., 2008
Cherimolacyclo peptide C	*Annona cherimola*	Plant	Human KB carcinoma	NR	Wele et al., 2004
Citropin 1.1	*Litoria citropa*	Frog	Leukemia, lung, colon, CNS, melanoma,ovarian, renal, prostate, breast	Carpet mechanism	Doyle et al., 2003
Cn-AMP1	*Cocos nucifera*	Plant	Colorectal adenocarcinoma	NR	Silva et al., 2012
Coibamide A	*Leptolyngbya sp.*	Cyanobacteria	Breast, CNS, colon, melanoma, leukemia, ovarian	Cell cycle arrest	Medina et al., 2008
CPAP	*Chlorella pyrenoidosa*	Algae	Human liver cancer	Condensation/ fragmentation of nuclear chromatin	Wang and Zhang, 2013
Cr-ACP1 and Cr-AcACP1	*Cycas revoluta*	Plant	Human epidermoid cancer, colon carcinoma	Cell cycle arrest	Mandal et al., 2012

(Continued)

Table 1 | Continued

Peptides	Source	Group	Application	Mechanism of action	References
CS5931	*Ciona savignyi*	Tunicate	Human colorectal carcinoma	Mitochondrial pathway of apoptosis	Cheng et al., 2012
Cyclotide	*Clitoria ternatea*	Plant	Lung cancer	NR	Sen et al., 2013
Cycloxazoline	*Lissoclinum bistratum*	Ascidian	MRC5CVI fibroblasts, T24 bladder carcinoma, leukemia	Cell cycle arrest/inhibition of cytokinesis	Hambley et al., 1992; Watters et al., 1994
Dianthins E	*Dianthus superbus*	Plant	Liver hepatocellular cells	NR	Hsieh et al., 2004
Didemnin	*Tridemnun solidum*	Ascidian	Leukemia, melanoma	Protein synthesis inhibition/apoptosis	Rinehart et al., 1983
Dolastatin 10	*Symploca sp.*, *Dolabella auricularia*	Cyanobacteria, mollusk	Murine leukemia cells, lung cancer	Bcl-2 phosphorylation/Caspase-3 protein activation	Bai et al., 1990; Kalemkerian et al., 1999
Gaegurins	*Rana rugosa*	Frog	Kidney, lung, colon, breast, stomach, liver, prostate, skin, ovary	Pore formation by carpet-model	Won et al., 2006
Geodiamolide H	*Geodia corticostylifera*	Sponge	Breast cancer	Altering the actin cytoskeleton	Freitas et al., 2008
Glidobactins A, B and C	*Polyangium brachysporum sp.*	Bacterium	Melanoma, leukemia, colon carcinoma	NR	Oka et al., 1988
Homophymines	*Homophymia sp.*	Sponge	Pancreatic cancer, human erythromyeloblastoid leukemia, breast, liver hepatocellular, human KB carcinoma, human colon adenocarcinoma, human ovarian, human prostate, glioblastoma, lung epithelial cells	NR	Zampella et al., 2009
Human alpha-defensin-1	*Homo sapiens*	Human	Human lung adenocarcinoma	Apoptosis by cytochrome c from mitochondria (mitochondrial pathway)	Xu et al., 2008
Human neutrophil Peptides (HNP-1: β-defensin)	*Homo sapiens*	Human	Leukemia and solid tumor	Induce membrane proteolysis	McKeown et al., 2006
Jamaicamide A	*Lyngbya majuscula*	Cyanobacterium	Human lung, neuroblastoma cell	NR	Edwards et al., 2004
Jaspamides	*Jaspis splendens*	Sponge	Lymphoma	Caspase-3 activation/decreasing in Bcl-2 protein expression	Ebada et al., 2009; Ghosh et al., 2010
Kahalalide F (KF)	*Elysia rufescens*	Mollusk	Colon, breast, non-small cell lung, prostate carcinoma, melanoma, hepatocellular carcinoma	Inhibit expression of genes involved in DNA replication/modifies lysosome membrane/apoptosis inducer	Hamann et al., 1996; Gracia et al., 2006; Singh et al., 2008
Keenamide A	*Pleurobranchus forskalii*	Mollusk	Leukemia, human lung adenocarcinoma, human colon adenocarcinoma	NR	Wesson and Hamann, 1996

(Continued)

Table 1 | Continued

Peptides	Source	Group	Application	Mechanism of action	References
Lactoferricin B	*Bos taurus*	Mammal	Human leukemia, fibrosarcoma, carcinoma, neuroblastoma	Mitochondria pathway of apoptosis/cytochrome c release/activation of the caspase cascade	Mader et al., 2005; Eliassen et al., 2006
LL-37	*Homo sapiens*	Human	Ovarian cancer	Pore formation by carpet-model	Chuang et al., 2009
Longicalycinin A	*Dianthus superbus*	Plant	Human liver carcinoma	NR	Hsieh et al., 2005
Lunasin	Soybean and other seeds	Plant	Breast cancer	NR	Hsieh et al., 2010
Lyngbyabellins	*Lyngbya majuscula*	Cyanobacterium	KB carcinoma	NR	Williams et al., 2003
Magainins	*Xenopus laevis*	Frog	Hematopoietic tumor, melanoma, ovarian cancer, bladder cancer, human cervical carcinoma	Mitochondria pathway of apoptosis/Pore formation by toroidal model/cytochrome c release/activation of the caspase cascade/Carpet mechanism	Zasloff, 1987; Cruciani et al., 1991; Jacob and Zasloff, 1994; Takeshima et al., 2003; Lehmann et al., 2006
Malevamide D	*Symploca hydnoides*	Cyanobacterium	Leukemia, lung cancer, human colon carcinoma	NR	Horgen et al., 2002
Melittin	*Apis mellifera*	Insect	Human hepatocellular carcinoma	Influx of Ca^{2+}/carpet mechanism/toroidal pore	Tosteson et al., 1985; Wang et al., 2009a
Mere15	*Meretrix meretrix*	Bivalve	Human lung adenocarcinoma	Induce release of cytochrome c/cleavage of caspases/poly ADP-ribose polymerase	Wang et al., 2012
Microcolin A	*Lyngbya majuscule*	Cyanobacterium	Breast carcinoma	Induction of apoptosis	Zhang and Longley, 1999
Mollamide	*Didemnum molle*	Ascidian	Leukemia, human lung carcinoma, human colon carcinoma	NR	Carroll et al., 1994
Pardaxinis	*Pardachirus marmoratus*	Fish	Human sarcoma	Elevation of caspase activities, disruption of the mitochondrial membrane	Huang et al., 2011
Phakellistatin 13	*Phakellia fusca*	Sponge	Human hepatoma	NR	Li et al., 2003
RA-XVII	Rubiaceous plants and *Aster tataricus*	Plant	Leukemia	Activation of caspase activity	Hitotsuyanagi et al., 2004
Sansalvamide A	*Fusarium ssp.*	Fungi	Pancreatic, colon, breast, and prostate sarcoma, melanoma	Cell cycle arrest	Vasko et al., 2010
Scopularide A and B	*Scopulariopsis brevicaulis* and *Tethya Aurantium*	Marine fungi, sponge	Pancreatic tumor, colon tumor	NR	Yu et al., 2008
*St*AP1 and *St*AP3	*Solanum tuberosum*	Plant	Leukemia	Induces apoptosis	Mendieta et al., 2010

(Continued)

Table 1 | Continued

Peptides	Source	Group	Application	Mechanism of action	References
Symplostatin 1	*Symploca sp.*	Cyanobacterium	Breast, colon tumor	Disrupts microtubules/mitotic arrest/induces apoptosis	Mooberry et al., 2003
Tachyplesin I	*Tachypleus tridentatus*	Japanese horseshoe crab	Human hepatocellular carcinoma, prostate carcinoma	Non-cytolytic mechanism	Chen et al., 2005
Tamandarins A and B	Family *Didemnidae*	Ascidian	Pancreatic carcinoma, prostatic cancer, head and neck carcinoma	Protein synthesis inhibition	Vervoort et al., 2000
Trapoxins A and B	*Helicoma Ambiens*	Fungi	Colorectal cancer	NR	Itazaki et al., 1990
Virenamides A–C	*Diplosoma virens*	Ascidian	Leukemia, human lung carcinoma, human colon carcinoma	Protein synthesis inhibition	Carroll et al., 1996
Viscotoxins	*Viscum coloratum, Viscum álbum*	Plant	Osteoblast-like Sarcoma, Yoshida sarcoma (rat)	NR	Xu and Jin, 1999
Vitilevuamide	*Didemnum cuculiferum* (Ascidians)	Ascidian	Colon tumor, lung cancer, melanoma, kidney cancer	Tubulin polymerization/cell cycle arrest	Edler et al., 2002

NR, Not reported. KB, nasophryngeal cancer; CNS, central nervous system.

Cationic antitumor peptides have been suggested as promising agents for antitumor therapy due to their numerous advantages over other chemical agents such as their low molecular masses, relatively simple structures, greater specific cytotoxicity to tumor cells over healthy cells, fewer adverse reactions, ease of absorption, a variety of routes of administration and low risk for inducing multi-drug resistance (Alberts et al., 1980; Mader and Hoskin, 2006; Hoskin and Ramamoorthy, 2008; Schweizer, 2009; Riedl et al., 2011; Liu et al., 2013). They can also function in combination with conventional therapies and other potential anticancer molecules, usually improving the results of therapy (Chuang et al., 2009; Wang et al., 2009a). The intrinsic relationship between their chemical structure, i.e., their cationic and hydrophobic features, and their high specificity to tumor cells is likely to be the key role for their cytotoxicity. These characteristics allow the cationic AMPs to bind to and invade cancer cells, quickly disrupting membranes and leading to the outflow of intracellular contents and consequent cell death (Yeaman and Yount, 2003; Leuschner and Hansel, 2004; Jenssen et al., 2006). This happens due to the negatively-charged tumor cell membrane [derived from a greater than normal expression of anionic molecules such as sialic acid-rich glycoproteins, phosphatidylserine (PS) or heparan sulfate], which present essential differences with the neutrally-charged healthy cell membranes. Therefore, these chemical differences aid the electrostatic interaction of the positively-charged peptide and the negatively-charged tumor cell membranes (Dobrzynska et al., 2005; Hoskin and Ramamoorthy, 2008). The membranolytic mechanism was first discovered in a study of magainin and its synthetic analogs against hematopoietic and solid tumors (Cruciani et al., 1991). Cationic antitumor peptides might also induce disruption of intracellular targets by so-called non-membranolytic mechanisms. For instance, there are reports of antitumor peptides that cause necrosis by triggering intracellular apoptotic pathways (e.g., by inactivating mitochondria; activating caspase cascade) (Mader et al., 2005; Chen et al., 2012; Paredes-Gamero et al., 2012). Cationic antitumor peptides can also impair the activity of proteins of the signal transduction pathways involved in oncogene activities (Sharma, 1992). Both membranolytic and non-membranolytic mechanisms are discussed in this review and are represented in **Figure 1**.

Cationic peptides that kill cancer cells are arranged into two groups: (1) AMPs which are effective against bacteria and cancer cells but not against normal mammalian cells such as cecropins from insects and magainins from amphibians and (2) AMPs which are cytotoxic for bacteria, cancer cells, and normal mammalian cells such as melittin, tachyplesin II, human neutrophil defensins, and insect defensins (Papo and Shai, 2005; Hoskin and Ramamoorthy, 2008; Schweizer, 2009). It is believed that the difference between both groups relay mainly on the difference of the membrane of tumorous and normal cells (Hoskin and Ramamoorthy, 2008), therefore it would be essential to detailed understand these important changes at the molecular level. To date, it is here understood that besides the difference on the electrostatic charge between tumorous and normal cells cited above, the membrane fluidity may also interfere on the susceptibility of the peptide to disrupt the cells (Hoskin and Ramamoorthy, 2008). The fluidity of cancer cells is greater than that of mammalian normal cells (Sok et al., 1999) which may facilitate membrane destabilization favoring the binding of cationic antitumor peptides, and the amount of cholesterol,

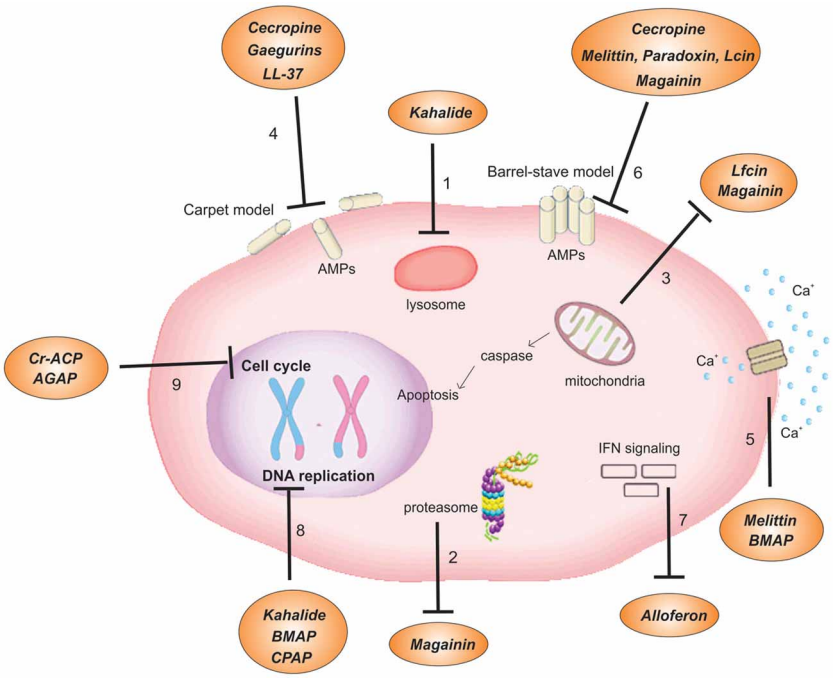

FIGURE 1 | Mechanisms of action of cationic antitumor peptides. (1) Modification of the lysosome membrane leading to an acidification of the intracellular environment and cell death. (2) Amplification of the proteasome activity. (3) Induction of mitochondrial pathway of apoptosis by either the cytochrome c release into the cytoplasm or activation of the caspase cascade. (4) Pore formation by the carpet model. (5) Increase of the influx of Ca^{2+}. (6) Formation of pore by either the toroidal or the barrel-stave models. (7) Activation of an immune modulatory pathway by induction of NK and IFN. (8) Inhibition of genes involved in DNA replication. (9) Arrest of cell cycle G0, G1, or S phases. Lfcin, lactoferrcin; BMAP, bovine myeloid antimicrobial peptide; Cr-AMP, Chlorella pyrenoidosa antitumor polypeptide.

the major component of eukaryotic cell membranes (Simons and Ikonen, 2000), alters the membrane fluidity, therefore protecting eukaryotic cells from cytolytic effects of antitumor peptides. For instance, membrane-insertion of the antitumor peptide cecropin is shown to reduced when synthetic lipid vesicles and Gram-negative bacteria have their cholesterol amount enhanced (Silvestro et al., 1999). Furthermore, the surface area of the membrane of tumorous cells, where the number of microvilli is higher than on non-tumorous cells (Chaudhary and Munshi, 1995), may also allow an increased number of peptides to bind to the membrane (Chan et al., 1998a,b).

In the following sections, cationic antitumor peptides from both groups which are derived from many sources including plants, vertebrates, and invertebrates will be presented (see **Table 1**).

CATIONIC ANTITUMOR PEPTIDES ISOLATED FROM PLANTS

High quantities of natural compounds and molecules from plants have been intensely studied for the treatment of a diverse number of diseases including cancer. Reviewed here is a specific class of cyclic peptides originally found only in plants as well as non-cyclic peptides with antitumor properties.

Clitoria ternatea is a rich source of a specific class of peptides named cyclotides, composed of a cyclic backbone combined with a conserved six cystine knot (Craik and Malik, 2013). A study carried out by Sen et al. (2013) has isolated five cyclotides named CT2, CT4, CT7, CT10, and CT12 (with an addition or removal of a net charge from −1 to +2) from this plant species. They showed significant cytotoxicity against human lung cancer cells (A549) and demonstrated a reduction of 2- to 4-fold of the IC_{50} (half maximal inhibitory concentration) value of cyclotides when compared to a mitotic inhibitor used in cancer chemotherapy. Moreover, these peptides were less cytotoxic against A549/paclitaxel (a sub-linage of A594) than only A549, which demonstrates a possible use of chemo-sensitization for treating cancer (Sen et al., 2013).

Along with cyclic peptides, many active non-cyclic antitumor peptides have also been isolated from different plants. For instance, the promiscuous peptide *Cn*-AMP1, isolated and purified from coconut water (*Cocos nucifera*), was tested against CACO-2- human epithelial colorectal adenocarcinoma cells and showed a 13% reduction of cell viability (Silva et al., 2012). Additionally, the peptide lunasin, isolated from soybeans and other seeds, was capable of suppressing *in vitro* and *in vivo* chemical carcinogen-induced tumorigenesis (Hsieh et al., 2010). *St*AP1 and *St*AP3, isolated from the potato *Solanum tuberosum*, were shown to induce apoptosis in Jurkat T leukemia cells (Mendieta et al., 2010; Guevara et al., 2011). Other examples are the peptides Cr-ACP, isolated from *Cycas revoluta*, and its acetylated-modified Cr-AcACP1, both repressors of cell proliferation of human epidermoid cancer (Hep2), and colon carcinoma through the induction of cell cycle arrest at the G0–G1 phase of Hep2 cells (Mandal et al., 2012).

Among chlorophyllous organisms, few studies have been developed. One example is the polypeptide *Chlorella pyrenoidosa* antitumor polypeptide (CPAP) isolated from the unicellular green algae *Chlorella pyrenoidosa*. It has shown the highest inhibitory activity on human liver HepG2 cancer cells (49%). CPAP induces apoptosis and necrotic death of HepG2 cells via membrane shrinkage, condensation and fragmentation of nuclear chromatin as well as formation of black apoptotic bodies (Wang and Zhang, 2013).

CATIONIC ANTITUMOR PEPTIDES ISOLATED FROM INVERTEBRATES

Bioactive peptides isolated from insects present many different activities and have strong potential as therapeutic agents (**Table 1**) (Chernysh et al., 2002). Cecropins, alloferons, and melittins are examples of AMPs isolated from insects which function as antitumor molecules with applications for various kinds of tumor cells (**Figure 1**). The AMP cecropin was first isolated from the giant silk moth *Hyalophora cecropia* (Steiner et al., 1981). Cecropins shares their potential antitumor activity with structural analogs from other families of AMPs such as magainins and defensins (Papo and Shai, 2005; Lehmann et al., 2006). It has been shown that two cecropin B analogs, cecropin B1 (CB1) which possesses two amphipathic helices, and cecropin B3 (CB3) which has two hydrophobic helices, exhibit strong cytotoxic activity against a number of human leukemia cell lines and do not lyse normal fibroblasts or erythrocytes (Srisailam et al., 2001). Both analogs exhibit drastically different mechanisms of actions on anionic lipid vesicles and show the importance of the structure and sequence of a cationic antitumor peptide and its potency toward different cancer cells (Srisailam et al., 2001). It has been claimed that the discontinuous helical segments in the structure of CB3 do not favor helix—helix interactions which are crucial for pore formation; these mechanisms are likely to be adopted by CB1 where the continuous helical conformation interacts with cell-surface structures such as microvilli in cancer cells. In a different study, CB1 was a more powerful cytolytic agent than cecropin B against HL-60 human promyelocytic leukemia cells (Chan et al., 1998a,b). Cecropin A and B have been shown to reduce the viability of bladder cancer cells (Suttmann et al., 2008) and to directly induce tumor cell lysis via cell membrane disruption, which stimulates cytolysis/necrosis. Furthermore, it was shown that these peptides are capable of imposing the disruption of mitochondrial membranes, consequently leading to the activation of apoptosis pathways (Suttmann et al., 2008).

Another important AMP isolated from insects is alloferon: a tridecapeptide isolated from the bacteria-challenged larvae of the blow fly *Calliphora vicina*. Synthetic alloferon I has been shown to act on tumor growth control in two different ways. It stimulates natural killer (NK) lymphocytes and interferon (IFN) *in vitro* by using mouse spleen lymphocytes and human blood mononuclear cells. The peptide was administrated in picomolar concentrations where its potential to stimulate natural cytotoxicity in these models was confirmed. Alloferon also induces IFN synthesis *in vivo* which was demonstrated using animal and human models, and consequently enhancing its antitumor activity (**Figure 1**). Based on these results, the researchers have suggested an interaction of alloferon anticancer activity with its immunomodulatory properties. The interferonogenic activity was more evident *in vitro* using human cells than *in vivo*, while in comparison to mouse cells the opposite result was observed. The NK-IFN network is well-documented and demonstrates the potential immune modulatory properties of this AMP. Furthermore, it has been recently shown that the combination of chemotherapy and alloferon I, referred to as pulse immune chemotherapy, demonstrated significant advantages compared to each treatment applied separately (Chernysh et al., 2002, 2012).

Also from the group of insects, the AMP melittin, isolated from *Apis mellifera*, is also an active molecule against antitumor cells. It is cytotoxic against human hepatocellular carcinoma (Tosteson et al., 1985; Wang et al., 2009a). Studies indicate that melittin damages cell membranes either via the barrel-stave mechanism, i.e., it acts under its membranolytic properties (Sui et al., 1994), or via its non-membranolytic properties through a mechanism that engages the hyperactivation of phospholipase A2 and the influx of Ca^{2+}, resulting in the destruction of the transformed cells (**Figure 1**) (Sharma, 1992, 1993).

Another antiviral AMP within the group of invertebrates is the peptide AGAP isolated from the scorpion *Buthus martensii*. It has been reported to possess both analgesic and antitumor activities. Recently, a heterologous expression system has been constructed using small ubiquitin-related modifier-AGAP (SUMO-AGAP) which is a product of recombinant AGAP (rAGAP) linked with a hexa-histidine tag from *Escherichia coli*. This recombinant system showed considerable inhibition of lymphoma and glioma propagation (Gu et al., 2013). Using SW480 human colon cancer cells, it was proposed that rAGAP induces cell cycle arrest in the G0/G1 phase, attended by the decrease in the S phase without significant change in the G2/M phase (Gu et al., 2013).

Other sources of cationic peptides isolated from invertebrates are found in the marine ecosystem. The biodiversity of this environment has been shown to be a rich source of biologically active molecules and has been considered an unlimited resource of new antitumor agents (Zheng et al., 2011; Malaker and Ahmad, 2013). Several AMPs with antitumor activity have been isolated from marine invertebrates such as cyclic depsipeptide didemnins. Isolated from the ascidian of the genus *Tridemnum*, it has shown antitumor activity against L1210 leukemia cells *in vitro* and P388 leukemia and B16 *in vivo* (Rinehart et al., 1983). The antitumor role of the peptide kahalaide F from *Elysia rufescens,* a marine gastropod mollusk, has also been reported. It has shown significant *in vitro* and *in vivo* activity against non-small cell lung cancer, colon and human breast tumor cell lines, melanoma, androgen-independent prostate cancer and hepatocellular carcinoma (Martin-Algarra et al., 2009; Malaker and Ahmad, 2013). This peptide acts on the liposome membrane of tumor cells and modifies its basal function (**Figure 1**) (Hamann et al., 1996; Singh et al., 2008). Moreover, it modifies the role of the lysosomal membrane, leading to intracellular acidification and cell death, a characteristic that discriminates it from all other known antitumor agents (Gracia et al., 2006). This peptide also appears to inhibit the expression of certain specific genes that are involved in DNA replication and cell proliferation, thereby inhibiting tumor spreading and growth. Recently another marine AMP isolated from the bivalve *Meretrix meretrix*, named mere15, has shown to

significantly inhibit the growth of human lung adenocarcinoma A549 xenograft in nude mice (Wang et al., 2012).

CATIONIC ANTITUMOR PEPTIDES ISOLATED FROM VERTEBRATES

Many peptides have been discovered from a variety of vertebrates that are responsible for improving the innate immune response (**Table 1**) They have been found at relatively low concentrations in the normal tissues of mammals and are usually present within the granules of neutrophils, in mucosal or skin secretions from epithelial cells, and as the degradation products of proteins (Boman, 1995; Hancock, 2001). Among the mammalian organisms, bovines have been a promising source of molecules which show a broad range of physiological activities, including immune function enhancement and defense against pathogenic bacteria and viruses. Among these molecules, three AMPs named BMAP-27, BMAP-28 and lactoferricin have been reported as active antitumor peptides. The bovine myeloid AMPs BMAP-27 and BMAP-28 (27 and 28 amino acid residues, respectively) have shown cytotoxic activity against neoplastic cells. These peptides, when tested against fresh tumor leukocytes from patients affected by myeloid or lymphoid leukemia, have shown to increase membrane permeabilization and the influx of Ca^{2+}, followed by DNA fragmentation, which is characteristic of programmed cell death (**Figure 1**) (Risso et al., 1998).

The cytotoxic activity of bovine lactoferricin (LfcinB) has been demonstrated *in vitro* with different rat and human cancer cell lines including leukemia, fibrosarcoma, various carcinoma, and neuroblastoma cells, and did not influence the viability of normal fibroblasts, lymphocytes, epithelial cells, endothelial cells, or erythrocytes (Yoo et al., 1997; Mader et al., 2005; Eliassen et al., 2006). It has been proposed that Lfcin targets tumor cells by the changes that occur in their cell membranes, such as the exposure of negatively-charged head-groups derived from the loss of phospholipid asymmetry in diseased cells (Gifford et al., 2005; Pepe et al., 2013). The activities against fibrosarcoma and neuroblastoma rat cells and human T-leukemia cells can be described by a mechanism that induces the formation of transmembrane pores allowing the peptide to enter the cytoplasmic compartment of the cancer cell, co-localize with negatively-charged mitochondria and consequently depolarize them, resulting in cytochrome C release or activation of the caspase cascade, thereby leading to cell death via apoptosis (**Figure 1**) (Mader et al., 2005; Pepe et al., 2013). Moreover, it may interfere with the interaction between growth factors and their receptors on the surface of endothelial cells, resulting in decreased endothelial cell proliferation and diminished angiogenesis (Mader et al., 2006). As reviewed by Gifford and colleagues, other mechanisms have been proposed. In brief, upon binding to the tumor cells, Lfcin is thought to trigger a Ca^{2+}/Mg^{2+} endonuclease and oxidant-dependent apoptotic pathway. Although the structural parameters that describe the antitumor effects of Lfcin are very similar to those that describe its antibacterial activity, a higher net positive charge (+7 when compared to +4 for antibacterial activity) is required for antitumor activity to promote a strong electrostatic interaction between the peptide and the membrane (**Figure 1**) (Gifford et al., 2005).

Among the human-derived mammalian AMPs, the amphipathic α-helical LL-37 has been extensively studied as an antibacterial peptide. Besides its strong activity against bacteria, it has also shown to be cytotoxic against ovarian cancer (Chuang et al., 2009) and is toxic to eukaryotic cells at a slightly higher concentration (25–30 mM) (Hoskin and Ramamoorthy, 2008). Regarding its mechanism of action, it is known that upon binding to membranes it changes the head group conformation of phospholipids, induces positive curvature strain on lipid bilayers, and significantly disorders the hydrophobic core of the membranes, exhibiting the carpet-like rather than the channel/pore-forming mechanism of cytotoxicity (**Figure 1**) (Henzler Wildman et al., 2003; Henzler-Wildman et al., 2004; Hoskin and Ramamoorthy, 2008).

Within the class of amphibians, the order Anura has been shown to be a rich source of AMPs. Their skin has diverse physiological activities and forms an essential part of their defense systems. In response to a multiplicity of stimuli, AMPs can be secreted from specific glands onto the dorsal surface and into the gut of the amphibian (Bevins and Zasloff, 1990; Barra and Simmaco, 1995; Doyle et al., 2003).

In comparison to synthetic magainins A, B, and G, the natural peptide magainin-2, isolated from the skin of the frog *Xenopus laevis*, is the most efficient peptide and causes fast lysis of hematopoietic and solid tumor cell lines including many human bladder cell lines (Cruciani et al., 1991; Jacob and Zasloff, 1994). Magainin-2 has been shown to enter the cell membrane of HeLa human cervical carcinoma cells by binding numerous magainin helices forming a toroidal pore in the lipid molecules of artificial membranes (Matsuzaki et al., 1996). It has also been reported that pore formation is followed by a dispersing membrane potential and leakage of intracellular molecules, consequently leading to cell death (Takeshima et al., 2003). Magainin are shown to access the cytosolic compartment of cancer cells and cause the mitochondrial pathway of apoptosis via a mechanism that involves cytochrome c release into cytoplasm and an amplified proteasome activity, confirming its apoptotic effect (all these mechanisms are shown in **Figure 1**) (Westerhoff et al., 1989; Cruz-Chamorro et al., 2006).

Another example of potential antitumor AMPs belonging to the order Anura are gaegurin and buforin. The peptide gaegurin was purified from the skin of the Korean frog *Rana rugosa*. It is a potent mediator of cytolysis using either the carpet or barrel-stave mechanism (Park et al., 1994; Hoskin and Ramamoorthy, 2008) and is also reported to follow the mitochondria pathway of apoptosis as described above for the magainin mechanism of action (Li et al., 2000; Mai et al., 2001).

Buforin II, a linear α-helical peptide similar to cecropins and magainins, was isolated from the stomach of the *Bufo bufo gargarizans* and is known to penetrate cell membranes through non-permeabilizing pore-like structures which allow its translocation into the cytoplasm without cell lysis, and consequently inhibits intracellular functions (Park et al., 1998). It has been shown that the cytotoxic activity of buforin II is low when compared with citropin 1.1, pexiganan MSI-78 and protegrin 1, and that it does not permanently disrupt the cell membrane like other molecules, i.e., its antitumor activity corroborates with the mechanism described above (Koszalka et al., 2011). A synthetic analog of buforin II named buforin IIb has shown greater cytolytic activity against

cancer cells (leukemia, breast cancer, non-small cell lung cancer, CNS cancer, melanoma, renal, ovarian, prostate, and colon cancer) than buforin II (Lee et al., 2008).

From the group of marine vertebrates, the cationic peptide pardaxin isolated from the small fish *Pardachirus marmoratus* has been shown to be a potential active antitumor peptide against human sarcoma (Huang et al., 2011). It has been reported that this peptide disrupts the membrane via the barrel-stave mechanism (Hallock et al., 2002), and changes its transmembrane orientation depending on membrane composition (Hoskin and Ramamoorthy, 2008).

CATIONIC ANTIVIRUS PEPTIDES

The success of viruses in evolution has been assured by four general attributes: genetic variation, variety in means of transmission, efficient replication within host cells, and the ability to persist in the host (Wagner et al., 1999). Due to these attributes, the control of viral diseases has not been an easy task. Despite the existence of antiviral drugs, there is a need to explore novel antiviral compounds in order to control emerging viral pathogens. In this perspective, AMPs are an alternative in drug design. Several cationic antiviral peptides from various sources have been isolated since the 1980s (**Table 2**) and they have shown strong potential for novel therapeutic drugs against many viral infections. Due to the promiscuity of these peptides, it is possible to verify a broad spectrum of antiviral activities within the same peptide. Moreover, this promiscuous activity can be extended to simultaneous cytotoxic activity against tumor cells (**Figure 2**). The first study reporting an antiviral role of a cationic peptide was published in 1986, in which the activity of α-defensin was described as inhibiting a number of viruses including herpes simplex virus types 1 and 2 (HSV), cytomegalovirus (CMV) as well as inhibiting the vesicular stomatitis virus with human neutrophil peptide 1 (HNP1) *in vitro* (Daher et al., 1986; Findlay et al., 2013). Since then, many reports have shown the antiviral activity of cationic host-defense peptides such as α-, β-, and θ-defensins, and the use of effective antiviral therapy with cathelicidins, as previously reviewed (Findlay et al., 2013). It is very promising that in the last years many new antiviral peptides have been either identified or synthesized in order to aid the development of new therapeutic antivirus therapies.

CATIONIC ANTIVIRAL PEPTIDES DRUGS APPLIED TO HUMAN HEALTH

Cationic antiviral peptides have been isolated from various sources and present broad antiviral activities against several viruses with different antiviral mechanisms of action (**Table 2**). They can either inhibit viral attachment by binding to viral targets on the host cell surface, or target viral proteins, therefore blocking viral fusion and entry into the host cell. Another mechanism of action is intracellularly driven where spreading of the virus is inhibited through the suppression of viral gene expression, inhibition of translation or by immune modulatory activities (**Figure 3**).

CATIONIC ANTIVIRAL PEPTIDES ISOLATED FROM INVERTEBRATES

AMPs isolated from the group of invertebrates which present strong antiviral activity against maladies that affect human health are well-represented by peptides such as melittin, cecropin, and alloferon. Described above as an antitumor peptide, melittin has also been reported to have inhibitory activity against enveloped viruses such as HIV-1, HSV-2, and the Junin virus (JV), an arenavirus, including this peptide under the concept of promiscuity (**Figure 2**). A proposed mechanism of action has suggested that melittin suppresses cell fusion mediated by HSV-1 syncytial mutants probably by interfering with the activity of the Na^+ K^+ ATPase, a cellular enzyme involved in the membrane fusion process (Albiol Matanic and Castilla, 2004). Analysis of the effect of melittin on the production of HIV-1 transcripts was assayed in acutely infected T-cells cultured with various concentrations of melittin. Levels of all HIV-1 transcript classes were suppressed (reduction of ∼30% when compared with cells without melittin) in a dose-dependent manner (Wachinger et al., 1998). Another mechanism whereby melittin interferes with viral gene expression has been proposed and involves intracellular immunization against HIV (**Figure 3**). Melittin interfere in the process of cellular signal transduction, such as the activation of phospholipase A2 for instance, and the decrease in activities of calmodulin and protein kinase C (Sharma, 1993; Fisher et al., 1994; Gravitt et al., 1994). These properties may therefore change the balance and activities of cellular stimulators of HIV transcription (as NFkB, AP-1 and NFAT) or induce inhibitory factors (interferon-induced cellular inhibitor) (Wachinger et al., 1998).

The peptides alloferon 1 and 2 have shown activity against the influenza virus, through the same mechanism described above for its role as an antitumor peptide, by induction of immune modulatory activities. Both activities against tumor and viral infections emphasize the mechanisms of cell-mediated natural cytotoxicity and IFN synthesis (**Figure 3**). Therefore, these peptides seem to be potential candidates as biopharmaceutical compounds containing the capability of improving important effector mechanisms of the innate immune response (Chernysh et al., 2002).

Another example of invertebrate peptides with antivirus activity against human virus-related diseases is the synthetic peptide T22 ([Tyr5, 12, Lys7]-polyphemusin II), which is associated with the promiscuous peptide tachyplesin (**Figure 2**) and the peptide polyphemusin, which are abundant in the hemocytes of the horseshoe crab *Tachypleus tridentatus* and *Limulus polyphemus*, respectively. It has shown potent antiviral activity against HIV-1 and HIV-2 *in vitro*. The inhibitory activity of this peptide is related to its specific binding to a chemokine receptor CXCR4, which serves as a co-receptor for the entry of HIV-1 into T cells (**Figure 3**) (Nakashima et al., 1992; Tamamura et al., 1998).

CATIONIC ANTIVIRAL PEPTIDES ISOLATED FROM VERTEBRATES

There are several AMPs derived from mammalian sources which have demonstrated strong activity against many viruses that compromise human health (**Table 2**). In this group are included defensins, Lfcin and LL-37 (**Figure 2**). Defensins are potent candidates for the development of antiviral drugs. They are peptides with conserved structures, usually with α-helix and antiparallel β-sheets stabilized by disulfide bonds (Terras et al., 1992; Bastian and Schafer, 2001; Franco et al., 2006), and belong to three subfamilies, designated α, β, and θ defensins. The HNP-1 is an α-defensin that have been widely tested for its antiviral activity. This

Table 2 | Cationic antiviral peptides from different sources, their application, and their mechanisms of action.

Peptide	Source (s)	Group	Application	Mechanism of action	References
HUMAN HEALTH					
Alloferon 1 Alloferon 2	*Calliphora vicina*	Insect	IAV	Immunomodulatory activity	Chernysh et al., 2002
Brevinin-1	*Rana brevipoda*	Frog	HSV	Viral inactivation	Yasin et al., 2000
Caerin 1.1 Caerin 1.9 Maculatin	–	Amphibian Skin	HIV	Disrupts the integrity of the virion membrane	Vancompernolle et al., 2005
CAP37	*Homo sapiens*	Human leococytes	HSV-1 AdV	Disrupts the envelope and/or capsid	Gordon et al., 2009
Cecropin	*Hyalophora cecropia*	Insect	JV HSV HIV	Suppresses viral protein synthesis Cellular target Suppresses viral gene expression	Wachinger et al., 1998; Albiol Matanic and Castilla, 2004
Circulin A	*Chassalia parvifolia*	Plant	HIV		Daly et al., 1999
Defensin	*Homo sapiens*	Human	HSV IAV HCMV VSV HIV AdV	Interacts with glycosaminoglycans Inactivates viral particle Cellular target Unknown	Daher et al., 1986; Nakashima et al., 1993; Gropp et al., 1999; Yasin et al., 2000; Bastian and Schafer, 2001; Sinha et al., 2003
Dermaseptin	Genus *Phyllomedusa*	Frog	HIV HSV	Disruptis viral membrane	Belaid et al., 2002
Didemnins A Didemnins B	Genus *Tridemnum*	Tunicate	HSV Parainfluenza Dengue virus	Inhibits RNA and DNA viral replication	Rinehart et al., 1981; Aneiros and Garateix, 2004
HNP-1 HNP-3	*Homo sapiens*	Human	HSV AdV	Blocks early steps of viral replication	Ganz et al., 1985; Bastian and Schafer, 2001; Hook et al., 2006
Hp1090	*Heterometrus petersii*	Scorpion	HCV	Disrupts viral membrane integrity	Yan et al., 2011
Indolicidin	*Bos taurus*	Bovine	HIV HSV	Inhibits integrase Targets viral glycosaminoglycans	Robinson et al., 1998
Lactoferricin	*Homo sapiens* *Bos taurus*	Human, Bovine	HCMV HIV HSV Papilloma	Activity at virus-cell interface Blocks heparan sulfate	Andersen et al., 2001; Jenssen et al., 2004; Mistry et al., 2007
LL-37	*Homo sapiens*	Human	HSV IAV	Viral receptor-based mechanisms	Yasin et al., 2000; Barlow et al., 2011
Magainin	*Xenopus laevis*	Frog	HSV HIV	Suppresses viral gene expression	Aboudy et al., 1994; Albiol Matanic and Castilla, 2004
Mellitin	*Apis mellifera*	Insect	HSV JV	Cellular target	Wachinger et al., 1998; Yasin et al., 2000; Albiol Matanic and Castilla, 2004
Microspinosamide	*Sidonops microspinosa*	Marine sponge	HIV	Inhibits cytopathic effect of HIV-1 infection	Rashid et al., 2001

(Continued)

Table 2 | Continued

Peptide	Source (s)	Group	Application	Mechanism of action	References
Pa-MAP	*Pleuronectes americanus*	Fish	HSV	Interacs with viral envelope	Migliolo et al., 2012
PAP	*Phytolacca americana*	Plant	HIV HBV HSV	Inhibits viral protein synthesis	Kaur et al., 2011
Polyphemusin	*Tachypleu tridentatus*	Horseshoe crab	HIV	Binds gp120 and CD4	Nakashima et al., 1992; Tamamura et al., 1996
Protegrin	*Homo sapiens*	Human	HIV HSV	Unknown Viral inactivation	Yasin et al., 2000; Steinstraesser et al., 2005
Tachyplesin	*Tachypleus tridentatus*	*Horseshoe crab*	HIV HSV VSV IAV	Virus-cell fusion Viral inactivation Viral envelope	Morimoto et al., 1991; Murakami et al., 1991; Yasin et al., 2000
θ-defensin	*Homo sapiens*	Human	HIV HSV	Binds glycosylated gp120 Binds gB and blocks viral attachment	Cole et al., 2002; Yasin et al., 2004
ANIMAL HEALTH					
Cecropin B CF17	*Hyalophora cecropia*	Insect Synthetic	IHNV VHSV SHRV IPNV	Disrupts the viral envelope Disintegrates the viral capsids	Chiou et al., 2002
Epinecidin-1 TH 1-5 cSALF	*Oreochromis mossambicus* *Penaeus monodon*	Fish Shrimp	NNV	Agglutinates NNV virions into clump	Chia et al., 2010
Pleurocidin MDPle	*Limanda limanda*	Fish	VHSV	Disrupts the viral membrane via toroidal pore formation model	Falco et al., 2009
AGRICULTURE					
Potide-G PAP	*Solanum tuberosum L* *Phytolacca americana*	Plant Plant	PVYO TMV CMV CaMV	Unknown Inhibit viral protein synthesis	Tripathi et al., 2006 Chen et al., 1991
Indolicidin	*Bos taurus*	Bovine neutrophils	TMV	Unknown	Bhargava et al., 2007
Peptamine	*Pseudomonas chlororaphis O6*	Bacteria	TMV	Unknown	Park et al., 2012
Analogs of melittin	*Apis mellifera*	Synthetic	TMV	Cellular target	Marcos et al., 1995

Adapted from (Jenssen et al., 2006); –Not reported. AdV, adenovirus; CaMV, cauliflower mosaic virus; CMV, cytomegalovirus; HBV, hepatitis B virus; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HIV, Human immunodeficiency virus; HSV, hermpes simplex virus; IAV, influenza; IHNV, infectious haematopoietic necrosis virus; IPNV, infectious pancreatic necrosis virus; JV, junin virus; NNV, nervous necrosis virus; PVYO, potato virus YO; SHRV, snakehead rhabdovirus; TMV, tobacco mosaic virus; VHSV, viral hemorrhagic septicemia; VSV, vesicular stomatitis virus; VSV, vesicular stomatitis virus.

peptide has been reported to deactivate HSV-1 and HSV-2, CMV, vesicular stomatitis virus, influenza virus and human respiratory adenovirus type-5 (AdV-5) (Daher et al., 1986; Yasin et al., 2004). Treatment with this peptide has shown a decrease of adenoviral infection by more than 95% in 293 cells infected with AdV-5) (Bastian and Schafer, 2001). θ-defensins are circular octadecapeptides with two antiparallel β-sheets that are bridged by a tri-disulfide ladder and connected by two β-turns (Yasin et al.,

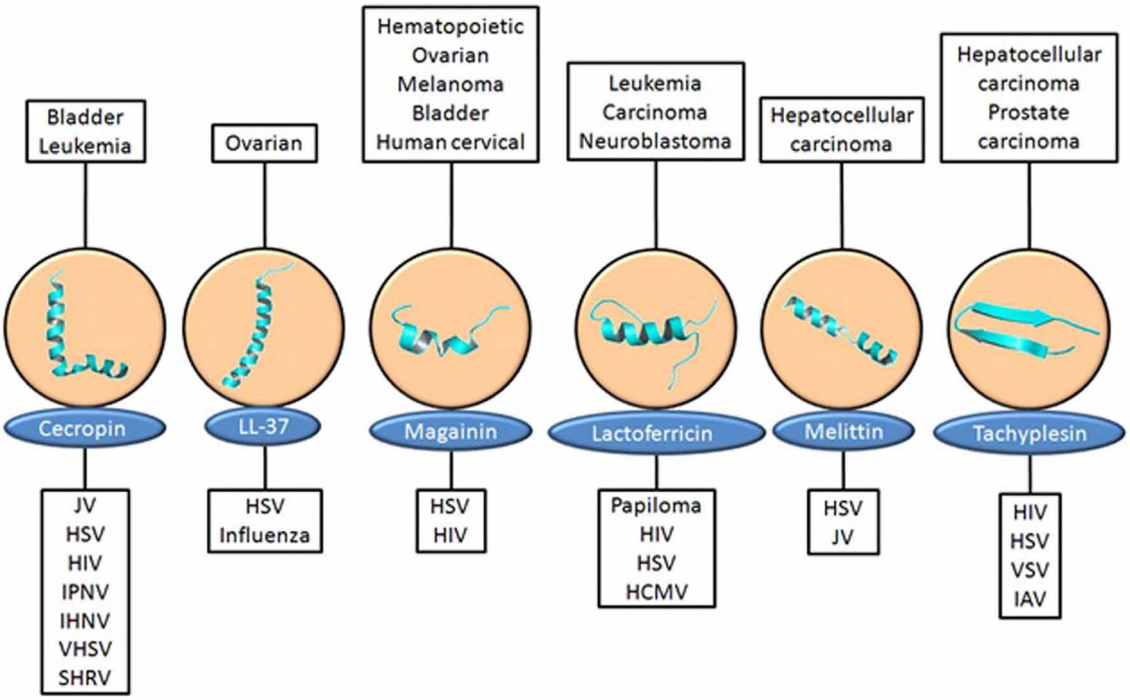

FIGURE 2 | Promiscuous cationic peptides with antitumor and antiviral activities. Each promiscuous peptide and their various tumors against which present cytotoxic activity **(top)** and viruses against which present antiviral activity **(bottom)**. #PDB from left to right, 2IGR, 2K6O, 2LSA, 1Z6V, 2MLT, 1WO1. HCMV, human cytomegalovirus; HIV, Human immunodeficiency virus; HSV, herpes simplex virus; IAV, influenza; IHNV, infectious hematopoietic necrosis virus; IPNV, infectious pancreatic necrosis virus; Jv, Junin virus; SHRV, snakehead rhabdovirus; VHSV, viral hemorrhagic septicemia; VSV, vesicular stomatitis virus.

2004). This class of peptide has been reported to be miniature lectins that bind to the protein gp120 of human immunodeficiency virus type 1 (HIV-1) with high affinity, blocking its entry into the host cell (**Figure 3**) (Munk et al., 2003).

The promiscuous peptide LfcinB (**Figure 2**) has shown inhibition against many viruses such as HIV-1, HSV-1, and HSV-2, human cytomegalovirus (HCMV), respiratory syncytial virus, hepatitis B and C viruses (HBV and HCV, respectively), adenovirus, and rotavirus (Van Der Strate et al., 2001; Andersen et al., 2003). The activity of Lfcin has been claimed to be attributed to the affinity of this peptide for carbohydrates, which are viral binding sites on the cell membrane, such as heparin sulfate (HS) and glycosaminoglycans (GAGs), thereby blocking viral entry (Andersen et al., 2003). The antiviral activity of LfcinB and LfcinH (human lactoferricin) against HSV has been verified with the ability of this peptide to interact with HS and block viral entry. It has been found that the positive net charges of the peptides are critical for affinity with HS which is due to the many negatively-charged sulfate groups present in the molecule (Jenssen et al., 2004). Together with Lfcin, the peptides human α-defensin, LL-37 and magainin have also been reported to bind to GAGs in order to perform their respective activities (**Figure 3**) (Jenssen et al., 2006). It has also been shown that a stabilized secondary structure is important for antiviral activity for both LfcinB and LfcinH (human lactoferricin). The higher potency of LfcinB against some viruses compared to LfcinH is attributed to the β-sheet conformation of LfcinB in solution compared with the α-helical structures of LfcinH (Jenssen et al., 2004).

Lactoferrin (Lf), the protein from which the peptide Lfcin is derived, shows antiviral activity against a number of viruses as much as seven times greater than that of Lfcin, proposing that either the size of the molecule is important or that other regions of LF contribute to the antiviral activity (Andersen et al., 2003; Gifford et al., 2005). The positive charge of Lf was found to be important for antiviral activity against human HCMV (Valenti and Antonini, 2005). When negatively-charged groups were added to Lf by succinylation, the antiviral potency was mostly decreased, whereas the addition of positive charges to Lf through amination of the protein resulted in increased anti-HCMV activity (Harmsen et al., 1995). On the other hand, when tested against HIV-1, a 4-fold stronger antiviral effect of Lf was observed when negatively-charged groups were added (Harmsen et al., 1995). The proposed mechanism of action for anti-HIV activity was that Lf and the charged-modified protein bind strongly to the V3 loop of the gp120 envelope protein, increasing the net negative electric charge of viral particles, and resulting in inhibition of virus-cell fusion and entry of the virus into cells (Puddu et al., 1998) Another report of direct interaction of viral proteins with Lf has been observed in HCV. Inhibition of virus-cell adsorption was verified in human hepatocytes PH5CH8 when bLf was mixed prior to the viral infection in serum containing HCV, and no antiviral activity of bLf was observed after

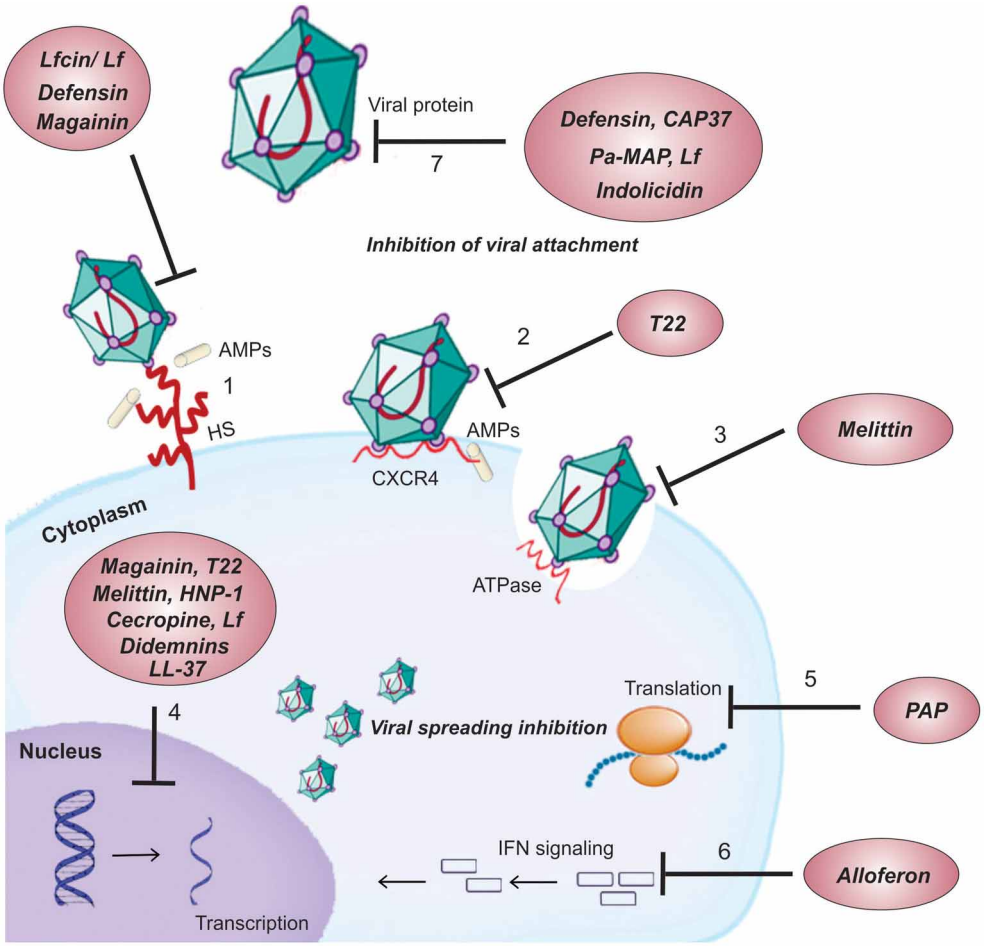

FIGURE 3 | Mechanisms of action of cationic antiviral peptides. *Cell surface targets*: (1) Interaction of peptides with different glycosaminoglycan (e.g., HS) present on the cell surface competing with the virus for cellular binding sites. (2) Blocking of viral entry into the cell by binding the peptide to viral CXCR4co-receptor required for its entry. (3) Suppression of cell fusion by interfering with the activity of ATPase protein. *Intracellular targets*: (4) Suppression viral gene expression. (5) Inhibition of peptide chain elongation by inactivating the ribosome. (6) Activation of an immune modulatory pathway by induction of NK and IFN. *Viral protein targets*: (7) Binding of peptides to viral proteins causing inhibition of adsorption/virus-cell fusion.

internalization of HCV in human hepatocytes (Ikeda et al., 1998). This activity has been reported to be involved in the ability of hLF and bLF to bind to E1 and E2 proteins of the viral envelope, indicating that neutralization occurs in order to prevent the adsorption of HCV in the hepatocytes (Yi et al., 1997). During *in vivo* experiments, bLF protected the host mice against infection of the mouse cytomegalovirus (MCMV) when the peptide was injected prior to the viral infection, but failed to protect the mice when the injection was performed after MCMV infection (Shimizu et al., 1996).

Another human-derived peptide which targets envelope/membrane is the promiscuous peptide CAP37, first isolated from the granule fractions of human PMNs. It has been shown to have potent activity against viruses (HSV-1 and Adenovirus), bacteria (*Pseudomonas aeruginosa, E. coli, Salmonella typhimurium, Staphylococcus aureus, and Enterococcus faecalis*), and fungi (*Candida albicans*). Its structure/function differs from the others peptides here mentioned. It is known that cystine residues forming intramolecular disulfide bridges are necessary for the antibacterial function of CAP37 but are not required for its antiviral activity, which has been suggested to be involved in the rupture of the envelope and/or capsid (Gordon et al., 2009).

The promiscuous peptide LL-37 (**Figure 2**) differs from the above defensins, Lfcin, and bLf in its mechanism of action. Instead of targeting cell surface molecules, it inhibits viral spreading by inactivating intracellular targets (**Figure 3**). LL-37 have nuclear localization signals and have been related to interact with DNA, directly influencing viral nucleic acid synthesis (Sandgren et al., 2004). The same is applied to indolicidin, a peptide isolated from the cytoplasmic granules of bovine neutrophils (Hsu et al., 2005). However, besides its DNA-binding ability, it has also shown activity against HIV and HSV through a membrane-mediated antiviral mechanism (Robinson et al., 1998).

Marine organisms are also included as a source of cationic antiviral peptides (**Table 2**). One example is the promiscuous

peptide Pa-MAP isolated from the fish *Pleuronectes americanus*, which beyond having shown broad antimicrobial activity against bacteria (*E. coli and S. aureus*), fungi (*Candida parapsilosis, Trichophyton mentagrophytes, and Trichophyton rubrum*) and tumor cells in culture (CACO-2, MCF-7, and HCT-116), also has activity against viruses (HSV-1 and HSV-2). The antiviral mechanism of this peptide has been suggested to involve its interaction with the viral envelope (Migliolo et al., 2012; Teixeira et al., 2013).

A group of cationic antiviral peptides from vertebrate and invertebrate sources has been tested by Carriel-Gomes et al. (2007). This study has shown *in vitro* evaluation of the cytotoxicity and antiviral activity of nine AMPs against many human viruses (Carriel-Gomes et al., 2007). They were PW-2 recombinant, tachyplesin-1 from limulid, gomesin from spider, clavanin A from tunicate, magainin from frog, synthetic HCTF, penaeidin-3 and ALF from shrimp, and mytilin A from mussel. These peptides have different structures, origins and antiviral activities against HSV-1, AdV-5, and rotavirus SA11 (RV-SA11). All evaluated peptides were cytotoxic and had antiviral activities in different degrees. The peptides PW-2, ALF and penaeidin-3 exhibited higher antiviral activity against HSV-1. The peptides ALF and clavanin A showed significant antiviral activity against AdV-5 with clavanin A exhibiting a greater inhibition of viral replication (Carriel-Gomes et al., 2007).

ANTIVIRAL PEPTIDES DRUGS APPLIED TO ANIMAL HEALTH

Several studies have reported the efficiency of AMPs active against viruses that cause diseases in humans. However, few studies have demonstrated the potential application of these peptides in animal health. Here we present studies showing the application of antiviral peptides in aquaculture. Viral diseases have emerged as the most serious infectious problems for the fish aquacultural industry, and studies have found promising cationic antivirus peptides being used against several viruses including rhabdoviruses such as viral hemorrhagic septicemia virus (VHSV) and infectious hematopoietic necrosis virus (IHNV), which are responsible for the greatest losses in aquaculture production.

The native cecropine B and its synthetic analog CF17, have demonstrated activity against major viral fish pathogens such as infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), snakehead rhabdovirus (SHRV) and infectious pancreatic necrosis virus (IPNV). The mechanism of action involved in the inhibition of viral replication by peptides is related to the direct disruption of the viral envelop and the disintegration of the viral capsids (Chiou et al., 2002).

Chia and collaborators reported that both peptides tilapia hepcidin 1-5 (TH 1-5) and cyclic shrimp anti-lipopolysaccharide factor (cSALF) exhibited noticeable antiviral activity *in vitro* against nervous necrosis virus (NNV), a virus that has caused mass mortality of numerous marine fish species at their larval stage. The antiviral mechanism of both peptides was by agglutinating NNV virions into clump and preventing viral entry into the cells (Chia et al., 2010). Together with hepcidin 1-5, the peptide hapcidin 1 has also shown antiviral activity *in vivo* against NNV in the Japanese rice fish medaka (*Oryzias latipes*). Pre-treatment, co-treatment or post-treatment with epinecidin-1 or hepcidin 1-5 has shown to be effective in promoting a significant increase in medaka survival when infected with NNV (Wang et al., 2010a).

Falco and collaborators (2009) have reviewed the role of many AMPs as antiviral agents in the fish farm industry. Among these molecules there are pleurocidin and the HNP1. Pleurocidin MDPle, isolated from the Mud dab fish (*Limanda limanda*), has presented antiviral properties against VHSV. It is likely that it disrupts the viral membrane under the same mechanisms adopted for its antibacterial activity (Falco et al., 2009). The HNP1 is a defensin that has demonstrated antiviral effects against the same virus as MDPle. It has been claimed that, as belonging to the class of defensins, HNP1 can deactivate the enveloped virus by interacting with GAGs present on the viral surface, altering the ability of these glycoproteins to bind to their receptors at the target cells. Moreover, this peptide has also been shown to stimulate the immune modulatory system of infected fish cells by modulating IFN-related mechanisms (Falco et al., 2007, 2009).

ANTIVIRAL PEPTIDE DRUGS APPLIED TO AGRICULTURE

Cationic antivirus peptides may also be applied to plant protection against viruses that cause diseases in crops. Among the various symptoms of viral infections in plants, stunting, mosaic patterns, yellowing, leaf rolling, ring spot, necrosis, wilting, and other developmental abnormalities can be observed (Hull, 2002). These symptoms consequently cause a decrease in production leading to economic losses. Strategies to combat viral diseases in plants are usually directed to the prevention or reduction of infection which may have, however, adverse effects on human health and the environment. Therefore, AMPs have become promising alternatives for protecting crops against viral diseases while simultaneously protecting human health and the environment.

A cyclic peptide of 7 amino acid residues called peptamine from *Pseudomonas chlororaphis* O6 has shown antiviral activity against the tobacco mosaic virus (TMV). Antiviral bioassays of tobacco plants suppressed 95% of TMV disease. The mode of action by which peptamine suppresses the disease is still unknown, but it has been speculated that this peptide may induce systemic resistance against TMV (Park et al., 2012). The peptide PAP, a ribosome inactivating protein (RIP) isolated from *Phytolacca americana*, was highly effective in inhibiting the formation of local lesions caused by TMV on tobacco leaves (Taylor et al., 1994). RIPs are known to cause damage to ribosomes by removing adenine residues from 28S rRNA through an N-glycosidase activity, and the removal of this base prevents binding of the elongation factor 2 (EF-2), consequently stoping the synthesis of proteins (Kaur et al., 2011). Another cationic antiviral with activity against TMV is subK7I, a synthetic analog of melittin. This peptide has sequence and structural similarity to an essential domain of the TMV coat protein and was found to possess highly specific antiviral activity. Bioassays of tobacco leaves upon addition of the analog to the solution before inoculation of the virus has demonstrated a reduction of more than 90% of infectivity of TMV with dependent doses (Marcos et al., 1995).

Tripathi and colleagues isolated a small antiviral peptide of 5.57 kDa called potide-G from potato tubers resistant to Potato Virus Y (PVY). Results of real-time PCR showed that the application of 10 μg of purified potide-G was sufficient to reduce

virus accumulation by 50% on average from the PVY infection in susceptible "Winter Valley" cultivars. The authors have reported that this peptide isolated from resistant potatoes offers new opportunities for the development of new biological pesticides against plant viruses (Tripathi et al., 2006).

PRODUCTION OF CATIONIC ANTITUMOR AND ANTIVIRAL PEPTIDES

While peptides have great potential for use in antitumor drugs, there are some limitations that need to be addressed. It is well-known that they can be used in a number of different ways in treating cancer such as vaccines, hormones, tumor targeting with cytotoxic drugs and radioisotopes, and anti-angiogenic peptides (Thundimadathil, 2012). Currently, there are about 60 approved peptide drugs on the market and it is expected to reach an estimated $12 billion USD in sales by the end of 2013 (Pichereau and Allary, 2005). All of these investments have resulted in more than 100 peptide-based drugs which are already available on the global market, representing about 1.5% of all drug sales (Lax, 2010; Craik et al., 2013). Out of four peptide drugs on the market which have reached global sales over $1 billion USD, three peptides are used in treating cancer directly or in the treatment of episodes associated with certain tumors: leuprolide acetate (Lupron; $2.12 billion), goserelin acetate (Zoladex; $1.14 billion), and octreotide acetate (Sandostatin; $1.12 billion) (Reichert et al., 2010; Thundimadathil, 2012). Mendoza and colleagues have summarized approximately 30 peptides which were discovered/developed as peptide-based anticancer drugs in order to provide foundations for therapies (Mendoza et al., 2005).

However, when it comes to the market of AMPs as antitumor drugs, the scenario is much different. Although over 1000 potential therapeutic AMPs have been isolated and characterized from different sources, only limited success has been achieved in clinical trials (Hu et al., 2011).

There are currently only around ten AMPs in either preclinical or clinical trial phases (Fox, 2013) and few of these present antitumor activity. Their major limitations are the poor bioavailability due to their instability and insolubility related to the intrinsic physicochemical properties, potential toxicity to host cells, tissue distribution, poor pharmacokinetic issues, and the cost of large scale production (Rotem and Mor, 2009; Hu et al., 2011). Despite these disadvantages, antitumor peptides have potential due to their high specificity and potency against malignant cells. Therefore, numerous studies have been performed in order to improve their bioavailability and reduce their cost of production. Much effort has been put into the mimicry of antitumor peptides to alter their features in order to achieve robustness and safety. These approaches have become important and promising for improving the therapeutic potential of antitumor peptides (Rotem and Mor, 2009).

HDP mimicry has been performed by constructing oligomers of acyl-lysyl and/or lysyl-acyl-lysyl (OAKs), which has turned out to be the first designed system to show antitumor potential *in vivo*. This system was able to translocate across the membrane and interact with multiple intracellular targets, including mitochondria, especially the inner membrane which contains a relatively high portion of negatively-charged phospholipids that might mediate interactions with these HDPs. Moreover, it was associated with an improved toxicity profile when compared to doxorubicin, an anthracycline antibiotic used in chemotherapy. It has also been observed that local administration of both OAK and doxorubicin resulted in a complete disappearance of tumors in 50% of treated mice, while in the remaining 50% tumors were minuscule, thereby suggesting that the synergic effect of this therapy was both potent and well tolerated. The OAK might also damage lysosome structure and/or interfere with the function of 50 hydrolases that normally process the cell's major macromolecules (Held-Kuznetsov et al., 2009).

In another study, sequence optimization and modification based on natural peptide sequences and traits was performed and it has shown that the system G(IIKK)nI-NH 2 (being $n = 3 - 4$) was effective against HeLa and HL60 cancer cell lines with 50% growth inhibition concentrations (Hu et al., 2011). Mai and colleagues have designed a novel antitumor peptide, DP1, derived from a synthetic AMP that significantly induces apoptosis in solid tumors by local injection. It was composed of a protein transduction domain (PTD), PTD-5, fused to the AMP $KLAKLAK_2$, an antimicrobial apoptosis-inducing peptide that upon internalization causes mitochondrial swelling and disruption of the mitochondrial membrane leading to apoptosis (Mai et al., 2001; Thundimadathil, 2012).

Rational design of novel peptides has also been performed to create six analogs of temporin-1CEa, a naturally occurring α-helical and amphipathic AMP derived from skin secretions of the Chinese brown frog *Rana chensinensis* (Yang et al., 2013). These analogs were synthesized with either increased cationicity or increased/decreased hydrophobicity generally by substituting neutral and acidic amino acids with lysine or leucine residues on the polar face and non-polar face of the α-helix in order to evaluate the correlation between anticancer activity and physical properties of these peptides. All peptides showed potent anticancer activities against three cancer cell lines (MCF-7, Bcap-37, and MDA-MB-231). This study has suggested that the strategy of increasing the cationicity and keeping moderate hydrophobicity of naturally occurring AMPs is suitable to improve their cytotoxicity against tumor cells and decrease their hemolytic activity (Yang et al., 2013).

Synthetic links between two functional domains, an AMP (KLAKLAKKLAKLA K) and the isoDGR, (isoAsp-Gly-Arg), a derivative of a targeted delivery tool, was performed to construct a novel antitumor peptide. It was shown that this novel construction can selectively kill $CD13^-/\alpha_v\beta_{3+}$ breast cancer cells in both *in vitro* and *in vivo* experiments. The mechanism of action was claimed to inhibit angiogenesis by binding to $\alpha_v\beta_{3+}$ which is upregulated on tumor cells and tumor endothelial cells (Hou et al., 2013).

These studies strongly suggest that the peptidomimetics is a potent tool for developing new antitumor peptides. Moreover, these molecules may have their potential increased when it functions as an adjuvant therapy in conjunction with radiotherapy, chemotherapy, or surgical procedures.

When antiviral therapy is considered, the major barriers to the development and use of effective therapy are the current expensive approaches to impairing the completion of the viral growth cycle in the infected cell without being toxic to the surrounding normal cells, and diagnosing the viral disease before it is too

late for effective therapy. It has also been claimed that the reason for the lack of progress in antiviral therapy is the selectivity, since the viruses are functionally incorporated into the host cells, and therefore it is difficult to select a proper tag (Kinchington et al., 1995; Abonyi et al., 2009). Furthermore, a relevant barrier is applied to the market of antivirus drugs, which concentrate efforts on a few viruses. It is known that out of 60 antiviral drugs that have thus far been approved by the US Food and Drug Administration (FDA), almost half of them target HIV-1 with the remaining half used for the treatment of HBV and HCV infections, HSV, CMV, varicella-zoster virus (VZV), and influenza (IAV) (De Clerq, 2008; Findlay et al., 2013). There are currently 15 peptide-based strategies against viruses in different stages of clinical trials as candidates for therapeutic drugs against many viruses (Thakur et al., 2012). However, none are cationic antiviral peptides, although reports of their high activity are exciting enough to include them as potential candidates, as is the case of alloferon. It has been shown that preventive and/or therapeutic administration of alloferon essentially increased the survival rate and suppressed virus reproduction in mice intracerebrally infected with HSV-2. Alloferon-based therapy of HSV, HBV, and HCV infections is now under extensive preclinical study (Chernysh et al., 2002).

Synthetic analogs of several naturally occurring AMPs have been made in attempt to identify important structural features contributing to their antiviral activity as well as to optimize these molecules in order to develop the shortest, cheapest, most stable and functional molecules at lower concentrations (Jenssen et al., 2006). Recently, the first antivirus peptide prediction method, based on the collected peptides which were experimentally proven for antiviral activity, was developed and named AVPpred (Thakur et al., 2012). This method is specific for antivirus peptides, where 25 physicochemical properties to develop antivirus peptide-physico models, amino acid composition, and sequence alignment implementing BLASTP algorithms for prediction of AVPs were used.

HETEROLOGOUS PRODUCTION OF ANTITUMOR AND ANTIVIRAL CATIONIC PEPTIDES

Beyond the use of peptide mimicry to develop synthetic peptides, the advance in the use of heterologous systems for production of cationic peptides has been a promising alternative. Many groups have been performing recombinant technology in order to optimize the production yield of cationic peptides using animal cells (Brocal et al., 2006), yeast (Wang et al., 2009b), plant (Lee et al., 2011), and bacteria systems (Wang et al., 2011).

For instance, the expression and purification of the recombinant human α-defensin 5 has been performed in *Pichia pastoris*, and it has been reported that this peptide was functionally expressed and it showed *in vitro* activity to block human papillomavirus infection (Wang et al., 2009b). The housefly cecropin peptide has also been expressed in this organism, however, neither antiviral nor antitumor activities have been tested in this specific system (Jin et al., 2006). The lack of both activity could also be observed for the expression of the peptide pleurocidin, which has been expressed in both yeast (Burrowes et al., 2005) and fish cell lines (Brocal et al., 2006), although only in the latter case antibacterial activity was tested.

The concept of plant-made biopharmaceuticals has also been lately explored for the expression of antiviral cationic peptides. For example, the peptide retrocyclin, an important AMP which can be used as therapeutic agent against HIV-1 viral infections, have been expressed and characterized in tobacco chloroplasts genome, and its antiviral activity was confirmed against TMV infection (Lee et al., 2011). The peptide cecropin was also expressed in both camelina (*Camelina sativa*) (Zakharchenko et al., 2013) and tomato (*Solanum lycopersicum*) (Jan et al., 2010). Nevertheless, in these studies the tests performed did not include antiviral or antitumor activities.

The most common organism for construction of heterologous expression systems is bacteria, and *E. coli* is the most used (Parachin et al., 2012). This bacterium has been used for expression of cecropin (Liang et al., 2006), lactoferricin (Luo et al., 2007), human α and β defensins (Wang et al., 2010b), buforin (Wang et al., 2011), indolicin (Morin et al., 2006), and LL-37 (Moon et al., 2006). Two different systems for expression of cecropin, one fused to enterokinase (Xu et al., 2007a) and other hybrid system fused to ubiquitin (Xu et al., 2007b) were constructed, and both systems were active against Gram-positive and negative bacteria, and fungi. Another two hybrid systems have also been constructed to actively express the peptide lactoferricin (Kim et al., 2006; Feng et al., 2011). Furthermore, many bioactive human defensin have been successfully expressed in *E. coli* such as the human α-defensin 6, which showed to inhibit HSV-2 infection (Wang et al., 2010b), the β-defensin 5 and 6 (Huang et al., 2008), the β-defensin 26 and 27 (Huang et al., 2009), β-defensin 2 (Zhong et al., 2006), and β-defensin 4 (Xu et al., 2006; Li et al., 2010).

As shown above, many studies have shown that the heterologous expression of cationic peptides is feasible, and in many cases, depending on their size and structure, is a better alternative for production of pharmaceuticals than the synthetic synthesis (Parachin et al., 2012). However, none of the studies cited above have optimized the large-scale production of the peptides expressed. Doubtless it is important to mention that many efforts have been made to efficiently develop or improve the cost-effective methodology (e.g., new expression systems and optimized fermentation processes) for production of AMPs, as well as to overcome barriers involving both recombinant technology processes and the economic scenario of biotechnology and pharmaceutical companies in order to change the drug-development current scenario.

NANOFORMULATION AS DRUG-DELIVERY SYSTEM

In order to overcome the restraints to the use of cationic peptides described above, nanoformulation techniques have emerged as a potent biological tool to improve the delivery and stability and of these molecules.

The primary goals of drug delivery for cancer therapy is to improve the therapeutic index of anticancer drugs by increasing the amount of drug delivered to the tumor site and decreasing its exposure to healthy tissues (McDaniel et al., 2010). Several microencapsulation technologies have been developed for use

in the pharmaceutical industry, such as hydrogels, liposomes, nanoemulsions, and nanoparticles (Onwulata, 2012). Therefore, the development and improvement of nanoparticles have shown great promise to overcome the delivery barriers of pharmaceuticals (Zhang et al., 2012).

Recently Wang and Zhang (2013) encapsulated a CPAP, a dose-dependent antiproliferation peptide and inducer of the post-G1 cell cycle arrest in gastric cancer cells. Two methods were used: complex coacervation (edible alginate, CaCl2 and chitosan involved) to perform a microencapsulation of CPAP, and ionotropic gelation (edible chitosan and sodium tripolyphosphate involved) to perform a nanoencapsulation process. The former refers to the phase separation of a liquid precipitate/phase when solutions of two hydrophilic colloids are mixed under suitable conditions, and the latter is based on the ability of polyelectrolyte counter ions to cross link to form hydrogels (Wang and Zhang, 2013). Their results demonstrated that the encapsulation efficiency of microencapsulation (74.5%) is much greater than that of nanoencapsulation (30.1%), but their polypeptides contents are similar (12.7 vs. 12.3%). The *in vitro* release tests revealed that CPAP was well-preserved against gastric enzymatic degradation after micro/nanoencapsulation and the slowly-controlled release in the intestine could be achieved. Although further *in vivo* studies are required to verify these findings, this study has provided a basis for the development of encapsulated antitumor peptides (Wang and Zhang, 2013).

Another study has performed encapsulation of an antitumor peptide in order to develop an effective gene-modified tumor cell vaccine. In this study, the gene of interleukin-27 (IL-27), a novel IL-6/IL-12 family cytokine, was transfected in LL/2 (Lewis lung cancer cell) by using a cationic liposome. This resultant tumor cell vaccine then containing both tumor associated antigen (TAA) of LL/2 cells and secreted mIL-27 (mouseIL-27) at a relative high level, could induce protective antitumor immunity in mice, which was claimed to be an attribute of high-cytokine production achieved by the liposome-DNA complex. It was also observed that LL/2-mIL-27 cell vaccine up-regulated IFN-γ in serum and improved local CD4+ and CD8+ T cell infiltration in mice. This study shows an important and promising strategy for AMP-based therapeutic approach against tumors (Zhang et al., 2013b).

Regarding cationic antiviral peptides, a nanoformulation technique has also been studied as a potent bio-tool to improve their delivery and stability. Nanoformulation of the amphiphatic α-helical peptide p41, a positively-charged analog of C5A peptide derived from the HCV protein, was performed to treat HIV/HCV co-infection. The cationic antiviral peptide was incorporated into anionic poly (amino acid)-based block copolymers prepared via eletrostetic coupling. The nanocomplexes were ca. 35 nm in size, stable at physiological pH and ionic strength. The *in vitro* antiviral activity against both HCV and HIV was retained and their intrinsic cytotoxicity was attenuated. The *in vivo* APN were able to decrease the viral load in mice transplanted with human lymphocytes and HIV-1-infected. Overall, these findings indicate the potential of these formulations for stabilization and delivery of antiviral peptides while maintaining their functional activity (Zhang et al., 2013a).

CONCLUDING REMARKS

Many natural cationic peptides from the immense biodiversity of several groups of organisms have been isolated and have demonstrated great potential as antiviral and antitumor agents. Furthermore, the development of novel synthetic analogs of these natural molecules has been an important biological tool for enhancing their activities and facilitating the screening process for new natural AMPs. Studies on antitumor and/or antiviral AMPs have demonstrated that these molecules work as excellent therapeutic agents and even lead to greater success when combined with traditional treatments. The number of antitumor and/or antiviral AMPs has been increasing and is expected to lead to a change in their current scenario, from their entry into clinical trial to their availability on the market. A crucial step for this change to occur is to continue researching and identifying their mechanisms of action and to discover new targets, followed by studies on developing new potential delivery systems.

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Antibiotic development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance

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Antimicrobial peptides (AMPs) are natural antibiotics produced by various organisms such as mammals, arthropods, plants, and bacteria. In addition to antimicrobial activity, AMPs can induce chemokine production, accelerate angiogenesis, and wound healing and modulate apoptosis in multicellular organisms. Originally, their antimicrobial mechanism of action was thought to consist solely of an increase in pathogen cell membrane permeability, but it has already been shown that several AMPs do not modulate membrane permeability in the minimal lethal concentration. Instead, they exert their effects by inhibiting processes such as protein and cell wall synthesis, as well as enzyme activity, among others. Although resistance to these molecules is uncommon several pathogens developed different strategies to overcome AMPs killing such as surface modification, expression of efflux pumps, and secretion of proteases among others. This review describes the various mechanisms of action of AMPs and how pathogens evolve resistance to them.

Keywords: antimicrobial peptides, mechanism of action, mechanism of resistance, bacterial, membrane permeability and intracellular targets

ANTIMICROBIAL PEPTIDES

Antimicrobial peptides (AMPs), an important part of the innate immune system, are small molecules that may present antibacterial, antifungal, antiparasitic, and antiviral activity (Hancock and Diamond, 2000; Jenssen et al., 2006). Usually these molecules are composed of 10–50 amino-acid residues, and arranged in different groups depending on the amino-acid composition, size, and conformation (Lai and Gallo, 2009; Nakatsuji and Gallo, 2012).

The largest group corresponds to cationic peptides, which is divided in three classes (Vizioli and Salzet, 2002; Brogden, 2005). The first class is composed by linear cationic α-helical peptides, such as Magainin and Cecropins that are linear before their interaction with the cell membrane, and then adopt an amphipathic α-helical secondary structure (Bechinger et al., 1993). The second class comprises cationic peptides enriched for specific amino acids like proline, arginine, and other residues. These peptides are linear, although some may exhibit extended coils (Brogden, 2005). The third class includes cationic peptides that contain cysteine residues and form disulphide bonds and stable β-sheets. Defensins, an example of this class, have six cysteine residues and are divided according to the alignment of their disulphide bridges (α-, β-, and θ-defensin) (Mehra et al., 2012).

Other groups of AMPs are described as non-cationic peptides, anionic peptides, aromatic peptides, and peptides derived from oxygen-binding proteins (Vizioli and Salzet, 2002). Just as the cationic peptides, anionic peptides (AAMP) are also an important part of the innate immune system and have been identified in vertebrates, invertebrates, and plants (Harris et al., 2009). Although the antibacterial activity of these peptides is considered weak, they could improve the activity of cationic peptides (Vizioli and Salzet, 2002).

Different studies revealed that besides peptide charge (cationic or anionic), other characteristics such as size, primary sequence, conformation, structure, hydrophobicity, and amphipathicity could be essential for antimicrobial activity and mechanism of action (Friedrich et al., 2000; Gennaro and Zanetti, 2000).

MAIN MECHANISMS OF ACTION

The classic action mechanism of AMPs involves their ability to cause cell membrane damage. AMPs can interact with microorganisms by electrostatic forces between their positive amino acid residues and negative charges exposed on cell surfaces. It has been suggested that the composition of cell surface drives the specificity of AMPs. In this sense, the sensitivity of prokaryotic and eukaryotic cells is directly related to the different physicochemical properties of the lipids found on both cell membranes (Dathe and Wieprecht, 1999; Matsuzaki, 1999). In mammalian membranes, the lipids most commonly found on the extracellular side of the bilayer are neutral phospholipids such as phosphatidylcholine and sphingomyelin. On the other hand, the bacterial cell membrane is essentially composed by negatively charged lipids,

such as phosphatidylglycerol (PG), cardiolipin, and the zwitter ionic phosphatidylethanolamine (PE) (Lohner, 2009). The overall negative charge found on the membrane of bacteria has an important role in the preferential binding of some peptides to those microorganisms. Moreover, Gram-negative bacteria contain lipopolysaccharides (LPS) on their outer membrane, and the cell wall of Gram-positive bacteria is enriched in acidic polysaccharides (teichoic and teichuronic acids). Those molecules that confer a negative charge to the bacterial surface were selected as targets for cationic AMPs (Brogden, 2005).

The interaction and action of AMPs with their target cells depends largely on the cell surface as well as on the amino acid composition of these peptides. This idea is supported by the high conservation of positively charged amino acid residues among peptides sequences from various organisms (Yeaman and Yount, 2003). In addition, the secondary structure adopted by the peptide is essential for the binding to negatively charged compounds in the target membrane, such as anionic phospholipids (Matsuzaki, 2009). Depending on the peptide/lipids ratios and affinity, these peptide molecules can be oriented perpendicularly, allowing their insertion into the lipid bilayer and the formation of transmembrane pores (Brogden, 2005; Melo and Castanho, 2012).

The mechanisms by which AMPs can traverse microbial membranes are not common to all peptides and seem to depend on the molecular properties of both, peptide addressed and lipid membrane composition. Several membrane defects can be induced by AMPs, among them we can highlight formation of pores, phase separation, and promotion of non-lamellar lipid structure or disruption of the membrane bilayer (Lohner and Prenner, 1999). Some models that may explain membrane disruption by AMPs have been proposed, such as barrel-stave, toroidal, and carpet models.

The first mechanism proposed, the "barrel-stave" model, suggests that peptides form transmembrane pores by their direct insertion into the lipid core of the target membrane (Ehrenstein and Lecar, 1977). In this model, AMP binds to the membrane surface as a monomer, which is followed by their oligomerization and pore formation. The recruitment of additional monomers can increase the pore size, allowing cytoplasmic content leaking with subsequent cell death. In this mechanism, peptide secondary structures, such as hydrophobic α-helix and/or β-sheet, are essential to pore formation (Breukink and de Kruijff, 1999). These peptide regions interact with the membrane lipids, while the hydrophilic peptide regions form the lumen of the channel (Brogden, 2005).

In contrast to the "barrel-stave" model, in the "toroidal" model, peptide molecules are inserted into the membrane forming a bundle, inducing the lipid monolayers to continuously bend through the pore (Yang et al., 2001). As a consequence, membrane lipids become interspersed with peptides forming the pore (Yeaman and Yount, 2003). Examples of AMPs toroidal pore forming are megainins, protegrins, and melittin (Brogden, 2005).

According to the "carpet" model, AMPs cover the membrane surface affecting its architecture, in a detergent-like manner (Rotem and Mor, 2009). Their interaction is first driven by electrical attraction and, when the amount of AMPs on the membrane surface reaches a threshold concentration, the membrane disintegrates, leading to cell lysis (Oren and Shai, 1998). However, it was described that some peptides might also form transmembrane pores at concentrations below the threshold, suggesting that the mechanism by which the peptide disrupts/permeates the membrane depends on its concentration (Lohner, 2009).

Although most AMPs interact directly with cell membrane lipids, it was reported that some peptides might require a bacterial receptor. This hypothesis was supported by the fact that the antimicrobial activities of the all-L and all-D enantiomers of some peptides, such as apidaecin and drosocin, are not the same (Casteels and Tempst, 1994; Bulet et al., 1996).

More recently it has been proposed that AMP driven microbial death can be caused by others mechanisms in addition to membrane disruption, followed by cell lysis. Many evidences indicate that some AMPs can interact with intracellular targets inducing cell damages, such as the inhibition of cell wall, DNA, RNA, and protein synthesis (Brogden, 2005; Straus and Hancock, 2006). Furthermore, one peptide can act upon multiple cell targets, involving a mixed multi-hit mechanism (Jenssen et al., 2006; Straus and Hancock, 2006). Finally, it is important to note that AMPs action may vary according to test conditions and can be influenced by external factors such as media pH, osmolarity, and temperature (Yeaman and Yount, 2003).

INTRACELLULAR TARGETS

INHIBITION OF CELL WALL SYNTHESIS

The cell wall is an essential bacterial structure responsible for the cell shape. Additionally, the cell wall prevents cell lysis due the high cytoplasmic osmotic pressure and allows the anchoring of membrane components and extracellular proteins, such as adhesins. In Gram-positive organisms, the main component of the cell wall is the peptidoglygan, present in multiple layers. In Gram-negative bacteria, an outer membrane, composed mainly by LPS, overlaps a thin layer of peptidoglycan. Since peptidoglycan is not found in eukaryotic cells, compounds that inhibit its synthesis are interesting targerts for therapeutics. In particular, lipid II, which is an important precursor of peptidoglycan synthesis, was shown to be an attractive target of antibacterial compounds, such as several lantibiotics (de Kruijff et al., 2008; Yount and Yeaman, 2013).

Class I bacteriocins, also known as lantibiotics, are a family of AMPs ribosomally synthesized and post-translationally processed to produce numerous molecules with unusual modified residues (Yount and Yeaman, 2013). Besides killing bacteria by targeting lipid II, and consequently inhibiting cell wall biosynthesis, it has been described that the lantibiotics can also form transmembrane pores, that allows the efflux of molecules and/or ions – ATP, K^+, and PO_4^{3-} (Islam et al., 2012). Several studies have demonstrated that nisin, an antimicrobial peptide widely used in food industry, first binds to lipid II and then acts by forming pores in the membrane, with consequent loss of amino acids, K^+, and ATP by the cell (Yount and Yeaman, 2013). A recent study also demonstrated that nisin binds to lipids III and IV, interfering with teichoic and lipoteichoic acids biosynthesis (Muller et al., 2012). In addition, nisin appears to stimulate the activity of the autolysin *N*-acetylmuramoyl-L-alanine amidase, as shown in *Staphylococcus simulans*. Similar stimulatory effect on this autolysin activity was

observed using Pep5, an antimicrobial peptide produced by *S. epidermidis* (Bierbaum and Sahl, 1987). The induction of autolysins might result in cell wall damage, leading to lysis and subsequent cell death.

Mersacidin, another well-characterized lantibiotic, is effective against methicillin-resistant *S. aureus* (MRSA) in a murine infection model (Chatterjee et al., 1992; Kruszewska et al., 2004). The binding site of mersadicin to lipid II differs from that described for nisin: while nisin binds to the phosphate, mersacidin probably binds to sugar residues. Despite of the different binding sites, both AMPs inhibit the formation of the peptidoglycan (Brotz et al., 1998;Islam et al., 2012).

Another group of lantibiotics is the two-peptide family, whose members are composed by two independently transcribed peptides that act synergistically in order to have an optimal antimicrobial activity (Lawton et al., 2007; Yount and Yeaman, 2013). It has been proposed that one of the peptides binds to lipid II while the other forms transmembrane pores (Wiedemann et al., 2006). Lacticin 3147, staphylococcin C55, plantaricin W, and haloduracin are examples of the two-peptide lantibiotic family (Lawton et al., 2007).

In addition to lantibiotics, other AMPs target the cell-wall synthesis. Lcn972, is a representative of class II bacteriocins that probably exerts an antimicrobial activity via binding lipid II, diverging from the most of other peptides from its class, that act preferentially via membrane disruption. Similarly, nonribosomally synthesized peptides, such as vancomycin, daptomycin, telavancin, and dalbavancin, as well as some defensins produced by eukaryotic organisms, target lipid II biosynthesis pathway, acting via a cell-wall inhibitory mechanism (Yount and Yeaman, 2013).

Because of the main role of bacterial cell wall in cell integrity, as well as its absence in mammalian cells, this structure becomes an excellent target in the search of new antimicrobial drugs. Moreover, the combinatory mechanisms of cell wall synthesis inhibition and cell membrane disruption observed for some AMPs could minimize the emergence of resistant microorganisms.

INHIBITION OF NUCLEIC ACID AND PROTEIN SYNTHESIS

Some AMPs can spontaneously traverse bacterial outer and inner membranes, targeting intracellular molecules such as nucleic acids and proteins. Buforin II, a 21 amino acid, cationic and linear molecule, is an example of an AMP able to cross the cell membrane without permeabilizing it, which later accumulates in the cytoplasm (Cho et al., 2009).

It is well known that this peptide has a proline hinge (Pro^{11}) that plays a critical role in promoting the peptide penetration into the cell (Cho et al., 2009; Xie et al., 2011). Once in the cytoplasm, buforin II binds to DNA and RNA, as showed for *Escherichia coli* (Park et al., 1998). The strong affinity of this peptide for nucleic acids *in vitro* (Park et al., 1998) might be explained by the sequence identity between buforin II and the N-terminal region of histone H2A (Cho et al., 2009). Curiously, buforin II has an antiendotoxin activity (Giacometti et al., 2002). The significant reduction of endotoxin plasmatic levels mediated by buforin II is remarkable since the endotoxin released in Gram-negative infections is responsible for strong inflammatory reactions, with the production of tissue-damaging cytokines, resulting in multiple organ failure and, sometimes, host lethality (septic shock syndrome). The capability to neutralize the toxic effects of endotoxin, preventing the septic shock, has been described for many other AMPs, such as temporins (Mangoni and Shai, 2009) and cathelicidins (Mookherjee et al., 2007).

Many other AMPs act by inhibiting nucleic acid synthesis. Indolicidin, one of the smallest natural cationic peptide, appears to act promoting significant membrane depolarization and inhibiting DNA synthesis (Subbalakshmi and Sitaram, 1998; Nan et al., 2009). As a consequence of the inhibitory effect on DNA synthesis, indolicidin induces filamentation of *E. coli* cells (Subbalakshmi and Sitaram, 1998).

Puroindoline, similarly to indolicidin, is a family of peptides rich in tryptophan residues, that also act by inhibiting DNA synthesis. Haney et al. (2013), using radioactive precursors for DNA, RNA, and protein biosynthesis, demonstrated that the PureB, a peptide of the puroindoline family, inhibits the transcription process and, therefore, the translation process. However, it is still unknown how indolicidin and puroindoline bind to the nucleic acid. Probably, the positive charges of these peptides interact with the phosphate groups of the nucleic acids (Park et al., 1998; Uyterhoeven et al., 2008).

Cathelicidins comprise a family of mammalian proteins with a wide spectrum of antimicrobial activity. One representative is PR-39, which is involved in several cellular processes, including wound repair, chemoattraction, angiogenesis, and inflammation (Zanetti, 2004; Kaneider et al., 2007). This AMP rapidly crosses the cell, without causing membrane damage, and blocks DNA and protein synthesis in bacteria (Boman et al., 1993; Chan et al., 2001; Bals and Wilson, 2003). In addition, some studies propose that PR-39 acts as a noncompetitive and reversible inhibitor of the 20S proteasome, blocking the degradation of NF-κB inhibitor, IκBα. As a result, NF-κB–dependent gene expression is suppressed thereby attenuating inflammation (Gao et al., 2000; Anbanandam et al., 2008). The multiple effects of PR-39 in the target cell might reflect its ability to selectively bind to cytosolic proteins containing SH3 domains (Kaneider et al., 2007).

Another family of cationic peptides with potent antimicrobial activity is the bactenecins, present in bovine neutrophil granules. Two members of this family, Bac5 and Bac7, act by increasing membrane permeability and inhibiting protein and RNA synthesis in *E. coli* and *Klebsiella pneumoniae* (Skerlavaj et al., 1990). Human neutrophils also produce large amounts of AMPs, such as defensins. Besides the ability to permeabilize cell membranes, the human defensin HPN-1 inhibits nucleic acid and protein synthesis in *E. coli* (Lehrer et al., 1989). More recently it was proposed that this AMP also targets the cell wall synthesis by binding to lipid II precursor (de Leeuw et al., 2010). These data support the idea that a single AMP can have multiple mechanisms of action that simultaneously contribute for microorganism death.

Microcins, a group of AMPs produced by some enterobacteria, can also inhibit intracellular targets. Microcin C (McC) is a non-hydrolyzable aminoacyl adenylate that is imported into bacterial cells by an outer-membrane porin and an inner-membrane ABC (ATP-binding-cassette) transporter. Once in the cytoplasm, McC inhibits an essential aminoacyl-tRNA synthetase (Nocek et al.,

2012; Rebuffat, 2012). Microcidin B17 is a modified bacterial peptide that targets DNA gyrase, with subsequent inhibition of DNA replication (Collin et al., 2013). A single mutation in DNA gyrase seems to be enough for bacterial resistance (del Castillo et al., 2001). Microcidin B17 penetration into bacterial cell is facilitated by the inner-membrane protein SbmA (Mathavan and Beis, 2012), which also helps microcidin J25 to cross the cell envelope, where it binds to the RNA polymerase, inhibiting bacterial transcription (Mathavan and Beis, 2012; Rebuffat, 2012). Despite using different mechanisms of action, most microcins employ the "Trojan-horse" strategy, interacting with a bacterial component that facilitates their active transport into the cell. Once inside de cell, the transport component is removed, and the microcin peptide is released to bind to its target (Rebuffat, 2012; Severinov and Nair, 2012).

INDUCTION OF CELL DEATH

Cell death occurs mostly in a programmed form, known as apoptosis, or in an unordered and accidental manner, known as necrosis (Kanduc et al., 2002). Although some mechanisms that cause the cell death are not clear yet, the importance of the caspase cascade in the induction of apoptosis is well known (Fink and Cookson, 2005; Chowdhury et al., 2006). These proteases, in particular caspase-3, catalyze reactions that induce a rapid disarrangement in signaling, homeostatic and repair enzymes, killing the cell (Nicholson and Thornberry, 1997).

Other signals have been described to provoke apoptosis and other mechanisms of death. Bortner and Cidlowski report that, when the concentration of intracellular potassium (K^+) is normal, the cell death process is repressed by the suppression of the caspase cascade and inhibition of the apoptotic nucleases activity. Thus, the efflux of K^+ could prompt cell apoptosis (Bortner and Cidlowski, 1999). In the same way, calcium ions (Ca^{2+}) and reactive oxygen species (ROS) work as pro-apoptotic second messengers (Chowdhury et al., 2006). These signals influence the mitochondrial homeostasis that activate the caspase cascade by releasing the cytochrome *c* (Li et al., 1997).

Currently, the number of studies in this area has increased due to the high frequency of various types of cancer. In this sense, the comprehension of these signals could improve the development of anti-tumoral drugs that could prevent the growth of tumors. Several reports have described antitumor activity for different AMPs. Magainin, a cationic peptide, induces apoptosis by increasing the levels of ROS and the caspase-3 activity in HL-60 cells, a cancer cell line from acute promyelocytic leukemia (Cruz-Chamorro et al., 2006). With the same cell line, other peptide, tachyplesin, that has a disulphide brigde and is stabilized in a β-hairpin structure, induces apoptosis by modifying the potassium intracellular concentration independent of caspase activation (Zhang et al., 2006).

With an erythro-leukemia cell line (K562), four β-hairpin AMPs (gomesin, protegrin, tachyplesin, and polyphemusin II) were tested and, despite of having the same structure, these peptides showed different intracellular mechanisms of cell death induction (Paredes-Gamero et al., 2012). Gomesin, for example, induce K562 cells apotosis by phosphatidylserine externalization and caspase-3 activation, with no cell membrane permeabilization (Paredes-Gamero et al., 2012), while in B16 melanoma, SH-SY5Y neuroblastoma and PC12 pheochromocytoma cells it causes apoptosis inducing Ca^+-dependent membrane permeabilization (Soletti et al., 2010). On the other hand, protegrin provokes cell death inducing a necrosis process by the increase of Ca^+ levels (Paredes-Gamero et al., 2012). Paredes-Gamero et al. also demonstrated that the mechanism of action of these four β-hairpin AMPs depend on their concentration correlated to their EC_{50} values. At low concentrations these peptides induced different intracellular mechanisms of cell death, as mentioned above. However, at high concentrations, these peptides interact with the membrane and form pores, killing the cell. The mechanism of its interaction to mammalian cell surface is not clear and does not fit to the models described above, since eukaryotic cells are quite different from bacterial cells. Finally, this corroborate that the structure of the peptide is not the only factor that determines the mechanism of action in a cell. Furthermore, the concentration used in an experiment could change the peptide interaction with the cell target and its death.

Similar to these antitumor peptides, some antifungal peptides could also induce fungal cell death by apoptosis, necrosis, or other mechanisms (van der Weerden et al., 2013). PvD(1), a plant defensin from *Phaseolus vulgaris* seed, is active against *Candida albicans* and *Fusarium oxysporum*, a human and a plant pathogen, respectively (Mello et al., 2011). This peptide kills the fungal cell by membrane permeabilization and the induction of oxidative stress damage, with the production of ROS and nitric oxide (NO; Mello et al., 2011). The link between endogenous ROS levels and apoptosis is also described to another plant defesin HsAFP1 (Aerts et al., 2011). This peptide could be found in coral bell seeds (*Heuchera sanguinea*) and inhibits several pathogenic fungi, such as *C. albicans* (Osborn et al., 1995; Thevissen et al., 2007). Although the membrane target has not been identified yet, but inside the cell this defensin induces several pro-apoptotics signals, such ROS accumulation, phosphatidylserine externalization, and DNA fragmentation, provoking cell death (Aerts et al., 2011).

To better understand the resistance against AMPs, in the next section, we will focus on this important issue.

MAIN MECHANISMS OF RESISTANCE TO AMPS IN BACTERIA

As discussed in the previous section we are now experiencing a rapid increase in the number of alternative mechanisms for AMPs microbial killing, showing that these molecules are more versatile than we have imagined. On the other hand, in spite of the crescent number of described AMPs active against multiple species of pathogenic organisms, several studies are also showing different strategies of natural or induced resistance to AMPs among different bacterial species, pointing us to the co-evolution of AMPs and AMP-resistance mechanisms (Peschel and Sahl, 2006). Although fungal species also present mechanisms of AMP resistance in the following sections we will focus on mechanisms of resistance in bacteria. In general, AMP resistant bacteria have developed multiple and frequently overlapping strategies of resistance, normally controlled by coordinated stress responses regulated by operons. The most common resistance mechanisms involve changes in bacterial cell surface and blockage of the AMP access to their targets by affecting its binding and/or its penetration into the cells and will be discussed in the following sections.

SURFACE REMODELING

Gram-positive and Gram-negative bacteria modify their cell wall components to reduce the net negative charge of their surfaces. One strategy in Gram-negative bacteria is masking the negative charge of the LPS lipid A by adding amine-containing compounds such as ethanolamine and 4-amino-4-deoxy-L-arabinose (Ara4N). It has been described that differences in polymyxin susceptibility between *Vibrio cholerae* O1 El Tor and classical biotypes could be explained by the ability of *V. cholerae* O1 El Tor to modify the lipid A anchor with glycine and diglycine residues (Hankins et al., 2012). These authors have also shown that the enzymes required for glycine addition are similar to the Gram-positive system for D-alanylation of teichoic acids. In addition to the binding of Ara4N to phosphates of lipid A, palmitoylation of lipid A also promotes resistance to AMPs. *Salmonella* sp., *E. coli, Yersinia enterocolitica*, and other enteric bacteria increase lipid A acylation in response to activation of the Pho-*P*-PhoQ regulon. On the other hand, mutants in the pathway responsible for this modification revealed increased permeability to AMPs (Guo et al., 1998).

Interestingly, the surface modification leading to cationic AMP resistance can occur in response to the presence of the cationic AMP itself. *S. enterica* serovar *typhimurium* (*S. typhimurium*) uses the two-component regulatory system PhoP-PhoQ and PmrA-PmrB to sense the presence of environmental factors in the host tissues such as CAMPs, which results in the activation of the enzymes responsible for LPS modification and CAMP resistance. In this case, when activated, the operon regulates the synthesis and attachment of positively charged Ara4N groups to lipid A. The surface of *S. typhimurium* cells growing *in vitro* in LB broth or on plates mainly present unmodified LPS while the highly modified version of LPS is predominant when the cells are grown *in vivo* (Strandberg et al., 2012). PhoP-PhoQ and PmrA-PmrB systems are also activated in *Pseudomonas aeruginosa* in response to AMPs or low concentrations of divalent cations resulting in the resistance to polymyxin B and other CAMPs (McPhee et al., 2003; Moskowitz et al., 2004; McPhee et al., 2006). Another pathogen, *Y. pestis,* also depends on a two-component regulatory system to respond to AMPs, since mutants of the PhoPQ pathway had decreased intracellular survival in PMN (O'Loughlin et al., 2010). Similarly, Pietiainen et al. found that, in *S. aureus*, AMPs induce the expression of two operons VraSR cell-wall regulon and the vraDE operon. Among the genes activated in this response there was an ABC transporter, a possible peptidase and a small unknown protein. The vraDE seemed to be involved mainly in bacitracin resistance, while VraSR was shown to act in the resistance to several cell wall-active antibiotics and other antimicrobial agents including AMPs (Pietiainen et al., 2009).

Helicobacter pylori is a Gram-negative bacterium that colonizes the stomach mucosa and can cause peptic ulcer and gastric cancer. The strategy of these bacteria to avoid AMPs consists in the dephosphorylation of the lipid A fraction of LPS followed by the addition of a phosphoethanolamine group (Tran et al., 2006). Cullen et al. showed that *H. pylori* mutants that are unable to remove the phosphate groups are highly sensitive to polymyxin and also more readily recognized by the innate immune Toll-like receptor 4 (Cullen et al., 2011).

The main strategy used by Gram-positive bacteria to reduce the negative charges in their surface is the D-alanylation of teichoic acids, mediated by the *dlt* operon and/or the incorporation of L-lysine into PG by the *mprF* gene product (Kristian et al., 2005; Andra et al., 2011; Saar-Dover et al., 2012). The *dlt* operon mediates the esterification of teichoic acids with D-alanyl esters while the membrane protein MprF modifies PG by the enzymatic transfer of L-lysine that changes the net charge of this lipid to become positive (Neuhaus and Baddiley, 2003; Andra et al., 2011). Mutations in genes of both pathways were shown to drastically increase the susceptibility of several Gram-positive strains to AMPs (Peschel et al., 1999; Kovacs et al., 2006; Thedieck et al., 2006; Abi Khattar et al., 2009; Andra et al., 2011; McBride and Sonenshein, 2011). Interestingly, Saar-Dover et al. suggested that the main mechanism involved in the AMP resistance mediated by the D-alanylation is an increase in the cell wall density impairing the penetration of AMPs instead of a major effect on the AMP binding to bacterial surface (Saar-Dover et al., 2012). However, further studies with other species are necessary to confirm if this mechanism can be generalized.

The work of Shireen et al. (2013) reinforce the idea that alterations in surface net charge are not always the main mechanism of surface modification leading to AMP resistance. They analyzed the effects of sub-lethal concentrations of two AMP, magainin 2 and gramicidin D, on the rise of resistant strains of *S. aureus*, and found that the two peptides induced different resistance strategies that result in an increase of membrane rigidity (Shireen et al., 2013).

Another surface modification that can be related to AMP resistance is capsule production. Capsular polysaccharide may acts as a shield, avoiding the interactions between the microbial surface and CAMPs. For example, Campos et al. (2004) have shown that *K. pneumoniae* capsule makes their cells more resistant to defensins, lactoferrin and polymyxin B, while acapsular mutants bound more AMP than wild-type strains. Interestingly, they also found that CPS expression is induced in the presence of AMPs, as previously observed with antibiotic treatment. Additionally to *K. pneumoniae*, CPS from *Streptococcus pneumoniae* serotype 3 and *P. aeruginosa* also increased resistance of a *K. pneumoniae* acapsular strain to polymyxin and for α-defensin (Llobet et al., 2008). The authors suggest that these bacteria can induce the release of capsular polysaccharide in response to AMPs in order to inhibit their interaction with their targets (Llobet et al., 2008). This phenomenon is also observed with *Neisseria meningitidis* where CPS contributes to LL-37 resistance (Jones et al., 2009). In *Campylobacter jejuni*, however, capsule expression was not essential to mediate AMPs resistance, but only to serum components. In this species, resistance to α-defensins, cathelicidins (LL-37) and polymyxin B seems to be related to lipooligosaccharides produced by this organism, as demonstrated by the increased susceptibility of the truncate lipooligosaccharide mutant, when compared to the wild-type strain (Keo et al., 2011). Shielding of AMP targets is also performed by alginate, a polymer secreted by

P. aeruginosa during biofilm formation that induces changes in AMP conformation, avoiding their interaction with microbial membrane (Chan et al., 2004).

MODULATION OF AMP GENES EXPRESSION

The highly contagious and invasive Gram negative rod *Shigella* spp. cause bacillary dysentery that can be often fatal to infants and children. *S. flexneri* was shown to subvert the immune system by down-regulating the expression of innate immune response genes, among them the genes of the cathelicidin LL-37 and the human β-denfensin-1 (Islam et al., 2001; Sperandio et al., 2008). This down-regulation probably facilitates bacterial adhesion and invasion as lower levels of AMPs correlates with deeper invasion of *S. flexneri* toward intestinal crypts (Sperandio et al., 2008).

Several species of the genus *Burkholderia* are inheritably resistant to AMPs. This genus includes several human pathogens among them the *Burkholderia cepacia* complex, which are particularly important for causing chronic opportunistic infections especially in patients with cystic fibrosis or chronic granulomatous disease (Mahenthiralingam et al., 2005; Loutet and Valvano, 2011). One of the explanations for the high resistance of *Burkholderia* sp. to AMPs is the constitutive production of Ara4N as part of their LPS molecule (Cox and Wilkinson, 1991; Isshiki et al., 1998; Loutet and Valvano, 2011). The other strategy used by this group is mediated by the alternative sigma factor RpoE, which controls several genes responsive to stress conditions, however the genes involved in this process are not know yet (Flannagan and Valvano, 2008; Loutet et al., 2011). Interestingly, RpoE was shown to be required for *B. cenocepacia* polymixin B resistance at 37°C, but not at 30°C (Loutet and Valvano, 2011).

Another example of modulation of host AMP production by the pathogen was observed in experiments using *K. pneumoniae* capsular mutant strains. Using acapsular mutants Moranta et al. found that the polysaccharide capsule is also involved in the inhibition of human β-defensins expression. They observed that an acapsular mutant induced the expression of β-defensins by engaging TLR 2 and 4 and activating NF-κb MAPK pathways necessary for β-defensins expression, while the wild-type strain or LPS O antigen mutant did not (Moranta et al., 2010).

BIOFILMS

Biofilms are microbial communities consisting of cells attached to biotic or abiotic surfaces, embedded in an exopolymeric matrix. These structures are well known for their remarkable resistance to diverse chemical, physical, and biological antimicrobial agents and are one of the main causes of persistent infections. Biofilms are also more resistant to AMPs and this feature is linked, although not restrict, to changes in gene expression of biofilm cells, the nature of exopolymeric matrix, and biofilm architecture.

Chan et al. have shown that alginate, a polysaccharide of *P. aeruginosa* biolfim matrix has an important role in *P. aeruginosa* resistance to AMPs (Chan et al., 2004). First, alginate mimics the microbial membrane by inducing conformational changes in AMPs similar to those that occur during the peptide insertion in the membrane bilayer. In addition, alginate also prevents AMPs diffusion because of its ability to bind and induce peptide aggregation, impairing their action (Chan et al., 2004, 2005).

DNA is also a major component of the biofilm matrix. Mulcahy et al. found that exogenous DNA chelates cations, inducing AMP resistance to aminoglycosides and cationic AMPs in *P. aeruginosa* (Mulcahy et al., 2008). The authors observed that extracellular DNA has antimicrobial activity, but that sub-inhibitory concentrations of exogenous DNA induced microbial resistance. The resistance was partially explained by the ability of DNA to chelate several divalent cations, activating the expression of antimicrobial resistance genes such those involved in LPS modification and also the release of genomic DNA, what can result in a DNA gradient inside biofilms (Mulcahy et al., 2008). Similar results were found in a study with *S. typhimurium*, showing that extracellular DNA also induced the expression of *pmr* genes, resulting an increased resistance to AMPs (Johnson et al., 2013).

Moreover, Folkesson et al. established that the differential organization observed in *E. coli* biofilms due to the presence of the transfer plasmids is crucial for AMP resistance. Bacterial strains lacking the transfer plasmid formed less organized biofilms, which were more susceptible to AMPs. The biofilm resistance was also dependent on a two-component regulatory system homolog to the *pmrA-pmrB*. Interestingly, the resistance was not a biofilm general property, being observed only in a subpopulation of cells within the biofilm, as previously observed for *P. aeruginosa* biofilm resistance to colistin (Haagensen et al., 2007; Folkesson et al., 2008).

PROTEOLYTIC DEGRADATION

Gram-positive and Gram-negative bacteria produce peptidases and proteases that degrade CAMPs. Linear peptides are the main target for those enzymes, since proteolytic sites are more exposed to cleavage. The human antimicrobial peptide, LL-37, is digested by proteases of many human pathogens, like *P. aeruginosa*, *Enterococcus faecalis*, *Proteus mirabilis*, *S. aureus*, and *S. pyogenes* (Schmidtchen et al., 2002; Sieprawska-Lupa et al., 2004; Johansson et al., 2008). The elastase of *P. aeruginosa* rapidly degrades LL-37, generating early intermediate fragments presenting residual bactericidal activity when incubated with *E. faecalis*. However, incubation with late digestion fragments shows no bactericidal effect, demonstrating the complete degradation and loss of function of LL-37. Notably, elastase preferentially cleaves LL-37 regions involved in its antimicrobial activity (Schmidtchen et al., 2002).

The metalloprotease ZapA produced by *P. mirabilis* is an important virulence factor involved in the degradation of AMPs (Belas et al., 2004). ZapA cleaves the β-defensin hBD1 and LL-37, partially cleaves PG-1, and is incapable to digest hBD2, in spite of the high similarity between hBD2 and hBD1. ZapA also cleaves LL-37 preferentially on sites inside the antimicrobial domains of this cathelicidin, as already described (Schmidtchen et al., 2002). ZapA digestion is not specific to AMPs, since it also degrades other molecules, like cell matrix components (actin, collagen, fibronectin, laminin), antibodies (IgA and IgG), and complement C1q and C3 (Belas et al., 2004). On the other hand, mutants of ZapA are no more susceptible to those AMPs than the wild-type strain, indicating that *P. mirabilis* may use multiple mechanisms to avoid the AMPs action. Similarly, culture supernatants of *Porphyromonas gingivalis* efficiently degrades α- and β-defensins (Carlisle et al., 2009) and other AMPs, but resistance to those peptides

seems to be independent of the proteolytic activity (Bachrach et al., 2008).

Species of *Bacillus* that produce metalloproteases rapidly degrade LL-37 and are more resistant to this CAMP than non producing species. Culture supernatants of *B. anthracis* also degrade LL-37 and are shown to increase LL-37 resistance in *B. subtilis*, a hypersensitive species, when grown together (Thwaite et al., 2006). Another example of protease-mediated AMP resistance occurs in *Burkholderia cenocepacia*, which produces two zinc-dependent metalloproteases (ZmpA e ZmpB) that inactivate different AMPs (Kooi and Sokol, 2009). Other proteases seem to mediate the resistance to lactoferrin B in *E. coli* and *S. aureus* (Ulvatne, 2002), like DegP in *E. coli*, a protease that also acts as a heat-shock protein.

Some members of the omptins family of outer membrane proteases present in Gram-negative bacteria are reported to mediate and contribute to resistance to AMPs. The PgtE protease of *S. enterica* Serovar *typhimurium* and CroP protease of *Citrobacter rodention* promote resistance to α-helical peptides (Guina et al., 2000; Le Sage et al., 2009). OmpT has an important role in the resistance of *E. coli* to protamine and urinary cationic peptides (Stumpe et al., 1998; Hui et al., 2010). In enterohemorrhagic *E. coli* (EHEC), OmpT contributes to the degradation of LL-37 more efficiently than in enteropathogenic *E. coli* (EPEC). The difference between EHEC OmpT and EPEC OmpT in providing defense against AMPs is due to the differences in the regulation of *ompT* genes in a promotor dependent manner, resulting in high levels of *ompT* transcripts and OmpT protein in EHEC (Thomassin et al., 2012). A posterior study with uropathogenic *E. coli* (UPEC) demonstrated that UPEC OmpT can cleave and protect the membrane from LL-37, but only when is expressed in high levels (Brannon et al., 2013), similar to those reported by Thomassin et al. (2012) for EPEC. Therefore, UPEC OmpT is not essential to LL-37 resistance. These data suggest that the role of OmpT in resistance to AMPs is specific to each pathogen.

In order to avoid killing by AMPs, pathogens may form complexes that retain their own proteases near the bacterial surface (Nyberg et al., 2004). This study shows that the host proteinase inhibitor α_2-macroglobulin (α_2_M) binds with high affinity to GRAB, a protein attached to *S. pyogenes* cell wall. The α_2_M, on the other hand, traps the SpeB protease secreted by the bacteria, but SpeB retains the ability to degrade LL-37 and, more interestingly, its proteolytic activity against that AMP molecule is enhanced.

EFFLUX PUMPS

Efflux pumps are energy-dependent transporters that extrude toxic compounds, including antibiotics, being one of the major mechanisms by which microbial pathogens resist to different classes of antibiotics (Piddock, 2006; Poole, 2007). Kupferwasser et al. (1999) demonstrated that a naturally occurring plasmid containing *qacA* gene, which encodes a proton motive force-dependent multidrug efflux pump, confers resistance to tPMP-1 in *S. aureus*, while constructions with deletions that compromise the *qacA* gene abolish tPMP-1 resistance. The *qacA*-mediated resistance seems to be specific to tPMP-1, because the expression of *qacA* did not confer resistance to other AMPs. It is possible that the efflux function of QacA protein itself is not responsible for the resistance to tPMP-1, but the alterations on the cytoplasmic membrane of strains expressing *qacA*. On the other hand, in *Neisseria gonorrhoeae* and *N. meningitidis*, the energy-dependent Mrt efflux pump mediates the resistance to antibacterial peptides PG-1, PC-8, LL-37, and TP-1 (Shafer et al., 1998; Tzeng et al., 2005). Notably, this pump recognizes structurally unrelated AMPs, such as PG-1, which folds into a β-sheet conformation, and LL-37, that assumes n α-helical structure. The importance of Mrt efflux pump system to avoid AMPs-killing during *in vivo* infections by *N. gonorrhoeae* was demonstrated by Jerse et al. (2003) where MtrCDE mutants were more susceptible to the host immune system. Warner et al. also showed that mutations upstream of *mrtC,* on the promoter of the *mtrR* gene encoding a repressor for MtrCDE, or in the binding site of MrtR, confer more resistance to LL-37 and CRAMP-8, a murine analogous to LL-37, by increasing the levels of MrtE and *mrt*CRNA stability (Warner et al., 2008). These mutants were also more fitted *in vivo* than wild-type strains, highlighting the importance of this pump in evasion of the immune system.

Resistance to polymyxin B in *Y. enterocolitica* and *K. pneumonia* involves a system composed of two proteins, RosA/RosB and AcrAB, respectively (Bengoechea and Skurnik, 2000; Padilla et al., 2010). The dissipation of the proton-motive force by the addition of Carbonyl cyanide *m*-chlorophenyl hydrazone CCCP reduced the resistance to polymyxin B, highlighting the importance of the energy-driven efflux pump of AMPs in resistance to these molecules. In *Y. enterocolitica,* RosA functions as a CAMP proton motive force-dependent efflux pump, while RosB has a potassium antiporter activity, involved in the regulation of intracellular pH, which is linked to CAMPs resistance by acidification of the cytoplasm. Both, RosA and RosB, are required for resistance to CAMPs and the presence of these defense molecules increases the expression of the *ros locus*, as observed in *S. pneumoniae* LL-37 resistance, mediated by MefE/Mel efflux pump (Zahner et al., 2010). Similarly, the protein TrkA of the potassium transport complex Trk is required for CAMP resistance in *Vibrio vulnificus* (Chen et al., 2004). It is hypothesized that protamine forms channels through which K^+ leaks from the cell; then, the high-rate K^+ uptake systems pump potassium inside the cell, preventing bacterial death until the protamin is degraded by extracellular proteases. Likewise, *sapG* mutants, a *S. typhimurium* gene that encodes a protein involved in potassium transport higlhy homologous to TrkA, exhibited hypersensitivity to protamin (Parra-Lopez et al., 1994). On the other hand, the overexpression of AcrAB, MexAB and NorA pumps in *E. coli*, *P. aeruginosa*, and *S. aureus*, respectively, did not increase resistance to CAMPs such HNP-1, HNP-3, HD-5, HBD-2, HBD-3, and LL-37, when compared to the wild-type strains and inactivated mutants. These results indicate that specific efflux pumps extrude specific types of AMPs (Rieg et al., 2009).

TRAPPING

Some secreted molecules can bind to and neutralize the microbicidal activity of AMPs. For example, SIC proteins produced by *S. pyogenes* M1 bind to several AMPs, blocking their antimicrobial activity in the early stages of infection (Frick et al., 2003; Fernie-King et al., 2004; Pence et al., 2010). SIC binds more efficiently to LL-37 than to the defensing human neutrophil peptide HNP-1

and SIC deficient mutants are more sensitive to LL-37 than wild-type strains. Other SIC variants from other serotypes also bind and interfere with the antimicrobial activity from AMPs, but SIC from M1 strain is more potent (Frick et al., 2003). In addition to the plasminogen binding activity, Staphylokinase (SAK) from *S. aureus* can also binds to HNP-1 and HNP-2. SAK induces HNPs release from neutrophils, and the formation of a complex of SAK and the α-defensin results in the loss of antimicrobicidal activity of those defense molecules. This complex abrogates bactericidal activity of HNPs in other species, when grown together.

Other trapping molecules can be found attached to the bacterial surface, like M1 protein from *S. pyogenes*. M1 mutant strains are killed more efficiently by cathelicidins, LL-37 or mCRAMP than the wild-type strain. It seems that M1 acts as a shield, binding, and trapping the AMPs before they can reach the cell membrane and kill the pathogen (Lauth et al., 2009). Pili also may be a trap to AMPs. Maisey et al. (2008) reported that PilB from Group B *S.* sequesters LL-37 and mCRAMP, increasing the resistance to those peptides. GRABs also mediate an important binding mechanism in resistance to LL-37 by *S. pyogenes* (Nyberg et al., 2004).

It is also described that the shedding of host molecules by proteases from pathogens are involved in sequestration and resistance to several AMPs. For example, extracellular proteases secreted by *P. aeruginosa*, and *E. faecalis* degrade decorin proteoglycan, producing dermatan sulphate that protects those bacteria from killing mediated by HPN-1 through directly binding to the α-defensin (Schmidtchen et al., 2001).

CONCLUSION

The increasing emergence of antibiotic resistance among human pathogens has led to the search for alternatives to overcome this alarming problem. Despite their ancient origin, only in the past decades the study of AMPs have gained increased attention as a promising therapeutic alternative against pathogenic microorganisms due their broad spectrum of activity against several species of bacteria, fungi, protozoa, and enveloped virus. Although the main target of AMPs was previously thought to be bacterial membrane several new and frequently, combined strategies of killing have been described in the past decades. Resistance to these molecules seems to be less common than to conventional antibiotics but was selected in several pathogens during the co-evolution of host and pathogens. A better comprehension of AMPs mode of action and counterpart resistance mechanisms is fundamental for the design of optimized AMPS that could be efficiently used as therapeutic drugs.

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Defensins: antifungal lessons from eukaryotes

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Over the last years, antimicrobial peptides (AMPs) have been the focus of intense research toward the finding of a viable alternative to current antifungal drugs. Defensins are one of the major families of AMPs and the most represented among all eukaryotic groups, providing an important first line of host defense against pathogenic microorganisms. Several of these cysteine-stabilized peptides present a relevant effect against fungi. Defensins are the AMPs with the broader distribution across all eukaryotic kingdoms, namely, Fungi, Plantae, and Animalia, and were recently shown to have an ancestor in a bacterial organism. As a part of the host defense, defensins act as an important vehicle of information between innate and adaptive immune system and have a role in immunomodulation. This multidimensionality represents a powerful host shield, hard for microorganisms to overcome using single approach resistance strategies. Pathogenic fungi resistance to conventional antimycotic drugs is becoming a major problem. Defensins, as other AMPs, have shown to be an effective alternative to the current antimycotic therapies, demonstrating potential as novel therapeutic agents or drug leads. In this review, we summarize the current knowledge on some eukaryotic defensins with antifungal action. An overview of the main targets in the fungal cell and the mechanism of action of these AMPs (namely, the selectivity for some fungal membrane components) are presented. Additionally, recent works on antifungal defensins structure, activity, and cytotoxicity are also reviewed.

Keywords: antimicrobial peptides, defensins, antifungal, resistance, host defense peptides

INTRODUCTION

Naturally occurring antimicrobial peptides (AMPs) probably represent one of the first successful forms of chemical defense of eukaryotic cells against bacteria, protozoa, fungi, and viruses (Ganz and Lehrer, 1998; Lehrer and Ganz, 1999; Zasloff, 2002; Mookherjee and Hancock, 2007; Lai and Gallo, 2009; Guo et al., 2012; Domingues et al., 2014), being also active against cancer cells (Hoskin and Ramamoorthy, 2008; Gaspar et al., 2013). Currently commercialized antibiotics are mostly of microbial origin or synthesized from those. These antibiotics are losing efficacy as a result of high selection pressure, leading to rapid emergence of resistance in many important human pathogens, thus threatening to put an end to the golden age of antibiotics (Clardy et al., 2009; Fisher et al., 2012). The use of antifungal treatments has increased as a consequence of the increase of immunocompromised patients, mostly due to improvements in oncology and transplant fields (Mehra et al., 2012), leading to more frequent resistances to the drugs used. A strategy to overcome this problem can be found in AMPs, which are part of the innate immune system of different living organisms (Hegedus and Marx, 2013), such as plants (Thomma et al., 2002; Lay and Anderson, 2005; Gonçalves et al., 2012b), fungi (Mygind et al., 2005; Schneider et al., 2010; Oeemig et al., 2012), bacteria (Zhu, 2007; Gao et al., 2009), invertebrates (Bulet and Stocklin, 2005; Ayroza et al., 2012), and vertebrates (Ganz, 2004; Sahl et al., 2005; van Dijk et al., 2008; Gonçalves et al., 2012a).

Although some AMPs have had their target unveiled, many are still unclear. Some mechanisms of action of antifungal peptides have been reported, such as binding to the cell wall, membrane permeabilization, receptor-mediated internalization inducing signaling cascades, and interaction with intracellular targets, inducing the formation of reactive oxygen species (ROS), leading ultimately to apoptosis (Hancock and Rozek, 2002; Oberparleiter et al., 2003; Thevissen et al., 2003a, 2004; de Coninck et al., 2013; van der Weerden et al., 2013). Apoptosis is a type of programed cell death, which is regulated by a complex network of proteins and metabolic pathways. The central core of this process is regulated by a family of proteins named caspases. Yeasts have at least one ortholog of mammalian caspases: the metacaspase YCA1 (yeast caspase 1; Madeo et al., 2002). Routinely used assays aiming at the detection of these apoptotic features are used for the identification of fungal cells undergoing apoptosis after treatment with antifungal agents.

Antimicrobial peptides have variable amino acid composition and size (ranging from less than 10 to more than 100 amino acid residues), commonly being cationic and amphipathic molecules (Brogden et al., 1996; Yang et al., 2003; Fontana et al., 2004; Glaser et al., 2005; Domingues et al., 2014). To date, more than 2200 natural or synthetic AMPs have been identified, as listed by the Antimicrobial Peptide Database (APD[1]), of which over 1900 have antibacterial activity and 800 have antifungal activity.

[1]http://aps.unmc.edu/AP/main.php

This discrepancy, however, may be redundant as antibacterial AMPs may also have antifungal activity, but this property is not systematically assessed.

Antimicrobial peptides may have linear structures, like indolicidin (Ladokhin et al., 1999), or they may have tertiary structures stabilized by disulfide bonds, with β-sheet (e.g., protegrin; Aumelas et al., 1996; and the defensin human neutrophil peptide 1, HNP-1; Zhang et al., 2011), α-helix (e.g., dermaseptin; Mor et al., 1991) or αβ-motif secondary structure (e.g., drosomycin; Landon et al., 1997; and *Pisum sativum* defensin 1, *Ps*d1; Almeida et al., 2002).

The most studied families of AMPs are cathelicidins, dermaseptins, magainins, cecropins, and defensins. Cathelicidins are found in the innate immune system of mammals, amphibians, and reptiles (Wang et al., 2008; Tsai et al., 2011; Hao et al., 2012); dermaseptins and magainins are found in amphibians (Morton et al., 2007); cecropins are found in insects (YiZeng et al., 1989); and defensins, which are the largest family of AMPs, have also the broader distribution across the majority of eukaryotic organisms (Wang et al., 2013). Besides having antimicrobial activity, defensins also have immunomodulatory functions in the organisms that produce these peptides. Defensins with antifungal properties are present in all eukaryotic kingdoms, pointing out to a common ancestor. This review is focused on defensins (as well as some defensin-like peptides) with antifungal activity. However, it is impossible to describe here all the defensins with this activity. Therefore, we highlight some of the most recent research made on this field. The chosen peptides are described taking into consideration their specific properties, evolutionary background, organism of origin, and antifungal mode of action.

Some databases have been created in order to provide useful information for the study of AMPs. Among the AMP databases, PhytAMP is a database dedicated to antimicrobial plant peptides[2] (Hammami et al., 2009). This resource contains valuable information on these AMPs, including peptide sequences, taxonomic, microbiological, and physicochemical data. Another database, Collection of Anti-Microbial Peptides (CAMP[3]), holds experimentally validated and predicted AMP sequences and structures of AMPs. These databases include several tools for AMPs analysis and prediction, helping in the design of new therapeutic peptides based on specific structure and functional features.

DEFENSINS

Defensins are the largest groups of AMPs. These peptides are cysteine-rich and have diverse sequences and structures, stabilized into compact shapes by three or four conserved cysteine disulfide bridges. They have at least two positive charges (lysine or arginine residues) and are small, ranging approximately from 12 to 50 amino acid residues (2–6 kDa; Ganz, 2003; Ren et al., 2011; Gao and Zhu, 2012).

Vertebrates' defensins are divided into three subfamilies: α-, β-, and θ-defensins. α-Defensins are present in mammals such as humans, monkeys, and several rodent species, being particularly abundant in neutrophils, certain macrophage subpopulations and Paneth cells of the small intestine (Ouellette and Selsted, 1996; Ganz and Lehrer, 1998; Lehrer and Ganz, 1999). β-defensins are found in a wide range of vertebrates, presenting a cysteine-stabilized αβ-motif composed of an antiparallel β-sheet and an α-helix. As an example, on bovine neutrophils, as many as 13 β-defensins have been identified (Yang et al., 2002a). However, in other species, β-defensins are mostly produced by epithelial cells lining different organs (e.g., epidermis, bronchial tree, and genitourinary tract). θ-Defensins, present only in Old World monkeys, are cyclic and derived from α-defensins (Lehrer, 2004; Lehrer and Lu, 2011; Semple and Dorin, 2012). In **Figure 1**, conserved cysteine residues among defensins from different kingdoms are shown.

Plants, fungi, and many invertebrates produce defensin-like peptides structurally similar to the β-defensins from vertebrates (Thomma et al., 2002; Bulet and Stocklin, 2005; Mygind et al., 2005; Sahl et al., 2005; Yount and Yeaman, 2006; van Dijk et al., 2008; Ayroza et al., 2012; Oeemig et al., 2012). These observations allowed to assume that defensins and defensin-like peptides all evolved from a common precursor. The relatively recent identification of three defensins in lower eukaryotes, plectasin from *Pseudoplectania nigrella* (Mygind et al., 2005), eurocin from *Eurotium amstelodami* (Oeemig et al., 2012), and bubble protein

[2]http://phytamp.pfba-lab-tun.org/main.php

[3]http://www.camp.bicnirrh.res.in/

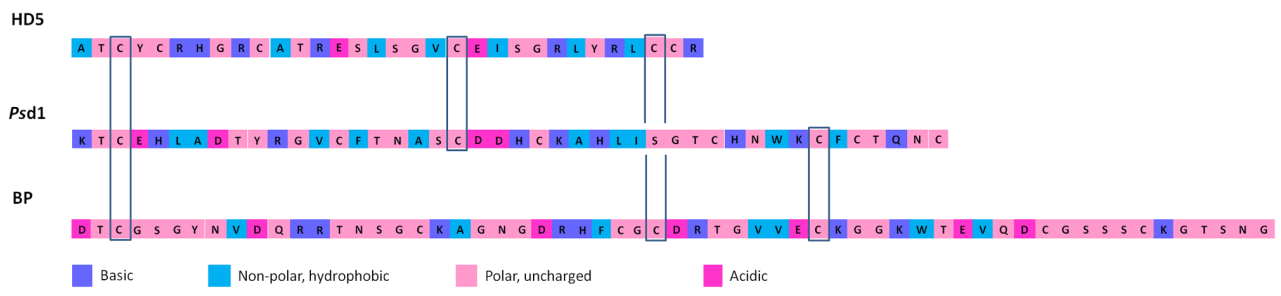

FIGURE 1 | Comparison of amino acid residues sequence of selected defensins with antifungal activity from animals, plants, and fungi: human defensin 5 (HD5), *Pisum sativum* defensin 1 (Psd1), and *Penicillium brevicompactum* Dierckx bubble protein (BP), respectively. Conserved cysteine residues among defensins from different kingdoms are indicated. The colors used in the sequences represent basic, non-polar hydrophobic, polar-uncharged and acidic residues.

(BP) from *Penicillium brevicompactum* Dierckx (Seibold et al., 2011), is important to demonstrate the wide distribution of these peptides over diverse eukaryotic lineages, which suggests that ancestral defensin genes existed over 1500 million years ago, before Fungi, Plantae, and Animalia kingdoms diverged (Wang et al., 1999). The wide distribution of these peptides in the Eukarya domain could suggest their uniqueness to eukaryotic cells, but it was possible to determine that these peptides may have had their ancestor in a prokaryotic organism after the discovery of the first defensin-like peptide in the bacteria *Anaeromyxobacter dehalogenans* (AdDLP; Zhu, 2007; Gao et al., 2009). This defensin-like peptide is proposed as an ancestor of eukaryotic defensins and defensin-like peptides due to the similarity of their structures, namely at the level of the cysteine-stabilized αβ-motif (Zhu, 2007). These findings further support the concept that AMPs may have been fundamental to the evolution of multicellular organisms within microbial-exposed environments.

Although defensins were initially identified only as AMPs, recent studies have demonstrated that they have a much broader range of action, including immunomodulatory function (issue further developed in the text, in Section "Immunomodulatory Function"; Ulm et al., 2012).

RECENTLY STUDIED ANTIFUNGAL DEFENSINS

When a new AMP is described, the most usual properties to assess are structure and peptide sequence, antimicrobial activity, expressed mainly in terms of minimal inhibitory concentration (MIC) or half maximal inhibitory concentration (IC_{50}), cytotoxicity and lytic activity against human cells (whenever the AMP origin is not mammalian), target, and mode of action toward the pathogen tested. The following AMPs were classified as defensins or defensin-like peptides due to their structural and sequence homologies with other defensins. They are examples of some of the recently studied antifungal defensins from fungal, plant, and animal origin that fulfill most of the properties expressed above. Further details and a list of some of these defensins can be found in **Table 1**.

FUNGAL SOURCES

Defensin-like antifungal peptides secreted by filamentous fungi have a low molecular mass (5.8–6.6 kDa), a basic character, presence of 4–10 cysteine residues and several disulfide bonds (providing resistance against temperature stress or adverse solvent conditions), and a β-barrel conformation (Hagen et al., 2007; Seibold et al., 2011). Proteins with such properties with antifungal activity have been isolated and investigated from several Ascomycota fungal species, such as *Penicillium chrysogenum*, *Penicillium nalgiovense*, *Penicillium brevicompactum* Dierckx, *Aspergillus giganteus*, and *Aspergillus niger* (Galgóczy et al., 2010; Seibold et al., 2011). Among these fungus-derived antifungal peptides, the most intensively studied are *Penicillium chrysogenum* antifungal protein (PAF) and *Aspergillus giganteus* antifungal protein (AFP), with six and eight cysteines, respectively. *Penicillium brevicompactum* Dierckx bubble protein structure is considerably similar to PAF and AFP.

Penicillium chrysogenum antifungal protein

PAF acts through a G protein-coupled signal transduction pathway, although this mechanism is not entirely understood (Marx et al., 2008). The G protein-coupled activity of PAF was confirmed by the study of the *fadA* mutant of *Aspergillus nidulans* which proved to be less sensitive to PAF treatment compared to the wild-type. The *fadA* gene encodes the heterotrimeric G protein α subunit, which dissociation from the $G_{\beta\gamma}$ complex is inhibited in the *fadA* mutant *Aspergillus nidulans* (Leiter et al., 2005). These results indicate that PAF toxicity requires active heterotrimeric G protein signaling (Leiter et al., 2005; Marx et al., 2008). Based on these observations, Marx et al. (2008) hypothesized that PAF interacts directly or indirectly with G protein signal transduction pathways. These authors also hypothesized that the cell wall could be a selective barrier for PAF, but *in vivo* chitin-binding activity of PAF has not been demonstrated yet. In the sensitive organisms, PAF exerts multiple detrimental effects: induction of plasma membrane polarization, increased exposure of phosphatidylserine (PS) on the cell surface, DNA fragmentation, membrane blebbing, cell shrinking, intracellular ROS production, and apoptosis-like phenotype (Kaiserer et al., 2003; Leiter et al., 2005; Marx et al., 2008).

Aspergillus giganteus antifungal protein

In susceptible organisms, AFP disturbs the polarized growth of hyphae by interfering directly or indirectly with the cell wall biosynthesis (Theis et al., 2003). Hagen et al. (2007) demonstrated that AFP can bind to chitin *in vitro*, and inhibits the cell wall chitin biosynthesis by the specific inhibition of chitin synthase III and V. These enzymes are unique among fungi and essential for the maintenance of the polarized growth and virulence of pathogenic fungi. Presence of chitin synthase III and V is confirmed in the AFP-sensitive fungi, while the insensitive fungal species do not have these enzyme classes. Sphingolipid membrane components in the sensitive fungi might serve as secondary receptors for AFP. This was further confirmed by the discovery that the depletion of cellular glucosylceramide (GlcCer) levels in AFP-sensitive fungal species (*Aspergillus fumigatus* and *Aspergillus niger*) resulted in reduced AFP susceptibility (Meyer, 2008). This, together with the observation that the sphingolipids are necessary to maintain the polarized hyphal growth, elucidates the mechanism of polarized growth degeneration effect of AFP (Li et al., 2006; Meyer, 2008). The species specificity of AFP may be related with the sphingolipid profile of the sensitive fungi (Meyer, 2008).

Penicillium brevicompactum Dierckx bubble protein

This fungal defensin was first described in 2003 (Olsen et al., 2004). It is found in the bright yellow–green fluorescent exudate bubbles of the ascomycete fungus *Penicillium brevicompactum* Dierckx. Similarly to other ascomycetes, BP produces a small antimicrobial molecule, mycophenolic acid, which gives the bubbles their yellow–green fluorescence. This combined production suggests a possible synergistic action between defensins and other antibiotic agents produced by this class of fungi (Seibold et al., 2011). BP has 64 amino acid residues, with high content of basic amino acids, β-barrel conformation (Olsen et al., 2004), and a cage-like pattern of four very stable disulfide bridges. In addition, it was discovered

Table 1 | Recently studied antifungal defensins from fungal, plant, and animal sources.

AMP	Organism of origin	Antifungal spectrum	Antifungal activity	Mode of action	Cytotoxicity	Reference
Fungi						
PAF	*Penicillium chrysogenum*	Several species of Zygomycetes, Ascomycetes, and Basidiomycota	MIC: 1–200 μg/ml	Interaction with G protein signal transduction pathways, leading to production of ROS and induction of apoptosis	Non-toxic to mammalian cells	Kaiserer et al. (2003); Marx (2004); Galgóczy et al. (2005, 2007, 2008); Barna et al. (2008)
AFP	*Aspergillus giganteus*	Several species of Ascomycetes	MIC: 1–200 μg/ml	Specific inhibition of the chitin synthase III and V, interfering with cell wall biosynthesis	Non-toxic to mammalian cells	Lacadena et al. (1995); Moreno et al. (2003); Moreno et al. (2006); Theis et al. (2003); Meyer (2008)
Bubble protein	*Penicillium brevicompactum* Dierckx	*Saccharomyces cerevisiae*	Growth inhibition in a dose-dependent manner	n.d.	n.d.	Olsen et al. (2004); Seibold et al. (2011)
Plant						
*Psd*1	Pea (*Pisum sativum*)	*Aspergillus* spp.; *Fusarium solani*; *Neurospora crassa*; *Candida albicans*	IC_{50}: 0.04–21.7 μg/ml; MIC *C. albicans*: 20 μM	Impairment of progression of cell cycle: cyclin F; S to G2 phase transition is blocked, resulting in endoreduplication; strong interaction with ergosterol and fungal sterol-rich membranes	Reduced interaction with cholesterol-rich mammalian membranes	Almeida et al. (2000); Lobo et al. (2007); de Medeiros (2009); de Medeiros et al. (2010); Gonçalves et al. (2012b)
*Rs*AFP2	Radish (*Raphanus sativus*)	*Candida* spp.; *Aspergillus flavus*; *Fusarium solani*	IC_{30} *C. albicans*: 10 μg/ml. Reduced cell viability in all *Candida* spp. tested with up to 10 μM of RsAFP2	Interaction with glucosylceramides; membrane permeabilization; ROS production; cell growth arrest; apoptosis induction; caspase activation; yeast-to-hypha transition blocking; septin localization; ceramide accumulation; altered cell wall shape	Non-toxic to mammalian cells	Aerts et al. (2007, 2009); Tavares et al. (2008); Thevissen et al. (2012)
*Hs*AFP1	Coral bells (*Heuchera sanguinea*)	*Neurospora crassa*; *Candida albicans*	IC_{50} *Neurospora crassa*: 4 μg/ml	Interaction with cell membrane (hypha); ROS formation; apoptosis induction	n.d.	Thevissen et al. (1997); Aerts et al. (2011)

(Continued)

Table 1 | Continued

AMP	Organism of origin	Antifungal spectrum	Antifungal activity	Mode of action	Cytotoxicity	Reference
Animal						
Arthropod						
Coprisin	Korean dung beetle (*Copris tripartitus*)	*Aspergillus* spp.; *Candida* spp.; *Malassezia furfur*; *Trichosporon beigelii*; *Trichophyton rubrum*	MIC: 5–20 μM	Apoptosis induction; ROS formation; disruption of mitochondrial membrane potential; cytochrome *c* release; intracellular metacaspase activation	No hemolytic activity on human erythrocytes	Lee et al. (2012)
Juruin	Amazonian pink toe spider (*Avicularia juruensis*)	*Candida* spp.; *Aspergillus niger*	MIC: 2.5–10 μM; fungicidal activity, rather than fungistatic	n.d.	No hemolytic activity on human erythrocytes	Ayroza et al. (2012)
Reptile						
Crotamine	South-American rattlesnake (*Crotalus durissus terrificus*)	*Candida* spp.; *Trichosporon* spp.; *Cryptococcus neoformans*	MIC: 12.5–50 μg/ml; fungicidal activity, rather than fungistatic	Pronounced ultrastructural alterations; membrane collapse; cytoplasmic coagulation	No hemolytic activity on human erythrocytes; $CC_{50} > 50$ μM against non-tumoral animal and human cells	Yamane et al. (2013)

n.d., not determined.

that the closely related fungus *Penicillium chrysogenum* encodes a BP homolog (in addition to PAF), indicating that fungi may have more than one defensin (Seibold et al., 2011).

PLANT SOURCES

Many fungi are phytopathogenic, with species such as *Fusarium* spp., *Cladosporium* spp., *Pythium* spp., *Curvularia* spp., *Aspergillus flavus*, and *Puccinia pittieriana* affecting potato, rice, corn, wheat, tobacco, and cotton crops by causing wilt, mold, crown rot, mildew, and rust, just to name a few plant diseases (The American Phytopathological Society, APS[4]). These diseases can deplete entire crops, bearing enormous costs for agriculture due to the difficulty in eliminating fungal infections from plants, once they appear. Soils harbor plants for most of their life cycle, but also a considerable amount of bacteria, fungi, and parasites, many of which can be phytopathogenic. For this reason, plants need to have good defenses against these microorganisms; thus, it is easy conceivable that plants are major AMPs producers, often with antifungal activity, but also antibacterial activity (Moreno et al., 1994; Segura et al., 1998; Thomma et al., 2002; Mayer et al., 2013).

In fact, a major research effort has been put forward on the screening for these molecules in plants. Besides defensins, other AMPs are also produced by plants, being exclusive to them. Examples of these plant exclusive AMPs are thionins, lipid transfer proteins and snakins, which were also demonstrated to have antifungal activity (Segura et al., 1999; Silverstein et al., 2007; Sun et al., 2008; Asano et al., 2013). Plant defensins with antifungal activity have been purified from several plants, such as *Pisum sativum* (Almeida et al., 2000; Lobo et al., 2007; de Medeiros, 2009; de Medeiros et al., 2010; Gonçalves et al., 2012b), *Raphanus sativus* (Aerts et al., 2007, 2009; Tavares et al., 2008; Thevissen et al., 2012), and *Heuchera sanguinea* (Thevissen et al., 1997; Aerts et al., 2011), which will be addressed below. Several other plant defensins with antifungal activity have also been studied. Specific information about some of those defensins can be found on the following references: *Medicago sativa* defensin 1 (*Ms*Def1) and *Medicago truncatula* defensin 4 (*Mt*Def4; Spelbrink et al., 2004; Ramamoorthy et al., 2007a,b; Sagaram et al., 2011); *Dahlia merckii* AMP 1 (*Dm*AMP1; Thevissen et al., 1999, 2000a,b, 2003b; Jha et al., 2009; Salahinejad et al., 2013); *Phaseolus vulgaris* defensin 1 (*Pv*D1; Games et al., 2008; Mello et al., 2011; Wu et al., 2011; Chan et al., 2012; Wong et al., 2012; Chan and Ng, 2013); *Nicotiana alata* defensin 1 (*Na*D1; Lay et al., 2003, 2012; van der Weerden et al., 2008, 2010; Hayes et al., 2013).

*Ps*d1

This garden pea (*Pisum sativum*) seed defensin, firstly characterized in 2000 (Almeida et al., 2000), has 46 amino acid residues. Its secondary structure comprises a globular fold composed of β-sheets and an α-helix stabilized by four disulfide bridges, i.e., a cysteine-stabilized αβ-motif (Almeida et al., 2002). As demonstrated by a yeast two-hybrid screening system, *Ps*d1 has affinity to a *Neurospora crassa* protein related to the cell cycle control, cyclin F (Lobo et al., 2007). Using a developing retinal tissue of neonatal rats as a model to study this interaction, it was proven that *Ps*d1 impairs the correct cell cycle progression, by blocking cyclin F role in the transition of S to G2 phases of the cell cycle, promoting endoreduplication and disturbing nuclear migration. Recently, it has been demonstrated through partition studies that *Ps*d1 has a high affinity with high specificity to model membranes enriched with ergosterol, the main sterol present in fungal membranes, and GlcCer (Gonçalves et al., 2012b). On the contrary, there is no interaction between the defensin and model membranes enriched in cholesterol (a characteristic of mammalian cells), reducing *Ps*d1 toxicity to human cells.

*Rs*AFP2

This defensin, isolated from radish (*Raphanus sativus*) seeds in 1992 (Terras et al., 1992), has 51 amino acid residues and is highly cationic. It has eight cysteine residues, forming four disulfide bridges that stabilize its αβ-motif structure. *Rs*AFP2 has fungal GlcCer as its target, as observed in experiments performed with wild-type *Candida albicans* and a mutant lacking GlcCer in the membrane (Δ*gcs*; Aerts et al., 2007). It does not need to be internalized to have its antifungal effect. After the initial contact, a signaling cascade is activated inside the cell and ROS are formed, leading to membrane permeabilization and consequent cell death (Aerts et al., 2007). Other effects of *Rs*AFP2 comprise the induction of apoptosis in *C. albicans* by triggering caspases activation, but not of metacaspases, implying that different apoptotic pathways can be induced in *C. albicans* (Aerts et al., 2009). *Rs*AFP2 also promotes an accumulation of ceramides in *C. albicans*, which can be lethal to the cell, and blocks the yeast-to-hypha transition (Thevissen et al., 2012). *In vivo* experiments were performed in murine models, proving that *Rs*AFP2 considerably reduces the fungal burden in kidneys of mice infected with *C. albicans*. This defensin has low susceptibility to serum peptidases, meaning that upon entering the bloodstream it will not be degraded, maintaining its antifungal activity. Lactate dehydrogenase (LDH) release levels are indicative of cell damage and tissue breakdown. Human brain endothelial cells incubated with *Rs*AFP2 show no release of LDH, hence supporting the conclusion that this defensin has limited toxicity to mammalian cells.

*Hs*AFP1

Firstly identified in 1995, this defensin found in the seeds of coral bells plant *H. sanguinea* (Osborn et al., 1995) was shown to have high affinity to specific sites in fungal membranes and to permeabilize cells of susceptible fungi (Thevissen et al., 1997). Unlike *Rs*AFP2, which relies on an interaction with GlcCer to exert its antifungal effect, *Hs*AFP1 has antifungal activity against *C. albicans* Δ*gcs* and its wild-type counterpart (Aerts et al., 2011). It was proposed that *Hs*AFP1 may interact with essential components of the fungal membrane, resulting in a low occurrence of resistance *in vitro*, an advantage for the use of *Hs*AFP1 as a novel antifungal agent (Aerts et al., 2011). Using sodium azide, a respiratory inhibitor, mitochondrial function is impaired and *Hs*AFP1 antifungal activity is affected, indicating that the defensin requires a properly working respiratory chain. This defensin induces ROS formation and apoptosis in yeast. It was also proposed that mitogen-activated protein kinase (MAPK) signaling pathways may

[4]http://www.apsnet.org/

be a possible strategy for yeast tolerance to *Hs*AFP1 (Aerts et al., 2011).

ANIMAL SOURCES

Mammal defensins

Antimicrobial peptides from animal sources have shown antifungal and immunomodulatory activities, being mammals major producers of defensins (Yang et al., 2002a,b; Sahl et al., 2005). θ-Defensins are the less studied defensin family, at least partially due to their source. To date, no antifungal activity was attributed to θ-defensins; as such, these defensins will not be further discussed in the present review. Being vertebrates, mammals possess an adaptive immune system, hence having a more complex network of signaling pathways, diverse responses against pathogens invading the organism and an array of AMPs produced in different organs and tissues, each with its particular function and mode of action (Ganz and Lehrer, 1998; Ganz, 2004; Lai and Gallo, 2009; Pasupuleti et al., 2012). Human β-defensins 1 and 2 are chemotactic for memory T cells and immature dendritic cells (Pazgier et al., 2006). Mammal defensins differ substantially in their antimicrobial specificities. For example, HNP-1, HNP-5 and human beta-defensins 1 and 3 (HBD1 and HBD3, respectively) have broad antimicrobial activities against Gram-negative and Gram-positive bacteria and yeasts (Ganz et al., 1985; Bensch et al., 1995; Porter et al., 1997; Harder et al., 2001; Hoover et al., 2003; Joly et al., 2004). HBD1 and HBD3 have been shown to be effective against *C. albicans* (Krishnakumari et al., 2009), while HBD2 has been shown to possess significant microbicidal activity against Gram-negative bacteria and *C. albicans* (Schroder and Harder, 1999). Recombinant human intestinal defensin 5 (rHD-5) exhibits microbicidal activity against *Listeria monocytogenes*, *Escherichia coli*, and *C. albicans*. Opposed to cryptdins, the mouse intestinal defensins, rHD-5 is active against both mouse-virulent wild-type *Salmonella typhimurium* and its isogenic, mouse-avirulent *pho*P mutant (Porter et al., 1997).

Mouse β-defensin 3 (MBD3), a HBD2 homolog, is an AMP expressed in the mouse epithelial and mucosal tissues (Jiang et al., 2010). The fungicidal properties of recombinant MBD3 suggest that similar peptide formulations can be used in the treatment of fungal and/or bacterial infections. MBD3 is expressed in footpads, skin, and mucosal membranes (tongue) of normal mice. Potent antifungal activity was observed against filamentous fungi, such as *Aspergillus fumaricus*, *Microsporum canis*, *Trichophyton rubrum*, *Trichophyton tonsurans*, and *Trichophyton violaceum* (all these species are primary human pathogens, meaning they cause infection whether or not the immune defenses are weakened, as opposed to opportunistic fungal infections), as well as yeast strains like *C. albicans* and *Cryptococcus neoformans*. This peptide also presents bactericidal activity against Gram-positive and Gram-negative bacteria, such as *Staphylococcus aureus*, *E. coli*, and *Salmonella typhi* (Jiang et al., 2010).

Arthropods defensins

Coprisin. This 43 amino acid residues beetle defensin-like peptide was described in 2009 as an antibacterial peptide (Hwang et al., 2009). Its structure comprises an αβ-motif, stabilized by three cysteine disulfide bridges (Lee et al., 2013). In 2012, the same authors investigated its antifungal activity against *C. albicans*, revealing that coprisin enters the fungal cell and localizes in the nucleus, which indicates that coprisin penetrated the membrane without disrupting the fungal plasma membrane, as confirmed with 1,6-diphenyl-1,3,5-hexatriene (DPH) analysis, calcein-leakage, and giant unilamellar vesicle assays (Lee et al., 2012). Using H_2O_2 as a positive control for apoptotic induction, coprisin proved to have the same effects in inducing early and late apoptosis, features shown by the annexin V conjugated with fluorescein and propidium iodide co-staining method. Apoptosis induced by this AMP is metacaspase-dependent. Concomitantly, coprisin compromises mitochondrial membrane potential and ROS production, in addition to the release of cytochrome *c* from the mitochondria to the cytosol. No hemolytic activity was observed for this peptide in human erythrocytes (Lee et al., 2012).

Juruin. This defensin-like peptide was discovered in 2012 by screening the venom on the theraposid Amazonian pink toe spider *Avicularia juruensis* (Ayroza et al., 2012). It has 38 amino acid residues, three disulfide bonds and, like neurotoxins reported to have antimicrobial activity, it has a putative inhibitory cysteine knot (ICK) motif, i.e., a fold common to venom peptides from spiders, scorpions, and aquatic cone snails (Smith et al., 2011). ICK-containing peptides of spider venom are likely to have evolved from β-defensins (Fry et al., 2009). Based on amino acid sequence and structure similarities with insecticidal peptides of other spiders, this peptide is likely to belong to a group of conserved toxins with voltage-gated ion channels inhibitory action. Juruin showed a fungicidal rather than fungistatic effect against *C. albicans* and *C. tropicalis*, without hemolytic activity (Fry et al., 2009).

Reptile defensins

Crotamine. This highly basic peptide, isolated from the venom of a South-American rattlesnake, was discovered in 1947 (Gonçalves and Polson, 1947). It shares structural similarity to β-defensins due to an identical disulfide bridge pattern (Fadel et al., 2005; Yamane et al., 2013). Crotamine structure comprises an antiparallel β-sheet and an α-helix stabilized by three disulfide bridges (Nicastro et al., 2003; Fadel et al., 2005). Recombinant crotamine displayed a more potent antimicrobial activity than native and synthesized crotamine (Yamane et al., 2013). This peptide induces extensive ultrastructural modifications in *C. albicans*. TEM studies showed deformed cell shape, irregular layering structure of cell wall and cytoplasmic contents coagulation, but without detectable hemolytic effects and low toxicity to mammalian cells (Yamane et al., 2013).

IMMUNOMODULATORY FUNCTION

Defensins may be produced constitutively or have their expression triggered when there is an inflammatory process, by the recognition of microbial conserved structures, such as lipopolysaccharide (LPS) and lipoteichoic acid, or inflammatory effectors, like cytokines. These AMPs are expressed differentially depending on the peptide itself and on the tissue or cell type (Ulm et al., 2012). Defensins, besides their antimicrobial action, can also be immunomodulatory and inhibitors of virulence factors. This ability is not exclusive of defensins, as other AMPs also

share this property. Thus, they can enhance the host's immune system, with this multifunctional character rendering these peptides lower probability of becoming tolerated by microorganisms (Mehra et al., 2012; Jarczak et al., 2013).

Pro-inflammatory mediation has been recognized in some of these molecules, as they can bind to chemokine receptors, being able to recruit immune cells, thus enhancing the immune response (Mookherjee and Hancock, 2007; Lai and Gallo, 2009; Alba et al., 2012; Semple and Dorin, 2012; Ulm et al., 2012; Zhu and Gao, 2013). β-Defensins were demonstrated to have the capability to induce chemoattraction of $CD4^+$ memory T cells, macrophages, and immature dendritic cells, by binding to receptors in the membrane (Yang et al., 1999; Wu et al., 2003; Taylor et al., 2008). This binding favors the attraction and migration of inflammatory cells to the inflammation site, in order to improve and speed up the inflammatory response. α and β-Defensins have also been shown to inhibit neutrophil apoptosis (Nagaoka et al., 2008). These authors showed that HBD3 binds to CCR6 at the neutrophil cell surface, initiating an increase in the levels of the antiapoptotic protein Bcl-xL and inhibiting caspase activity. This increases neutrophils life span and is an inflammatory event that is beneficial to eradicate invading microorganisms (Nagaoka et al., 2008), thus promoting the production of proinflammatory cytokines and chemokines, which in turn, amplifies the immune system response. Defensins have been shown to have a proinflammatory effect on human keratinocytes (Niyonsaba et al., 2007). Treatment of these cells with HBD2 HBD3 or HBD4 leads to the increase of the expression of proinflammatory mediators, like monocyte chemoattractant protein-1, macrophage inflammatory protein-3, and some interleukins (Niyonsaba et al., 2007).

Surprisingly, some defensins are also able to attenuate proinflammatory responses whenever these can be harmful to the organism (Lande et al., 2007; Yamasaki et al., 2007). These antagonistic effects depend on the level of expression, disease state, and pathogen exposure. It has been previously described for α-defensins that mice having a matrilysin deficiency (hence without mature α-defensins in the intestine) are more susceptible to chemically induced colitis than wild-type controls. Interleukin-1β (IL-1β), a cytokine with an important role in mediation of inflammation, reaches level significantly increased in the deficient mice and it was ultimately shown that α-defensins are able to inhibit the production of IL-1β (Shi et al., 2007).

It has been demonstrated that HBD3 (mainly expressed in epithelial cells), when in basal concentration, has an immunosuppressive effect in the presence of LPS, contributing to the maintenance of a non-inflammatory environment over continual low-level exposure to microorganisms, commensal or pathogenic (Semple et al., 2010). Concentrations of HBD3 ranging from 0.5 to 1 μM are able to suppress the induction of tumor necrosis factor α (TNFα), a proinflammatory effector of the immune system, and IL-6, an interleukin that acts both as pro and anti-inflammatory. At these concentrations, proinflammatory proteins are not induced and there is no proinflammatory gene expression (Semple et al., 2010). The proinflammatory effects of β-defensins were observed at slightly higher concentrations of the defensin, in the 4–6 μM range (Funderburg et al., 2007; Niyonsaba et al., 2010). This was not the first case observed of opposite effects in immunomodulating AMPs. Cathelicidin LL-37 has been shown to have also a duality in inflammatory effects, being proinflammatory at concentrations above 20 μg/ml but anti-inflammatory at 1–5 μg/ml (Scott et al., 2002). Defensins were also shown to have a role in other biological processes, namely wound healing (Hirsch et al., 2009), dog coat color determination (Candille et al., 2007), fertility (Li et al., 2001), plant development (Stotz et al., 2009), and carcinogenesis regulation (Donald et al., 2003; Gambichler et al., 2006; Joly et al., 2009).

It is clear that defensins have many functions that are determined by the level of expression. Whereas higher expression of defensins takes place at the pathogen's site of entry, with a proinflammatory response and the chemoattraction of macrophages and other immune cells, defensins expressed at lower levels may be involved in the resolution of the immune response. When the danger is neutralized and defensins and other proinflammatory molecules decrease in the inflammation site, defensins may then have a role in resolving inflammation (Semple and Dorin, 2012).

Due to this multifunctionality, AMPs have also been referred to as host defense peptides (HDPs; Steinstraesser et al., 2010; Ulm et al., 2012).

RESISTANCE

Like other antibiotics resistance, it is easily conceivable that AMPs resistance is a key characteristic for increased virulence of pathogenic strains. Despite this fact, and contrary to antibiotics that act through a single approach (meaning that microbes can evade them through a single resistance system), AMPs follow a multidimensional strategy against microbial invasion (Lai and Gallo, 2009). Therefore, selective pressures on microbes are avoided, reducing the development of resistant strains (Zasloff, 2002).

A synergistic effect between different host AMPs is also possible, as evidenced by the fact that the MIC of AMPs *in vitro* are usually higher than the physiological concentrations of those AMPs *in vivo* (Lai and Gallo, 2009). Two distinct AMPs may have their combined MIC much lower than when acting isolated, strongly suggesting heterologous HDP interactions (Westerhoff et al., 1995).

Microorganisms have evolved their own strategies for evading the antimicrobial action of the compounds used against them. AMPs frequently have the ability to disrupt microbial membranes and to inhibit the synthesis of some of their components; thus, strategies to escape the action of those AMPs follow the redesign of cell membranes, as described for both Gram-negative and Gram-positive bacteria (Gunn et al., 2000; Li et al., 2007). Other evasion mechanisms include affecting the correct function of the AMP by turning off its expression, releasing plasmid DNA in epithelial cells, a strategy adopted by highly contagious bacteria from the *Shigella* genus that cause dysentery (Islam et al., 2001). As AMPs frequently rely on transmembrane potential to interact with microbial pathogens and exert their mechanism of action against them, it is probable that another microbe strategy for evading AMPs could be to change their transmembrane potential status (Yeaman and Yount, 2003).

Candida albicans resistance to some AMPs is regulated by the protein Ssd1, combined with the transcription factor Bcr1 (biofilm and cell wall regulator; Nobile and Mitchell, 2005; Gank et al., 2008; Jung et al., 2013). Ssd1 is an RNA-binding protein and a component of the regulation of morphogenesis pathway (Saputo et al., 2012). In *C. albicans*, this pathway governs multiple processes, including filamentation and cell wall integrity (Song et al., 2008; Bharucha et al., 2011). This combination yields resistance to protamine, RP-1 and HBD2 by maintaining mitochondrial energetics and reducing membrane permeabilization, thus allowing the fungus to counteract the negative effects of these AMPs (Jung et al., 2013). Protamine is an α-helical cationic polypeptide, frequently used to screen for AMP susceptibility (Yeaman et al., 1996), and RP-1 is a synthetic AMP modeled upon the C-terminal α-helical domain existent in the human platelet factor-4 kinocidins; this domain is responsible for RP-1 microbicidal activity (Bourbigot et al., 2009). *C. albicans* mutant strains in Bcr1 and Ssd1 proteins are more susceptible to the AMPs described above; thus, Jung et al. (2013) were able to conclude that these proteins are necessary for the resistance to protamine, RP-1 and HBD2. Further studies are necessary to clarify the roles of Bcr1 and Ssd1 in early *versus* late mechanisms of resistance to AMPs.

The Hog1 (high osmolarity glycerol) MAPK pathway, which provides a response to osmotic, oxidative, and heavy-metal exposure stresses in fungal cells, was shown to be activated in the presence of AMPs, such as *Na*D1, HBD2, HBD3, and histatin-5 (a salivary cationic AMP that has a role in keeping *C. albicans* in its commensal state; Yeaman et al., 1996; Vylkova et al., 2007; Argimon et al., 2011; Hayes et al., 2013). The injuries imposed on *C. albicans* by these defensins seem to share common features with osmotic and/or oxidative stress (Argimon et al., 2011). Upon exposure to these defensins, the Hog1 MAP kinase is activated, triggering a transcriptional response aimed to rescue the cells from the source of injury, i.e., the core and osmotic-stress transcriptional responses (Enjalbert et al., 2006; Argimon et al., 2011).

Another strategy for evading AMP function is to enzymatically degrade these peptides before they exert their effects. This is possible by producing proteases and peptidases involved in tissue degradation, as described for *C. albicans* secreted aspartic proteases (Saps). Namely, histatin-5, present in human saliva, is a host-specific substrate of Sap9, enabling the transition of the fungus from commensal to pathogenic in HIV^+ individuals. These patients, who have lower levels of this isoenzyme in the saliva, have an increased incidence of oral candidiasis (Meiller et al., 2009; Khan et al., 2013). Also regarding histatin-5, a transport mechanism of efflux mediated by the flu-1 transporter has been described for *C. albicans*, rendering the pathogen the ability to reduce the isoenzyme cytosolic concentration and fungicidal activity (Li et al., 2013). The LL-37 cathelicidin and histatins bind to cell wall carbohydrates, preventing adhesion of *C. albicans* to host cells; thus, the release of AMP-binding proteins acts as a decoy for these AMPs, diverting them from binding to fungal cell surface (den Hertog et al., 2005, 2006; Mochon and Liu, 2008). For example, Msb2 (multicopy suppressor of a budding defect) is a *C. albicans* surface protein (a mucin) highly soluble and proteolytically stable, which is shed to the extracellular environment, acting as a basal AMP-resistance decoy by binding to LL-37 and histatin-5, avoiding the antimicrobial action of these AMPs (Szafranski-Schneider et al., 2012).

The characteristics described above are associated with a decrease in microbes' susceptibility to AMPs, indicating that microbial pathogens have developed some structure-specific and energy-dependent mechanisms to subvert the action of these host defense systems.

FUNGAL CELL MEMBRANE

Fungi possess a unique cell wall and cell membrane that can serve as specific targets for antifungal agents. The fungal cell membrane is similar to those of other eukaryotic cells, composed of a lipid bilayer with proteins embedded within it (Katzung et al., 2011). Sterols (absent in prokaryotes) are major components of fungal membranes. The sterol present in higher eukaryotic membranes is cholesterol, but in fungal membranes the main sterol present is ergosterol, providing stability and flexibility to the cell membrane (Thevissen et al., 1999, 2003a).

Glycosphingolipids (GSLs) are a family of lipids that act as key components of biological membranes. They exist in animals, plants, and fungi (Leipelt et al., 2001; Halter et al., 2007; Daniotti and Iglesias-Bartolome, 2011). GSLs were initially described as components of the architecture of cell membranes, straightly connected with fluidity and stability (Feinstein et al., 1975; Aaronson and Martin, 1983; Campanella, 1992). Recently, however, it was demonstrated that their role goes clearly beyond the initial concept, since these molecules are major components of specialized membrane domains called lipid rafts (Bagnat et al., 2001; Hakomori, 2003, 2008). GSLs have been characterized as important structures in cell–cell interaction, cell signaling, and protein sorting (Bagnat et al., 2000; Bagnat and Simons, 2002; Nimrichter et al., 2008; Staubach and Hanisch, 2011). Lipid rafts are more ordered and tightly packed than the surrounding bilayer, serving as organizing centers for the assembly of signaling molecules, influencing membrane fluidity and membrane protein trafficking (Chiantia and London, 2013).

The most common GSL found in fungi is GlcCer, present in the membranes of most fungi, such as *Pichia pastoris*, *C. albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Sporothrix schenckii* and *Neurospora crassa* (Saito et al., 2006). Large amounts of this GSL have also been found in the fungal cell wall (Nimrichter and Rodrigues, 2011). GlcCer has been identified as a fungal component decades ago. Its functions during fungal growth/dimorphism, lipid raft formation, and correlation with virulence have been reported (Rittershaus et al., 2006). In fact, it was recently shown to be required for virulence in *C. albicans* (de Medeiros et al., 2010; Noble et al., 2010; de Coninck et al., 2013).

Work published by Thevissen and colleagues strongly suggested that fungal GlcCer targeting by the AMPs *Rs*AFP2 and *Hs*AFP1 could initiate a cell signaling response in fungi, with formation of ROS and subsequent cell death by apoptosis (Thevissen et al., 2004; Aerts et al., 2007, 2009, 2011). The use of anti-GlcCer antibodies was shown to block germ tube formation in *C. albicans*, *Colletotrichum gloeosporioides*, and *Pseudallescheria boydii* (Pinto et al., 2002; da Silva et al., 2004), and also to protect mice upon

the potentially lethal infection by *C. neoformans* (Rodrigues et al., 2007).

The crescent knowledge of GlcCer functions in eukaryotes (may these be related to virulence, growth or morphological transitions), together with the findings described above, can be connected to specific and essential structural features and particular biosynthetic steps to validate this GSL, as well as other fungal specific membrane lipids and sterols, as potential targets on the development and discovery of new antifungal drugs (Nimrichter and Rodrigues, 2011; Gonçalves et al., 2012b). Besides GlcCer, fungal membranes are also rich in phosphomannans and in the negatively charged phospholipids PS, phosphatidylinositol (PI) and diphosphatidylglycerol (DPG), which confer a highly negative surface charge to these membranes (Pasupuleti et al., 2012).

MODELS OF MEMBRANE ACTIVITY – MECHANISM OF ACTION

The biological activity of AMPs is strongly influenced by peptide–membrane interactions. To explain how some AMPs show differential membrane affinity, their biological activities, and modes of action have been assessed on studies of defensins interaction with fungal membrane model systems, which showed a strong dependence on membrane lipid composition and on the concentration of specific components (de Medeiros et al., 2010; Gonçalves et al., 2012a,b). As with other AMPs, the mechanisms of action of some plant defensins with antifungal activity involve membrane binding, binding to the cell wall, interaction with intracellular targets leading to apoptosis, membrane permeabilization, and receptor-mediated internalization (van der Weerden et al., 2013).

The mechanisms of action of some defensins have been studied by using synthetic lipid vesicles mimicking the lipid composition of fungal, bacterial and mammal membranes (de Medeiros et al., 2010; Wimley and Hristova, 2011; Gonçalves et al., 2012a,b). The permeabilization models used to explain the mode of action of defensins could be classified into two main groups: transmembrane pore formation, such as the barrel-stave and toroidal models, and non-pore formation, such as the carpet, aggregate channel, Shai–Matsuzaki–Huang, lipid clustering, and interfacial activity models (Alba et al., 2012). The carpet model can evolve to disrupt the membrane through pore formation models or through a detergent-like mechanism, with partial micellization of the membrane (Bechinger and Lohner, 2006; Chang et al., 2008; Hoskin and Ramamoorthy, 2008). There are currently at least three different commonly accepted models describing possible AMPs mode of action: the barrel-stave pore model, the toroidal pore model, and the carpet model (Shai, 2002; Chang et al., 2008; Hoskin and Ramamoorthy, 2008; Alba et al., 2012).

Most defensins are amphipathic molecules with clusters of positively charged amino acid residues side chains and hydrophobic amino acid side chains (Lehrer and Lu, 2011). This structural behavior allows them to interact with microbial membranes both at the level of the negatively charged phospholipid head groups and of the hydrophobic fatty acid chains. The orientation of the peptide on the membrane surface depends on the specific peptide–lipid system, but it is common for the AMP to stay at the membrane interface until a threshold peptide concentration is reached (Yang et al., 2000; Yount and Yeaman, 2005; Pasupuleti et al., 2012). In the barrel-stave model (**Figure 2**), once the critical threshold concentration of peptide is reached, peptides self-aggregate in the membrane resulting in the formation of a transmembrane pore lined by peptide, which dissipates proton and ionic gradients (Ehrenstein and Lecar, 1977; Reddy et al., 2004), but the membrane thickness and homogeneity do not change (Chang et al., 2008). The toroidal pore model is a variant of the barrel-stave model, claiming that, at some critical peptide concentration, curvature strain induces membranes to curve inward, resulting in the formation of a pore that is lined by both peptides and lipid headgroups (**Figure 2**). Toroidal pores seem to have varying lifetimes and longer-lived pores may have a lethal effect similar to barrel-stave pores, with dissipation of proton and ion gradients. This type of mechanism of AMPs action also causes a decrease in membrane thickness and a slightly decreased surface homogeneity (Chang et al., 2008). In the carpet model (**Figure 2**), peptides bind to phospholipid head groups by electrostatic interactions and align themselves parallel to the membrane surface in a carpet-like fashion until a critical threshold concentration is reached. When a detergent-like membrane micellization takes place, a strong decrease of membrane homogeneity occurs (Chang et al., 2008; Hoskin and Ramamoorthy, 2008; Epand et al., 2010; Hazlett and Wu, 2011; Li et al., 2012; Pasupuleti et al., 2012).

Besides targeting fungal membranes' specific components, defensins may also have other mechanisms of action, as previously referred. These mechanisms comprise binding to the cell wall, membrane permeabilization, receptor-mediated internalization inducing signaling cascades and interaction with intracellular targets, which would cause the formation of ROS, leading ultimately to programed cell death. To address these mechanisms the reader is directed to some relevant references on this topic (Hancock and Rozek, 2002; Oberparleiter et al., 2003; Thevissen et al., 2003a, 2004; Schroeder et al., 2011; de Coninck et al., 2013; De

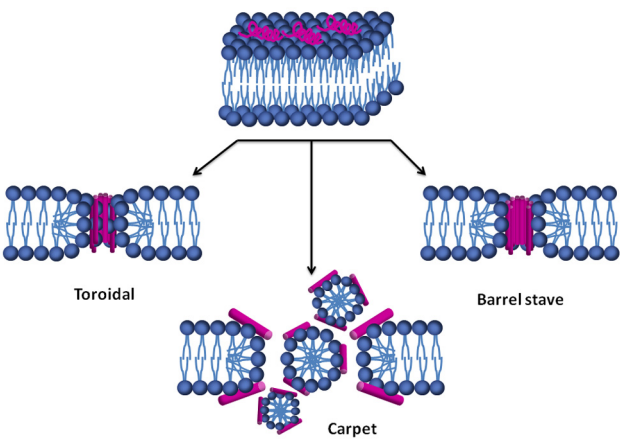

FIGURE 2 | Models of lipid membrane permeabilization by AMPs. Initially, the peptide (magenta) is adsorbed at the membrane surface. After an initial recognition of the surface, a conformational change of the peptide occurs. Once a threshold concentration of peptide on the membrane is reached, it is followed by membrane disruption by one of these three mechanisms.

Paula et al., 2013; Jaeger et al., 2013; van der Weerden et al., 2013; Zhang et al., 2013).

CONCLUSION

The knowledge on AMPs has been increasing considerably during the last 20 years. This increased knowledge shows that AMPs have much more than only antimicrobial activity, presenting a broad spectrum of physiological functions. Defensins are the most represented AMPs across the eukaryotic domain, and in all types of eukaryotic organisms we can find defensins not only with antifungal activity but also with other potential applications.

Despite this relevance, defensins may have limitations in terms of new drug development, due to their cationic, amphiphilic, and protease labile nature, leading to a low serum half-life that limits their systemic administration (Maisetta et al., 2008). This limitation can be overcome by the use of peptidomimetics, like the substitution of natural occurring L-amino acid residues by D-amino acid residues or unusual amino acids (Oren et al., 1997; McPhee et al., 2005). Defensins bare a favorable characteristic against this problem, as their disulfide-stabilized structure confers increased protease-resistance (Wu et al., 2003). Nonetheless, defensins combine targeted antimicrobial activity with the capacity to positively modulate the immune system, and have proven to be effective across life evolution, making these peptides highly appealing as an anti-infective strategy.

Defensins have evolved as successful barrier of defense not only against bacteria, but also pathogenic fungi, present among plants, animals, and fungi. This ability may serve as a "lesson" on how selective pressures that shape organisms and their components served and continue to serve as a lever for the evolution of better defenses. Most antibiotics used nowadays are from bacterial origin or synthetic (Clardy et al., 2009; Fisher et al., 2012). The molecular design and synthesis of new molecules inspired on defensins or on other AMP structures and sequences seem to be a promising approach to develop a new and extensive field of applications, ranging from antimicrobial therapy, to their possible use as vaccine adjuvants. Therefore, a better understanding of function and mechanism of action of HDPs, specially defensins, is highly relevant for the development of new anti-infective and immunomodulatory therapeutics (Guaní-Guerra et al., 2013).

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Antifungal defensins and their role in plant defense

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Since the beginning of the 90s lots of cationic plant, cysteine-rich antimicrobial peptides (AMP) have been studied. However, Broekaert et al. (1995) only coined the term "plant defensin," after comparison of a new class of plant antifungal peptides with known insect defensins. From there, many plant defensins have been reported and studies on this class of peptides encompass its activity toward microorganisms and molecular features of the mechanism of action against bacteria and fungi. Plant defensins also have been tested as biotechnological tools to improve crop production through fungi resistance generation in organisms genetically modified (OGM). Its low effective concentration towards fungi, ranging from 0.1 to 10 μM and its safety to mammals and birds makes them a better choice, in place of chemicals, to control fungi infection on crop fields. Herein, is a review of the history of plant defensins since their discovery at the beginning of 90s, following the advances on its structure conformation and mechanism of action towards microorganisms is reported. This review also points out some important topics, including: (i) the most studied plant defensins and their fungal targets; (ii) the molecular features of plant defensins and their relation with antifungal activity; (iii) the possibility of using plant defensin(s) genes to generate fungi resistant GM crops and biofungicides; and (iv) a brief discussion about the absence of products in the market containing plant antifungal defensins.

Keywords: plant defensins, antifungal, phytopathogens, peptide structure, peptide function, transgeny

INTRODUCTION

Plants are constantly exposed to several pests and pathogens in nature. They have developed complex defense mechanisms to protect themselves against the attack of pathogens (Gachomo et al., 2003, 2010). To circumvent these occurrences, defense factors are produced, including, hydrogen peroxide, phenolics, terpenoids, alkaloids, polyacetylenes, and a diverse array of pathogenesis-related (PR) defense proteins (Broekaert et al., 1997; Garcia-Olmedo et al., 1998; Osbourn, 1999; Van Loon et al., 2006; Benko-Iseppon et al., 2010) and plant defensins (Terras et al., 1995).

Defensins are small cationic peptides of 45–54 amino acid residues with a conserved signature of cysteines, which can form three to four disulfide bridges. Plant defensins exhibit a conserved tertiary structure that consists of a triple-stranded antiparallel β-sheet and one α-helix that are stabilized into a compact shape by the disulfide bridges. These bridges form a cysteine-stabilized α-helix β-sheet motif (CSα/β) (Kobayashi et al., 1991; Zhu et al., 2005). In addition to the CSα/β motif, two additional conserved motives, named α-core, encompassing the loop connecting the first β-strand to the α-helix, and the γ-core containing the hairpin loop that links β-strands 2 and 3 (Lβ2β3) were also present in the defensin structure (Yount and Yeaman, 2004; Yount et al., 2007). Despite the low level of amino acid sequence identity between defensins, their three dimensional structures are remarkably similar between different plant defensins (Pelegrini and Franco, 2005). Variations in the amino acids are reflected by small conformational changes in the tertiary structure that contribute to the broad range of biological activities in these proteins. Only one amino acid substitution can change the spectrum of activity exhibited by these peptides (Carvalho and Gomes, 2011).

Since the beginning of 1990s, lots of cationic plant cysteine-rich antimicrobial peptides (AMP) have been studied. Plant defensins were first described in the seeds of wheat (*Triticum turgidum*) and barley (*Hordeum vulgare*) (Colilla et al., 1990; Mendez et al., 1990). They were characterized as a new member of the thionine family due to their similarity in molecular mass, amino acid sequence and number of cysteines. However, subsequent studies performed by Bruix et al. (1995) revealed the existence of differences in the pattern of the disulfide bridges, demonstrating that these two peptide families are unrelated. Broekaert et al. (1995) renamed these peptides as "plant defensins," after comparing their structural and functional resemblance to previously characterized AMPs found in insects and mammals.

DEFENSINS AND THEIR CONTRIBUTION TO PLANT DEFENSE

HOW CAN DEFENSINS HAVE A ROLE IN PLANT DEFENSE?

The role of defensins in the preformed defense of plants is well reported. Several reports show that defensins are an integral part of the plant innate immune system. Most plant defensins already characterized show a constitutive pattern of expression with up regulation in response to pathogen attack, injury and some abiotic stresses (de Beer and Vivier, 2011).

Several features make clear that defensin peptides are involved in plant defense (Selitrennikoff, 2001). Their distribution is consistent with their putative defense role. They have been

identified in leaves, tubers, flowers, pods and seeds, playing an important role in the protection of germinating seeds and developing seedlings (Garcia-Olmedo et al., 1998). In addition, plant defensins are also localized in the xylem, stomata, and stomata cells, parenchyma cells, and other peripheral areas (Kragh et al., 1995; Segura et al., 1998; Chen et al., 2002). The presence in the different tissues is consistent with a defensive role of such peptides, once it is believed that such sites are the place of the first contact with a potential pathogen (Carvalho and Gomes, 2011).

Moreover, plant defensins have a broad spectrum of *in vitro* antimicrobial activity and, currently, there are several reports describing the production of transgenic plants constitutively expressing foreigner defensins. Hence, they possess an enormous multiplicity of biological activities, such as antimicrobial, insecticidal, inhibiting protein synthesis, mediating abiotic stress, and Zn tolerance, and as inhibitors of digestive enzymes (Carvalho and Gomes, 2009, 2011). According to Franco (2011), these defense peptides are classified as promiscuous proteins, as they show numerous biological activities. As an example, there is the family of defensins isolated from *Vigna unguiculata*, in which different homologous forms may act as antifungal, antibacterial, and enzyme inhibitors (Franco, 2011). Although they present multiple functions, the antimicrobial activity of plant defensins is mainly observed against fungi.

Therefore, the present review explores the current knowledge about the structure and mechanism of action of plant defensins with emphasis on its activity against phytopathogenic fungi. Furthermore, we describe the current use of these peptides as biotechnological tools in the production of transgenic plants that could result in the future release of agronomically important crops resistant to various diseases.

STRUCTURAL CONFORMATION AND MECHANISM OF ACTION

Plant defensins present a well-conserved three-dimensional structure composed by a cysteine-stabilized α/β (CSαβ) motif, which forms one α-helix followed by three anti-parallel β-sheets. The amino acid sequence is also quite conserved, especially due to the presence of six to eight cysteine residues, which form three to four disulfide bridges in the sequence of Cys1-Cys8, Cys2-Cys5, Cys3-Cys6, and Cys4-Cys7 (Lay and Anderson, 2005). Nevertheless, plant defensins with five disulfide bonds have been described, such as the peptide from *Petunia hybrida* (PhD1), whose cysteine residues interact in the following order: Cys1-Cys10, Cys2-Cys5, Cys3-Cys7, Cys4-Cys8, and Cys6-Cys9 (Janssen et al., 2003). The additional disulfide bond does not affect the typical three-dimensional structure of the defensin, which is located after the α-helix and the first β-sheet (Janssen et al., 2003).

Furthermore, plant defensins with alternative structures have been identified in the literature, including defensins from *Nicotiana alata* (NaD1), *Petunia hybrida* (PhD1 and PhD2), and ZmESR6 isolated from developing maize kernels. These defensins contain an extra acidic C-terminal prodomain whose function is still unknown, although it has been suggested that it is involved in vacuolar targeting or in eliminating potential detrimental effects caused by the basic nature of the defensin (De Coninck et al., 2013).

As they are peptides consisting of 45–54 amino acid residues, structural studies on crystallography and nuclear magnetic resonance (NMR) have been widely extended during the last few years. Among the peptides with antifungal activity, whose structures have been elucidated, are included the defensins from *Nicotiana alata* (NaD1), *Pachyrrhizus erosus* (SPE10), *Petunia hybrida* (PhD1), *Pisum sativum* (Psd1), *Raphanus sativus* (Rs-AFP1), and *Saccharum officinarum* (Sd5) (Fant et al., 1998; Almeida et al., 2002; Janssen et al., 2003; Lay et al., 2003; de Paula et al., 2011; Song et al., 2011; Van der Weerden and Anderson, 2013; **Figure 1**). An amino acid sequence alignment of antifungal defensins from plants shows that they do not present conservative amino acid sequences, except the cysteine residues and a glycine residue positioned in the second β-sheet (Pelegrini and Franco, 2005; Van der Weerden and Anderson, 2013). According to their structural features, plant defensins show a conserved γ-core signature classified as the dextromeric isoform, which is related to the amino acid sequence conservation of the region $NH_2...[X_{1-3}]$-[GXC] = $[X_{3-9}]$-[C]...COOH (**Figures 1** and **2**). This preservation in the primary sequence gives them a three-dimensional conformation denominated γ-core motif, consisting of two antiparallel β-sheets, with an interpolated turn region. Earlier studies classified plant defensins as belonging to the β-γ-α Group, according to their relative structural γ-core (Yount and Yeaman, 2004). It has been described that the γ-core motif is important for antimicrobial activity in disulfide-stabilized peptides (Yount and Yeaman, 2004), not only for their cysteine content, but especially due to the presence of positively charged residues at the second β-turn of their structure (Fant et al., 1998). This characteristic was first observed when the structure of *R. sativus* defensin 1 (Rs-AFP1) was determined by ^{1}HNMR, and mutation analyzes was also performed using the peptide isoform Rs-AFP2 (De Samblanx et al., 1997; Fant et al., 1998). In both cases, it was demonstrated that positively-charged amino acids located at the γ-core motif were essential for the antifungal activity of theses peptides, and the substitution of neutral residues inside this γ-core by other positively-charged amino acid residues increased their activity towards pathogenic fungi. Spelbrink et al. (2004), while studying defensins from *Medicago trunculata*, verified that the antifungal activity of MtDef1 was due to the presence of four positively-charged amino acids, also located in the γ-core region, which was lacking in the structure of the non-antifungal peptide MtDef2. Moreover, *in vitro* assays revealed that this region might be involved in the ability of MtDef1 to block L-type Ca++ channels in mammalian cells.

There are two major hypothesis that tries to explain the mechanism of action of antimicrobial defensins: (i) the carpet model and (ii) the pore model. In both models, defensins are described to interact with the negatively charged molecules present at the cell membrane of pathogens, causing an increase of its permeabilization, leading to cell leakage and death by necrosis. While the carpet model emphasizes the pore formation of several peptides into the membrane, the pore model shows that peptides form oligomers that, then, form multiple pores into the cell membrane. However, there is an alternative hypothesis, where defensins do not damage

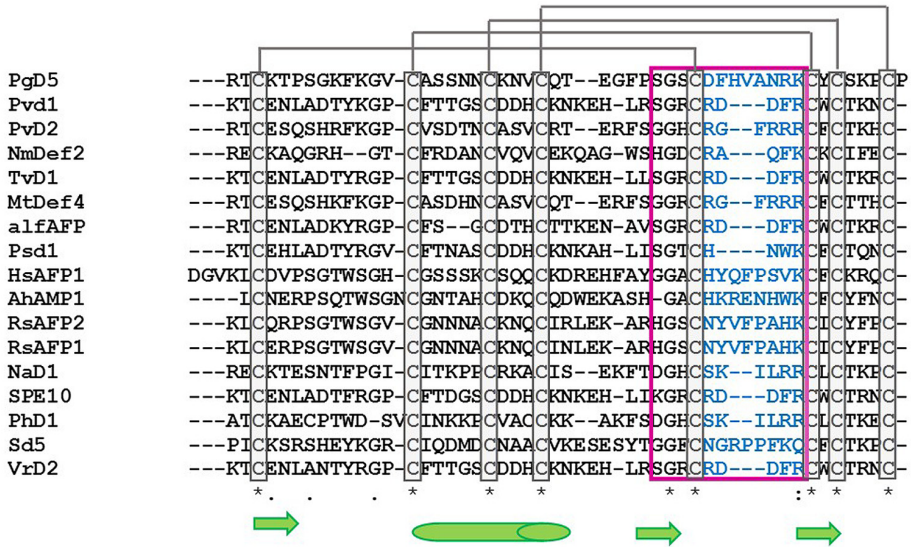

FIGURE 1 | Alignment of the amino acid sequence of antifungal plant defensins. PgD5: *Picea glauca* defensin (Accession: AAR84643); Pvd1: *Phaseolus vulgaris* defensin 1 (Accession: ADR30066); PvD2: *Phaseolus vulgaris* defensin 2 (Accession: ADR3006); NmDef1: *Nicotiana megalosiphon* defensin (Accession: ACR46857); TvD1: *Tephrosia villosa* defensin (Accession: AAX86993); MtDef4: *Medicago trunculata* defensin 4 (Accession: 2LR3_A); alfAFP: *Medicago sativa* antifungal peptide 1 (Accession: AAG40321); Psd1: *Pisum sativum* defensin 1 (Accession: 1JKZ_A); HsAFP1: *Heuchera sanguinea* antifungal peptide (Accession: P0C8Y5); AhAMP1: *Aesculus hippocastanum* antimicrobial peptide 1 (Accession: AAB34970); RsAFP1: *Raphanus sativus* antifungal peptide 1 (Accession: 1AYJ_A); RsAFP2: *Raphanus sativus* antifungal peptide 2 (Accession: P30230); NaD1: *Nicotiana alata* defensin 1 (Accession: 4ABO_A); SPE10: *Pachyrrihizus erosus* peptide (Accession: 3PSM_A); PhD1: *Petunia hybrida* defensin 1 (Accession: 1N4N_A); Sd5: *Saccharum officinarum* defensin 5 (Accession: 2KSK_A); VrD2: *Vigna radiata* defensin 2 (Accession: 2GL1_A). Asterisk indicates conserved cysteine amino acid residues among antifungal defensins (gray boxes). Gray lines represents the disulfide bridges between cysteine amino acid residues. Pink box and blue amino acid residues correspond to the γ-core region. Green arrows indicate β-sheet region and green cylinder indicate α-helix region. Alignment was done using ClustalW2 Tool.

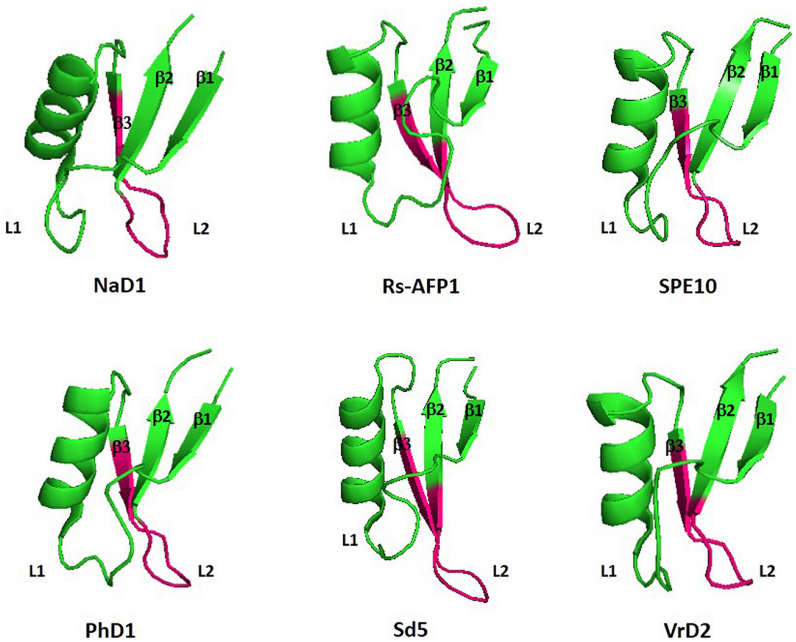

FIGURE 2 | Three-dimensional structure of six antifungal defensins from plants. Pink region highlight the γ-core motif of each peptide. β1: β-sheet 1; β2: β-sheet 2; β3: β-sheet3; L1: Loop1; L2; Loop 2: NaD1: *Nicotiana alata* defensin 1 (Accession: 4ABO_A); Rs-AFP1: *Raphanus sativus* antifungal peptide 1 (Accession: 1AYJ_A); SPE10: *Pachyrrihizus erosu* peptide (Accession: 3PSM_A); PhD1: *Petunia hybrida* defensin 1 (Accession: 1N4N_A); Sd5: *Saccharum officinarum* defensin 5 (Accession: 2KSK_A); VrD2: *Vigna radiata* defensin 2 (Accession: 2GL1_A). All figures were designed using PyMol Molecular Graphic System Version 1.2r3pre, Schrödinger, LLC.

the cell membrane, but interact with the phospholipids, leading to an increase of ion permeability, or even to the transportation of these peptides to the intracellular environment (Wilmes et al., 2011; Hegedus and Marx, 2013). Hence, they can also enhance reactive oxygen species (ROS) and activate programmed cell death (PCD; Wilmes et al., 2011; Hegedus and Marx, 2013).

Moreover, positively charged amino acid residues were described to be important for antifungal activity, when located at loops and β-sheet regions. Hence, it was observed that the concave side of the VI β-turn from Rs-AFP1 was positively-charged, leading to the suggestion that the contact of this peptide with pathogenic fungi may occur through electrostatic interactions (De Samblanx et al., 1997; Fant et al., 1998). Other studies on the structural analyses of plant defensins, such as NaD1, described the importance of positively-charged amino acid residues at the loop region between β2 and β3, not only for antifungal activity, but for also functioning as a specificity factor towards different pathogens (Lay et al., 2003). Recently, it was reported that the amino acid residues located in the γ-core motif of MtDef4 are key tools for its antifungal activity and its specificity towards pathogenic fungi (Sagaram et al., 2011). First, *in vitro* assays using only the γ-core sequence of Mtdef4 and MsDef1 (alfAFP) showed that the high content of positively charged residues with the core of MtDef4 could, alone, provide antifungal activity, in contrary to the core of alfAFP, which was inactive against filamentous fungi (Sagaram et al., 2011). Later, mutagenesis studies on the region RGFRRR from MtDef4 showed that the substitution of the hydrophobic and positively-charged residues, Phe and Arg, at positions 3 and 4, respectively, by Ala residues decreased intensely its activity against fungi. Furthermore, it was shown that both defensins present differences on their kinetics of permeabilization, when assayed against *Fusarium graminearum*, as MtDef4 was able to induce a more potent antifungal activity and could take up the molecular probe SYTOX Green (SG) at a dependent concentration, indicating physical damage of cell membranes. In comparison, alfAFP induced a less effective membrane permeabilization, and did not induce a concentration dependent SG uptake (Sagaram et al., 2011).

Further reports displayed a comparison between the electrostatic potential surfaces of different defensins with their potential antimicrobial activities (Almeida et al., 2002). However, although there was no pattern of charge distribution among defensins, there was a high indication that plant defensins may act as potassium channel inhibitors, due to their similarities with neurotoxins, which contains residues for such activity (Almeida et al., 2002). **Figure 3** shows the electrostatic surface area of three antifungal plant defensins (Phd1, Rs-AFP1, and VrD2), in which the site related to the second loop of the defensins that contains the γ-core region is described as the most important site for their antifungal activity. This is highly positively-charged in the cartoons where electrostatic surfaces were designed in vacuum. Therefore, it corroborates with the antifungal assays and the *in silico* studies performed by many researchers over the last 20 years.

A structural study on sugarcane defensin, Sd5, provided new information about the mechanism of action for those antifungal peptides. It was described that the hydrophobic core at the C-terminal of the defensin is also important for membrane interaction and permeabilization (de Paula et al., 2011). In addition, evaluations on the backbone conformational dynamics of Sd5 suggest that the mechanisms of its structural exchange is related to modifications in the hydrogen bond distances of the β-sheet and α-helix of the peptide, giving it the ability to bind to membranes. Hence, membrane permeabilization and vesicle leakage induced by Sd5 may occur through the interaction of the side chains of residues of three serines and the glycosyl part of the membrane model with glucosylceramide extracted from the hyphae of *F. solani* (de Paula et al., 2011). Recent studies on dynamics of Sd5 structure revealed that this peptide displays many dynamic properties. It was able to interact with a sphingolipid glycosylceramide (CMH) membrane in a conformational selection process, which involved a specific binding, while other flexible regions of Sd5 showed to interact with the interface in a nonspecific manner (Valente et al., 2013).

Recent reports described the structural conformation of dimeric defensins being highly significant for its antifungal activity (Song et al., 2011; **Figure 4**). In this way, analyses of the defensin from *Pachyrrihizus erosus*, SPE10, provided the selection of the binding pattern Arg36-Trp42-Arg40 as essential for dimer formation. Moreover, it was demonstrated that Trp42 is fundamental for antifungal activity of plant defensins, as it is absent in non-antifungal peptides Therefore, dimers of SPE10 are arranged in a side-by-side manner with the α-helix of one monomer interacting with the β-sheet of the second monomer, leading to a stretched

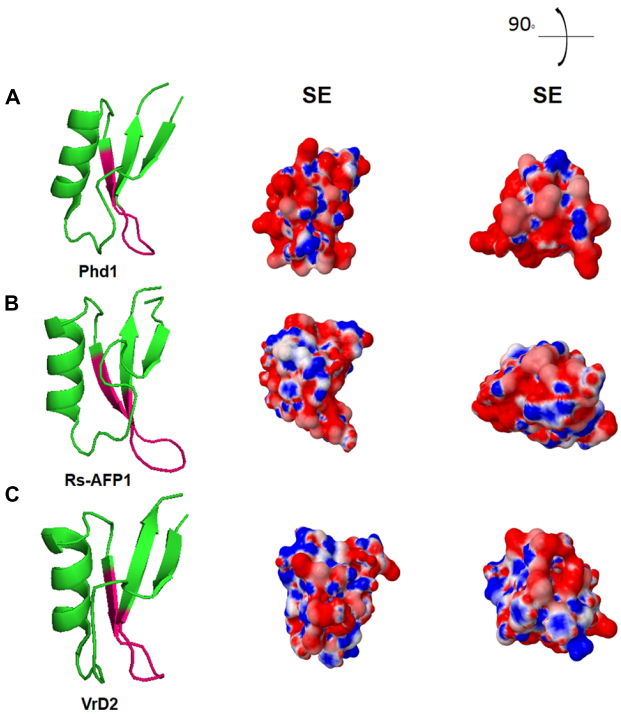

FIGURE 3 | Electrostatic surface of three plant defensins under vacuum environment. Three-dimensional structures of **(A)** Phd1; **(B)** Rs-AFP1; **(C)** VrD2. SE: solvent excluded electrostatic surface. 90° and the illustration and the right top of the figure indicates the angle deviation for new visualization of the peptides structures. All figures were designed using PyMol Molecular Graphic System Version 1.2r3pre, Schrödinger, LLC.

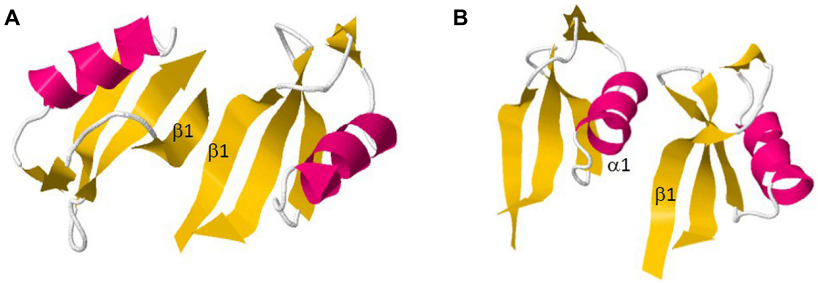

FIGURE 4 | Dimer formation of two plant defensins. (A) NaD1; **(B)** SPE10. All figures were designed using PyMol Molecular Graphic System Version 1.2r3pre, Schrödinger, LLC.

and twisted molecular surface. Conformational changes on Arg36 and Trp42 would alter the dimeric interface of SPE10, destabilizing the dimer (Song et al., 2011). In addition, the dimerization of the defensin NaD1 was performed in order to evaluate the relation between structural conformation and antifungal activity. In contrary to what was observed by SPE10 dimer, monomers of NaD1 were connected by a β-sheet/β-sheet configuration, although the antifungal activity was maintained (Lay et al., 2012; **Figure 4**). Hence, plant defensins that form dimers coupled with their positively charged surface area become highly efficient molecules against pathogenic fungi, as they can strongly interact with the negatively charged glycoproteins located at the fungal cell walls (Lay et al., 2012).

TARGETED FUNGI AND EFFECTIVE CONCENTRATIONS

One of the first studies that attempted to highlight this class of plant antimicrobial defensins was carried out with two peptides isolated from Radish seeds, Rs-AFP1 and Rs-AFP2. Both peptides were assayed against 20 different plant pathogenic fungi and the lower protein concentration required for 50% inhibition of fungal growth (IC_{50}) was obtained by Rs-AFP2, when assayed against *Pyricularia oryzae*. Its IC_{50} ranged from 0.08 to 5 μM. Since that lots of defensins were reporter to show high biological activity in the range of micromolar to nanomolar as will soon be shown. Terras et al. (1992) were the first ones to report the importance of disulfide bonds to defensins stabilization and the role of inorganic ions in its antifungal activity. They also showed how thermostable defensins are, once they found that heating Rs-AFP1 and 2 at 100°C for 10 min did not affect antifungal properties of such molecules. The stability of such molecules is an important feature which allows wondering a wide range of biotechnological applications to plant defensins.

Few years after Terras report, Osborn et al. (1995) increased the knowledge about plant defensins and their effects under fungi. They assayed four AMP isolated from *Aesculus hippocastanum* (Ah-AMP1), *Clitoria ternatea* (Ct-AMP1), *Dahlia merckii* (Dm-AMP1), *Heuchera sanguinea* (Hs-AFP1) against eight different fungi in the presence, or absence, of inorganic ions. The lower IC_{50}, around 0.1 μM, was acquired when Ah-AMP1 was tested towards *Cladosporium sphaerospermum*, *Leptosphaeria maculans*, and *Septoria tritici*. Hs-AFP1 presents the same antifungal activity when assayed against *Septoria tritici*. In all the studies, inorganic ions decreased IC_{50}. When visualized under a microscope, it was possible to see that such antifungal peptides caused distinct morphological changes during germ tube elongation and hyphae development, like multiple hyphae buds or the diminished of the rate of germ tube elongation (Osborn et al., 1995; **Table 1**).

A great number of the earlier studies about the mechanism of action of plant defensins agree on the membrane permeabilization outcome (Thevissen et al., 1996, 1999). More recently, two peptides similar to plant defensins were reported to show such disruption power. The first one, from *Phaseolus vulgaris*, permeabilizes *Mycosphaerella arachidicola* membrane, among other fungi (Wong et al., 2012). The second, from *Picea glauca*, was reported to act on permeabilization of *Verticillium dahlia* membranes (Picart et al., 2012). Membrane permeabilization seems to be just one of a huge variety of mechanism of action for such molecules. While some results point to cellular membranes as the point of action, others suggest intracellular targets (Thevissen et al., 2000).

The use of antifungal peptide genes to generate important agronomical traits resistant to fungal disease have been seen with some skepticism by the biotechnological thinkers. Plant defensins proved to be useful for biotechnological purposes in the year of 2000, when Gao et al. (2000) showed that AlfAFP, an antifungal peptide from *Medica sativa* and active towards *Verticillium dahliae*, was expressed in a transgenic potato, increasing resistance against such filamentous fungus. The IC_{50} of AlfAFP towards *Verticillium dahlia* was determined at 1 μM, around ten times higher than the previous AMP described here (Gao et al., 2000). However, the resistance of transgenic potato expressing AlfAFP towards *Verticillium dahliae* showed to be more effective in greenhouse conditions and in the field than the chemical methods, what make of it a useful choice to plant transformation aiming resistance to phytopathogenic fungi, which will be discussed in detail later in this review.

Almeida et al. (2001) reported the heterologous expression of a *Pisum sativum* defensin (Psd1) in a eukaryotic expression, system based on the methilotrophic yeast *Pichia pastoris*. The high amount of Psd1 produced by *P. pastoris* expression system (13.8 mg/L), allowed investigations about the conformational features between wide type and recombinant form of Psd1 (rPsd1). Besides being active towards filamentous fungi, such as *Neurospora crassa*, Psd1 did not demonstrate any activity against yeasts, even at high (20 μM) concentrations (Almeida et al., 2000). According

Table 1 | Short sample of plant defensins and its IC_{50} concentration against its fungal targets.

Plant defensin	Organism	Target organism	IC_{50} (μM)	Reference
PgD5	*Picea glauca*	*Verticillium dahliae*	0,4	Picart et al. (2012)
Defensin-like peptide	*Phaseolus vulgaris*	*Mycosphaerella arachidicola*	3,9	Wong et al. (2012)
NmDef02	*Nicotiana megalosiphon*	*Fusarium oxysporum*	1	Portieles et al. (2010)
Pdc1	*Zea mays*	*Fusarium graminearum*	0,75	Kant et al. (2009)
Limyin	*Phaseolus limensis*	*Fusarium solani*	8,6	Wang et al. (2009)
TvD1	*Tephrosia villosa*	*Pheaoisariopsis personata*	1,9	Vijayan et al. (2008)
MtDef4	*Medicago truncatula*	*Fusarium graminearum*	0,75	Ramamoorthy et al. (2007)
MsDef1(alfAFP)	*Medicago sativa*	*Fusarium graminearum*	1,2	Spelbrink et al. (2004)
Psd1	*Pisum sativum*	*Neurospora crassa*	2	Almeida et al. (2001)
alfAFP	*Medicago sativa*	*Verticillium dahliae*	1	Gao et al. (2000)
HsAFP1	*Heuchera sanguinea*	*Septoria tritici*	0,1	Osborn et al. (1995)
AhAMP1	*Aesculus hippocastanum*	*Leptosphaeria maculans*	0,1	Osborn et al. (1995)
RsAFP2	*Raphanus sativus*	*Pyricularia oryzae*	0,08	Terras et al. (1992)

to the report, the heterologous expression in *Pichia pastoris* did not significantly affect the defensin conformational features, and all post-translational modifications needed to its activity had been done. One of the small differences between Psd1 and rPsd1 was their N-terminal sequences. rPsd1 kept four amino acids residues from the recombinant signal peptide, and this seemed to be related to the 5-fold decrease on its activity towards *F. solani* and *Aspergillus niger*, in comparison to the wide type peptide. rPsd1 activity towards *N. crassa* was not affected, which suggests distinct modes of action of Psd1 against fungi belonging to different classes (Almeida et al., 2001). Furthermore, the *Pichia pastoris* system was also used to produce the recombinant *Nicotiana megalosiphon* defensin (NmDef02) active against *F. oxysporum* (Portieles et al., 2010).

Plant defensins have also been expressed in prokaryotic system and tested against fungi. TvD1, a defensin from *Tephrosia villosa*, was expressed in *Escherichia coli* and assayed towards *Pheaoisariopsis personata* (Vijayan et al., 2008; **Table 1**). A comparison between the expression of Pdc1, a corn defensin, in yeast and *E. coli* was done and in both cases the peptide kept its antimicrobial activity, however, Pdc1 expressed in yeast (IC_{50} 7.5 μM) was more efficient than when expressed in *E. coli* (IC_{50} 30 μM) in arrest *F. graminearum* growth. The presence or absence of a His-tag also influences its activity, suggesting that defensins are sensible to covalent modifications on its terminal ends (Kant et al., 2009).

Different from some results, which suggest the importance of N-terminus in defensin activity (Almeida et al., 2001), Spelbrink et al. (2004) demonstrated that the major determinant of antifungal activity of a defensin from *Medicago sativa* (MsDef1) resides in the carboxy-terminal region. They evaluated six different defensin chimeras obtained from molecular combinations of MsDef1, active towards *F. graminearum*. They also analyzed MtDef2, a defensin from *Medicago truncatula*, which did not have any activity towards *F. graminearum*. Among the six chimeras, only the ones harboring the MsDef1 portion on the C-terminal displayed some activity against *F. graminearum* (Spelbrink et al., 2004). The divergence among results pointing to C-terminus and to N-terminus as essentials to plant defensin activity, expose the uncertainty about the relation between structure and function of such molecules and even more on its modulation mechanism of activity.

Three years after Spelbrink findings, Ramamoorthy et al. (2007) tried to go a little deeper into the cellular mechanisms of activity modulation using Medicago defensins against *F. graminearum*. They have demonstrated that mutants of *F. graminearum* can react differently to Medicago defensins MsDef1 and MtDef4. *F. graminearum* mutant, whose MAP Kinase cascades were disrupted, were hypersensitive to MsDef1. However, it did not show any difference on its sensitivity to MtDef4. MAP kinase signaling cascades seemed to provide protection towards MsDef1, but not to MtDef4, which suggests that these plant defensins utilize specific signaling pathways to alter fungal growth (Ramamoorthy et al., 2007).

Besides antimicrobial activity, a plant defensin from *Phaseolus limensis* named Limyin and active against *F. solani*, were also reported to show antiproliferative activity towards human tumor cells (Wang et al., 2009), suggesting there are lots of things to be discovered about the cellular targets and mechanisms of action of plant defensins.

Plant defensins encompass a class of biomolecules with the potential to be explored as biotechnological tools towards phytopathogenic fungi, which nowadays, are controlled only by chemicals. The wide natural sources of these molecules and the heterogeneity of their action on different targets allow hundreds of possible biotechnological approaches that, together with their low effective concentration, as shown in **Table 1**, could lead to phytopathogeninc fungi control with less environmental impact.

BIOTECHNOLOGICAL APPLICATIONS AND TRANSGENY

Although there are many transformed plants in the market with additional genes coding to proteins that confer resistance towards herbicides and insect-pests, there is still no transgenic plant available against phytopathogenic fungi, nor even containing

Table 2 | Antifungal defensins from plant sources used for transformation into foreign species.

Peptide	Origin of peptide	Transformed plant	Pathogenic fungi tested	References
Rs-AFP2	Radish	Tobacco	*Alternaria longipes*	Terras et al. (1995)
		Apple	*Fusarium culmorum*	De Bondt et al. (1999)
		Tomato	*Alternaria solani*	Parashina et al. (2000)
			Fusarium oxysporum	
			Phytophtora infestans	
			Rhizoctonia solani	
		Pear		Lebedev et al. (2002)
		Rice	*Magnaporthe oryzae*	Jha and Chattoo (2010)
			Rhizoctonia solani	
Pea defensin	Pea	Canola	*Leptosphaeria maculans*	Wang et al. (1999)
D4E1	Synthetic	Tobacco	*Aspergillus flavus*	Cary et al. (2000)
			Verticilium dalhia	
BSD1	Stamen	Tobacco	*P. parasitica*	Park et al. (2002)
BjD	Mustard	Tobacco	*F. moniliforme*	Anuradha et al. (2008)
			P. parasitica	
		Peanut plants	*Cercospora arachidicola*	
			Pheaoisariopsis personata	
Wasabi defensin	Wasabi	Rice	*Magnaporthe grisea*	Kanzaki et al. (2002)
alfAFP	Alfalfa	Potato	*V. dalhiae*	Gao et al. (2000)
		Tomato	*R. solanacearum*	Chen et al. (2006)
MsDef1	Medicago sativa	Tomato	*F. oxysporum*	Abdallah et al. (2010)

plant defensins as the resistant factor. Nevertheless, several studies describe the efficient activity of antifungal defensins when transformed into different host plants (**Table 2**). Therefore, plant defensins with antifungal activity have become the first molecule for the development of transgenic crops resistant to phytopathogens.

The first attempt to evaluate of transgenic plants containing foreigner antifungal defensin genes was done in tobacco plants expressing Rs-AFP2, a peptide from radish. High levels of peptide expression were observed in the transformed tobacco plants, as well as an increasing resistance towards the phytopathogenic fungus *Alternatia longipes* (Terras et al., 1995). Four years later, the same defensin was used for studies with transgenic apple plants and evaluation against pathogenic fungi species (De Bondt et al., 1999). Hence, after transformation through *Agrobacterium tumefaciens*, the transgenic plants were selected and the expressed peptide was isolated and quantified. *In vitro* assays showed that the recombinant peptide was able to inhibit the germination of *Fusarium culmorum* spores (De Bondt et al., 1999). Tomato lines have also been transformed with Rs-AFP2, generating the increase in their antifungal activity. In this study, leaves of tomato plants overexpressing the radish defensin were extracted and tested against some phytopathogenic fungi, including *Alternatia solani*, *F. oxysporum*, *Phytophthora infestans*, and *Rhizoctonia solani* (Parashina et al., 2000). It was demonstrated that the crude extract of tomato leaves containing the radish defensin could decrease the activity of all the fungi cited above.

Furthermore, in 2002, Rs-AFP2 was again evaluated in transgenic plants, this time using two pear cultivars – Burakovka and Pamyat' Yakoyleva. After transformation, leaves of pear plants were collected for PCR and Western Blot Hybridization analyses. The presence of the foreigner gene and recombinant peptide were detected through the respective techniques, confirming the success of plant transformation (Lebedev et al., 2002). Nevertheless, *in vitro* and *in vivo* assays against pathogen fungi are still to be done in order to check the antifungal activity of transgenic pear plants expressing Rs-AFP2. The most recent work on Rs-AFP2 was published in 2010, when Jha and Chattoo (2010) transformed this peptide into rice (*Oryza sativa* L. cv. *Pusa* basmati 1). The transgenic plants were tested *in vitro* and *in vitro* against *Magnaporthe oryzae* and *Rhizoctonia solani*, the main causes of rice losses in agriculture, revealing that overexpression of Rs-AFP2 can control the rice blast and sheath blight diseases (Jha and Chattoo, 2010).

In addition, other works on transgenic plants expressing an antifungal defensin were published. Hence, it was demonstrated that pea defensins transformed into *Brassica napus* cultivars enhanced their resistance against *Leptosphaeria maculans*, which causes blackleg diseases in plants (Wang et al., 1999). Tobacco plants transformed via *Agrobacterium tumefasciens* and containing a synthetic antifungal gene was also performed. The expressed peptide, named D4E1, provided an increasing resistance of tobacco against *Aspergillus flavus* and *Verticillium dahlia* (Cary et al., 2000). Tobacco was also used for transformation of the stamen

defensin BSD1, where the expressed peptide provided higher tolerance to the plant against the attack of *Phytophtora parasitica* (Park et al., 2002). Transformation of tobacco with the mustard defensin – BjD – once more validated the potential of these peptide-family members as excellent antifungal agents, as transgenic plants displayed improved resistance towards *F. moniliforme* and *Phytophtora parasitica* (Anuradha et al., 2008). More recently, a defensin purified from maize, ZmDEF1, when transformed into tobacco plants, showed increased tolerance against *Phytophtora parasitica* (Wang et al., 2011). Transgenic peanut plants, expressing the same mustard defensin, also provided an enhancement of tolerance against *Cercospora arachidicola* and *Pheaoisariopsis personata*, which mutually cause the late leaf spot disease (Anuradha et al., 2008).

Kanzaki et al. (2002) performed a successful attempt of expressing a defensin from *Wasabia japonica* into rice plants, as an effort to increase the plant resistance against the phytopathogenic fungus *Magnaporthe grisea*. Moreover, they showed that T3-generation transformed rice plants could still overexpress the wasabi defensin and maintain its ability to control *Magnaporthe grisea in vivo*. Earlier, it was demonstrated that transgenic potato expressing an antifungal defensin from alfalfa (alfAFP) was more resistant to the attack of *Verticillium dahliae*, when compared to non-transformed plants (Gao et al., 2000). A summary of information of expressed plant defensins into plant cultivars can be seen at **Table 2**.

An attempt at transforming two different genes at the same time in tomato plants was performed using genetic material of a defensin and a glucanase from alfalfa, in order to analyze their efficiency towards phytopathogenic fungi. Therefore, T1-generation transgenic plants revealed enhanced tolerance to *R. solanacearum*, when compared to non-transformed plants, indicating the existence of a synergic effect of both proteins as antifungal molecules in tomato cultivars (Chen et al., 2006). Further efforts using other plant defensins into transformed tomato plants were carried out. In this way, Abdallah et al. (2010) inserted the *Medicago sativa* defensin gene into *Licopersicum esculentum* cultivar CastleRock and evaluated the transformed plants against the pathogenic fungus *F. oxysporum* f. sp. *Lycopersici. In vivo* assays demonstrated that T1- and T2-generations of transgenic tomato plants presented increased resistance against the fungal pathogen, when compared to non-transformed plants.

Plant defensins have also displayed indirect responses towards phytopathogenic fungi in transgenic plants, when other foreigner genes are being overexpressed (Murad et al., 2007). Hence, earlier reports showed that a peptide from *Arabidopsis thaliana*, named AtPep1 stimulated the transcription activation of the defensin gene *pdf1.2* (Huffaker et al., 2006). When AtPep1 precursor gene PROPEP1 was expressed into transgenic *Arabidopsis* plants, the transcription of PDF1.2 was also observed. Moreover, the expressed defensin stimulated root development, which, consequently, improved plant resistance against the filamentous fungus *Pythium irregular* (Huffaker et al., 2006).

Similar results were obtained when an ionotropic glutamate receptor (RsGluR) was transformed into *Arabidopsis* plants. The expression of RsGluR led to an up-regulation of defensins, causing an increase of the plant resistance towards *Botrytis cinerea* (Kang et al., 2006). Microarray analyses later confirmed that up-regulated defensins and jasmonic acid-responsive genes were produced after overexpression of RsGluR in *Arabidopsis*. Furthermore, the same plant species was transformed with a cotton non-symbiotic hemoglobin for tolerance against fungal pathogens. However, the foreigner gene could also induce a constitutive expression of the PR protein K (PR-1) as well as the defensin PDF1.2, providing an enhanced resistance to *Verticillium dahliae* (Qu et al., 2006).

CONCLUSION AND PERSPECTIVES

Plant defensins correspond to a world of possibilities for defense mechanisms, and new peptides with different activities are still to be discovered, as well, studies with thousands of plant species need to be performed. Nowadays, several peptides already show satisfactory efficacy against such pathogens with strong potential to be applied for the production of a commercial fungicide or application into transgenic plants. But, the question remains. Why is there still no product containing antifungal plant defensins – in its natural form or in nanocapsules – already available in the market?

It is interesting that plant defensins with antifungal peptides are mostly studied for pathogens located in tropical areas, including Latin American, African, and some Asian countries. Moreover, the loss of commercially important crops due to the attack of phytopathogenic fungi is considered worldwide, until now, less detrimental than the losses caused by drought stress and insect-pests. Therefore, the efforts focused on the release of novel plant varieties resistant to drought stress and insect-pests are more significant, as there is mounting pressure to control these adversities in order to provide an increase in crop production. However, the development of transgenic plants expressing antifungal defensins or the production of defensin-based biofungicide depends, mainly, on the determination of regional research teams focusing on specific fungal targets, so these products can reach the market.

Furthermore, there is a long process required for analyzing the efficiency, environmental risks, safety towards animal and human consumption, and reproducibility of transformed plants expressing certain molecules, as well as the need for having an extremely stable, effective, and easy-to-produce peptide to be used in the fabrication of a biofungicide. Therefore, it is possible that there are already plant defensin-based products on the horizon that will soon be released on to the market.

Also, it is expected that, in the near future, antifungal defensin-based commercial agro-products be targeted as essential for the increase of crop production. This will stimulate and accelerate the transition between biotechnological research and field application of bioproducts.

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Peptide array based discovery of synthetic antimicrobial peptides

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ANTIMICROBIAL PEPTIDES AS A MEANS TO COMBAT ANTIBIOTIC RESISTANCE

The rise of antibiotic resistance has emphasized the shortcomings in antibiotic drug development (Boucher et al., 2013). The move from biological based discovery methods to chemical approaches to identify candidates has left the antibiotic pipeline painfully dry (Lewis, 2013). The paucity of compounds that are effective against antibiotic resistant pathogens has led to great interest in antimicrobial peptides (AMPs) as potential solutions to the rise of resistant organisms (Hancock and Sahl, 2006; Fox, 2013). AMPs are short (5–50 amino acid) peptides that are produced by virtually all organisms as part of an innate immune system. There are 2,398 AMPs that have been reported (Antimicrobial Peptide Database—September 2013) and over 80% are cationic AMPs (CAMPs). Most positively charged AMPs interact with anionic bacterial membranes (Schmidtchen and Malmsten, 2013) which leads to a rapid breakdown in membrane function and subsequent cell death (Wimley, 2010). It is this mechanism of action that is of interest as it should be difficult for bacteria to develop resistance against lethal concentrations of CAMPs.

However, many AMPs have poor drug-like properties and questions remain about that their ultimate utility as antibiotics (Brogden and Brogden, 2011). Great strides have been made in improving the protease stability; pharmacokinetics and therapeutic profile of peptide drugs and these methods have been used to improve the drug-like properties of AMPs. Despite the significant developments that have been made to advance AMPs through the clinical pipeline there has yet to be an approved AMP therapeutic (Vila-Farres et al., 2012). Clearly there is an ongoing need for additional AMP candidates as a tool in the fight against antibiotic resistant bacteria.

DISCOVERY OF SYNTHETIC AMPs

Many groups are turning to non-natural sources to discover the next generation of AMPs. These efforts are focused on computational design of AMPs or by screening large libraries of peptides for new candidates. There have been significant advances in computational design of AMPs and progress continues in this field, illustrated by the recent work of Deslouches et al. (2013). However, these studies are guided by rules learned from natural AMPs and could be limiting in terms of designing peptides that function like natural AMPs with all of their inherent strengths and weaknesses. Other groups have used peptide discovery systems to screen large libraries of peptides with the aim of identifying synthetic AMPs and potentially novel classes of AMPs. Display technologies, such as phage display, are capable of producing large libraries of peptides ($\sim 10^7$ peptides) that can be used to discover AMPs (Huang et al., 2012). However, display techniques can be difficult to adapt to whole bacteria screening and require multiple rounds of selection to identify peptides with activities similar to natural AMPs.

A promising approach to discover antibacterial candidates is to screen a target bacterium against a peptide library arrayed on a solid surface. This approach uses two different types of peptide libraries: *in situ* synthesized peptide arrays and libraries of peptides prepared as spotted peptide microarrays. Seminal work in the use of *in situ* peptide microarrays for AMP development was demonstrated for small libraries of variants of natural AMPs (Hilpert et al., 2005, 2007, 2009; Hilpert and Hancock, 2007). In this method, hundreds to thousands of peptides are synthesized on a nitrocellulose membrane, then chemically cleaved into micro well-plates where there are then tested for activity. In contrast to this approach, spotted peptide microarrays are prepared by the synthesis of thousands to tens of thousands of peptides, which are printed on glass slides using standard microarray printing technology. Peptide microarrays have been used for ligand discovery by many groups and assays have been developed to screen whole cells against immobilized ligands (Papp et al., 2012).

DISCOVERY OF ANTIBACTERIAL PEPTIDES DIRECTLY ON PEPTIDE MICROARRAYS

We have recently introduced an activity based assay that enables the selection of peptides with antibacterial activity directly on peptide microarrays (Domenyuk et al., 2013). In this assay, the target bacteria is labeled with an internal dye that fluoresces while the cell is metabolically active (e.g., Cell-Tracker Orange) and the exterior of the bacteria (outer membrane for Gram-negative or peptidoglycan layer for Gram-positive bacteria) is labeled with an amine-reactive dye, such as AlexaFluor-555 (**Figure 1A**). Fluorescently labeled

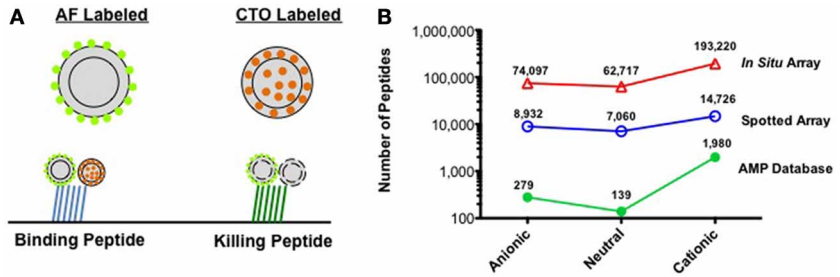

FIGURE 1 | (A) Activity based bacterial screening to identify peptides that bind and those that kill a specific pathogen. AF, AlexaFluor-555; CTO, Cell-Tracker Orange. **(B)** Number of peptides based on net charge for AMPs described in the AMP Database, the 30,000 peptides used in spotted peptide arrays, and the 330,000 peptides used in the first generation *in situ* peptide array produced in our laboratory.

bacteria are then screened against a spotted peptide microarray of 10,000, 20 amino acid long peptides to identify peptides that bind the bacteria and those that kill the bacteria. Peptides that bind but do not kill the bacteria produce two fluorescent colors, while those that bind the bacteria and disrupt the membrane produce one signal. Using this system, we were able to identify peptides that inhibited growth of both Gram-positive and Gram-negative bacteria with minimum inhibitory concentrations (MIC's) in the 20 μM range.

This approach has several important advantages as a source of antibacterial peptides. First, the system can be used to screen a wide variety of bacteria. Laboratory strains or clinical isolates can be easily labeled and do not require genetic modification to express a fluorescent or colorimetric indicator. The labeling procedures are robust and effective for Gram-positive and Gram-negative bacteria. Second, the method is rapid; bacteria can be labeled, screened, and analyzed in the same day. Additionally, the convenience of solid phase synthesis enables the incorporation of non-natural amino acids, such as D-amino acids or β-amino acids, into peptide libraries, enabling the direct screening of protease stabilized peptides. The *in vitro* assay format is very flexible in terms of the screening conditions, buffers, sera or media that can be used. Finally, peptide libraries can be designed without the inherent biases present in natural AMPs, potentially enabling the discovery of active peptides that function with novel mechanisms of action.

FUTURE DIRECTIONS FOR PEPTIDE ARRAY BASED DISCOVERY—SELECTION OF PEPTIDES THAT SPECIFICALLY TARGET AMP RESISTANT BACTERIA

In our opinion, the future of array based AMP discovery lays in the selective targeting of antibiotic and AMP resistant bacteria over normal flora. This could be possible due to the convergence of several parallel avenues of technical and scientific development. Recently, robust methods for the *in situ* synthesis of peptide microarrays with medium densities, $>10^3$–10^4peptides (Loeffler et al., 2012; Price et al., 2012), and those with much higher densities, 10^5–10^6 peptides per array (Legutki, submitted) have been reported. This significantly expands the peptide sequence space that can be explored in a single experiment. This could be an especially important development in the search for peptides that are active against either intrinsically AMP resistant bacteria, such as *Burkholderia cepacia* complex (Loutet and Valvano, 2011), or those that acquire AMP resistance after treatment with CAMPs (Anaya-López et al., 2013; Fernández et al., 2013; Napier et al., 2013; Pelletier et al., 2013; Shireen et al., 2013). Acquired AMP resistance generally involves membrane modifications that increase the charge of the surface of the bacteria that prevents binding of CAMPs to the cell surface. This potentially is a troubling development as the vast majority of AMPs (>80%) discovered to date are cationic and there is some evidence that acquired resistance for one CAMP can extend to host AMPs (Napier et al., 2013). The latter report argues for the selection of AMPs that are not related to natural ones. For these pathogens, it is possible that neutrally charged or anionic AMPs will be effective against AMP resistant bacteria that have membranes modified with cationic groups.

It is here that the recent advances in *in situ* peptide array synthesis could become important. As peptide libraries can be designed with a more even distribution of charged peptides, large numbers of neutral and anionic peptides can be screened against a resistant pathogen. This is illustrated in **Figure 1B**, where the number of anionic, neutral, and cationic peptides is plotted for: the peptides in the AMP database, the 30,000 peptides we have used in our spotted peptide libraries, and the 330,000 peptides of our recent high-density *in situ* peptide arrays. As can be seen, peptide arrays offer the opportunity to screen thousands to over 130,000 neutral and anionic peptides in a single experiment. It is possible that by screening both the AMP resistant and sensitive phenotype of a pathogen against this array, one could identify reduced charge peptides that inhibit the resistant phenotype with little effect on the sensitive phenotype. It is also possible that a peptide that selectively targets the AMP resistant bacteria would have a much narrower spectrum of activity toward normal flora. The selective targeting of the pathogen should help limit the spread of resistance to other species in the microbiome and maintain normal flora. It is likely that as the understanding of the host microbiome increases, the importance of targeted therapeutics will be even more evident. The use of molecular methods to quickly identify

bacteria from clinical specimens is rapidly being adopted and should enable physicians to match a targeted antibiotic with the correct pathogen. Advances in peptide array discovery assays could provide a system to develop pathogen-specific antibiotics (Casadevall, 2009; Lemon et al., 2012) and lead to the discovery of the first generation of targeted antibiotics.

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Potential of known and short prokaryotic protein motifs as a basis for novel peptide-based antibacterial therapeutics: a computational survey

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Short linear motifs (SLiMs) are functional stretches of protein sequence that are of crucial importance for numerous biological processes by mediating protein–protein interactions. These motifs often comprise peptides of less than 10 amino acids that modulate protein–protein interactions. While well-characterized in eukaryotic intracellular signaling, their role in prokaryotic signaling is less well-understood. We surveyed the distribution of known motifs in prokaryotic extracellular and virulence proteins across a range of bacterial species and conducted searches for novel motifs in virulence proteins. Many known motifs in virulence effector proteins mimic eukaryotic motifs and enable the pathogen to control the intracellular processes of their hosts. Novel motifs were detected by finding those that had evolved independently in three or more unrelated virulence proteins. The search returned several significantly over-represented linear motifs of which some were known motifs and others are novel candidates with potential roles in bacterial pathogenesis. A putative C-terminal G[AG].$ motif found in type IV secretion system proteins was among the most significant detected. A KK$ motif that has been previously identified in a plasminogen-binding protein, was demonstrated to be enriched across a number of adhesion and lipoproteins. While there is some potential to develop peptide drugs against bacterial infection based on bacterial peptides that mimic host components, this could have unwanted effects on host signaling. Thus, novel SLiMs in virulence factors that do not mimic host components but are crucial for bacterial pathogenesis, such as the type IV secretion system, may be more useful to develop as leads for anti-microbial peptides or drugs.

Keywords: short linear motifs (SLiMs), virulence factor, motif mimicry, antibacterial, bioinformatics, pathogen

INTRODUCTION

Short linear motifs (SLiMs) are functional microdomains in proteins that play a critical role in many distinct biological processes such as cell signaling and regulation, post-translational modifications, proteolytic cleavage, and protein trafficking (Davey et al., 2011b; Mooney et al., 2012). These motifs are typically found in eukaryotic disordered protein regions and vary in size from 3 to 12 amino acids (Fuxreiter et al., 2007). In general, SLiMs have less than five defined amino acid positions and frequently these positions have some degree of flexibility in amino acid composition. Their shortness makes them evolutionarily plastic, allowing them to evolve convergently in unrelated proteins. This can allow proteins to rapidly acquire new protein interaction functions (Neduva and Russell, 2005; Diella et al., 2008; Davey et al., 2010, 2012b). Their short length also presents a challenge for SLiM discovery both experimentally and computationally, since there may be many false positive findings using both methods.

The presence of SLiMs in eukaryotes and viruses has been well-established. Several pioneering viral studies were crucial for the original characterization of SLiMs (Davey et al., 2011b). Viruses use SLiMs as a principal mechanism of hijacking cells by binding to host proteins and recruiting them to process viral proteins. A viral genome can contain various short motifs, many of which are necessary for the viral life cycle, providing a plethora of ways for the virus to take over the molecular machinery of the host cell (Kadaveru et al., 2008; Davey et al., 2011b). Like viruses, pathogenic bacteria are extremely proficient in intercepting host cell functions and in many cases it is still poorly understood how bacteria carry out the manipulation of the host cells. SLiMs have been documented in a number of cases to play a role in bacterial pathogenicity. However, bacterial linear motifs are not as well-characterized as in eukaryotes.

Most of the known instances of bacterial motifs are involved in pathogenicity including signals in effector proteins or host motif

mimicry (Cornelis and Van Gijsegem, 2000; Alto et al., 2006). The tripeptide RGD motif is a known host extracellular matrix adhesion factor that is also used by bacteria to attach onto host cells (Tegtmeyer et al., 2010; Zimmermann et al., 2010; Zhang et al., 2012). RGD based anticancer and antithrombotic drugs are currently being developed but their direct impact on limiting bacterial adhesion and infectivity has not been investigated. A second example of a bacterial motif is the EPIYA motif found in several bacterial type III or IV secretion system effector proteins, which mimics SH2 binding peptides of the host (Hayashi et al., 2013). A third example of a bacterial motif has evolved to antagonize host proteins, but does this using a motif for which there is no eukaryotic equivalent. This W…E motif (where "." indicates any amino acid) in bacterial effector proteins has been proposed to mimic host G-proteins (Alto et al., 2006; Jackson et al., 2008; Ham et al., 2009). Other motifs found in prokaryotes which are not simply mimicking known eukaryotic motifs play roles in transport, modification and proteolysis of the bacterial proteins (**Table 1**).

Since SLiMs are used in a plethora of cellular processes in eukaryotes and are utilized by both pathogenic bacteria and viruses, discovering and characterizing new linear motifs is of great importance. As well as shedding light on the mechanisms of fundamental cellular processes they also hold promise as future therapeutic targets. There is an urgent need for new classes of antimicrobial therapeutics that are effective against multidrug resistant bacteria. Conventional antibiotics are becoming increasingly ineffective against pathogenic bacteria, such as methicillin resistant *Staphylococcus aureus* (MRSA) which presents a severe threat to public health.

We were interested in whether SLiMs may be valuable when developing new antimicrobial peptides or drugs. Compared with recombinant proteins, the smaller size of peptides makes them easier to manufacture and deliver. The use of chemically synthesized peptides in pharmacological and clinical applications is relatively limited by their low systemic stability and high clearance, poor membrane permeability, negligible activity when administered orally and their high cost of manufacture in comparison to small chemical compounds. However, to date more than 100 peptide-based drugs have already reached the market and of these, the majority are at the smaller end of the size spectrum at 8–10 amino acids (Craik et al., 2013).

Here, we conducted a study to discover SLiMs computationally in bacterial virulence factor datasets. We surveyed the distribution of these novel motifs, and compared their distribution with that of known motifs observed in prokaryotic proteins. The list of motifs given here represents a useful resource for experimental scientists interested in targeting SLiMs that may be important for the pathogenicity of bacteria.

MATERIALS AND METHODS

We utilized data from a virulence factor database MvirDB (Lawrence Livermore National Laboratory), which integrates DNA and protein sequence information from Tox-Prot, SCORPION, the PRINTS database of virulence factors, VFDB, TVFac, Islander, ARGO, CONUS, KNOTTIN, a subset of VIDA and sequences derived by means of literature searches (Zhou et al., 2007). MvirDB can be accessed at http://mvirdb.llnl.gov. The MvirDB browser tool was used to search the database to retrieve virulence factors by functional categories (**Table 2**) and to download sequences of interest. Protein sequence identifiers for the downloaded sequences for each functional category are available in **Table S1**.

The recovered protein sequences in each functional category thought to be associated with pathogenicity were searched for SLiMs using SLiMFinder (Davey et al., 2010) both locally, and on a webserver that is available at http://bioware.ucd.ie. The default settings provided in SLiMFinder without any extra masking were used in the analysis. This method finds sets of three or more unrelated proteins in a dataset of proteins that share a motif. Chemotaxis and enzyme protein sequence datasets were filtered to contain only sequences longer than 20 amino acids and lipoprotein and Exotoxin datasets sequences longer than 40 amino acids prior to the analysis.

The motifs identified by the SLiMFinder analysis were further examined for similarity to known SLiMs from literature motifs using CompariMotif, which takes two lists of protein motifs and compares them to each other, identifying and scoring similarities between short motifs in the sets (Edwards et al., 2008).

Motifs were visualized using the MEME Suite (Bailey et al., 2009), by taking a stretch of 10 amino acid residues containing the motif of interest from each protein sequence where the motif was found. MEME represents motifs as position dependent letter probability matrices which describe the probability of each possible letter at each position in the pattern. These are displayed as "sequence LOGOS," containing stacks of letters at each position in the motif. The total height of the stack is the "information content" of that position in the motif in bits. The height of the individual letters in a stack is the probability of the letter at that position multiplied by the total information content of the stack.

Datasets comprised of protein sequences obtained from UniProtKB that are predicted to be effector proteins from a selection of 60 organisms represented in the MvirDB were used to assess the distribution of prokaryotic protein motifs. The presence of both known and novel motifs in these datasets was investigated using the predictive computational tool SLiMSearch which can be used to determine the occurrences of predefined motifs in protein sequences (Davey et al., 2011a). Heat maps were generated to visualize the incidences of motifs in the protein datasets where the frequency of the heat map represents the logarithm of the normalized N_UPC (Number of incidences of a motif in an Unrelated Protein Cluster) value returned in the SLiMSearch results. The N_UPC for an individual motif in a specific organism was normalized by dividing the value by the total amount of UPCs (Unrelated Protein Clusters) in the specific organism and the average N_UPCs of a motif across all 60 organisms. For motifs where there were no incidences in a specific organism the frequency was set to an arbitrary value lower than the minimum actual observed value.

The organisms in **Figures 2**, **3** which cover the motif sequences were presented in a phylogenetic tree (**Figure 4**). The Taxonomic IDs for all the organisms are used as input in NCBI's Taxonomy Common Tree tool (http://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi). The "phenogram" taxonomic tree (*.phy format) obtained from the NCBI server was fed into

Table 1 | Examples of known instances of Short Linear Motifs in bacterial virulence factors.

Virulence factor	Motif	Function	References
ADHERENCE			
CagL, Mce	RGD*, NGR	An integrin binding cell adhesion motif	Conradi et al., 2012a,b; Zhang et al., 2012
CagL	**FEANE**	Participates in integrin binding	Conradi et al., 2012b
YadA	**[SG][VI][AS][IVT]G..S**	Repeated collagen binding motif	Tahir et al., 2000
InlJ, InlA, nanA, CspA	**LP.TG**	Cell wall anchor motif	Harris et al., 2003; Sabet et al., 2005; Banerjee et al., 2010
Eno	**[LF]Y[DNK]. . . [KG][KV]Y[VD]***	Plasminogen binding motif	Bergmann et al., 2003; Nogueira et al., 2012
CBPA-G, LytA-C	**W[WFY][FY]. . . .G.M**	Repeated cholin (cell wall) binding motif	Garau et al., 2005
SpsA	**YRNYPT**	Host secretory immunglobulin A (SIgA) and secretory component (SC) binding motif	Hammerschmidt et al., 2000
EFFECTOR			
SipA, SopA	DEVD*, [DSTE][^P][^DEWHFYC]D[GSAN]	Caspase 3 cleavage site motif	Srikanth et al., 2010; Dinkel et al., 2012
AvrPto, HopF2, AvrB, AvrRpm1	MG..C*,G. . . [STC], ^M{0,1}(G)[^EDRKHPFYW]..[STAGCN][^P]	*N*-myristoylation/*S*-palmitoylation motif	Shan et al., 2000; Robert-Seilaniantz et al., 2006; Dinkel et al., 2012; Hicks and Galan, 2013
WtsE, AvrE1, IpgB2, IpgB1, Map, EspM, EspT, SifA, SifB	**W. . . E***	Host Rho GTPase activation/modulation motif	Alto et al., 2006; Ham et al., 2009
WtsE,	[LR][KQVS][KQLR][EST][GQR][FLKS][EGPK] [MLVAS][KNAL][SGIE]*	Putative endoplasmic reticulum membrane retention/retrieval motif	Ham et al., 2006, 2009
SifA, AnkB	CLCCFL*, (C)[^DENQ][LIVM].$	CAAX box, putative prenylation motif (addition of farnesyl or geranylgeranyl group)	Boucrot et al., 2003; Hicks et al., 2011; Dinkel et al., 2012
YopE, SptP, ExoS	G.LR. . . T(YopE*)	Arginine finger motif, essential for Rho GAP function of virulence factors	Black and Bliska, 2000; Wurtele et al., 2001
PopB, PopP2, AvrBs3	[^DE]((K[RK])|(RK))[KRP][KR][^DE], [KR][KR].{7,15}[^DE]((K[KR])|(RK))(([^DE][KR]) |(KR][^DE]))[^DE], [^DE]((K[RK])|(RK))(([^DE][KR])|([KR][^DE]))(([PKR]) |([^DE][DE])), (([PKR].{0,1}[^DE])|([PKR]))((K[RK])|(RK)) (([^DE][KR])|([KR][^DE]))[^DE]	Nuclear localization signal (NLS) motifs	Szurek et al., 2002; Deslandes et al., 2003; Dean, 2011; Dinkel et al., 2012
CagA, Tarp, AnkA, LspA	E[PNS][IV]Y[AEG]	Membrane targeting/phosphorylation motif	Higashi et al., 2005; Suzuki et al., 2009; Hayashi et al., 2013
SspH2, SseI	. . . .GSGC. . . .., G(C)M[GS][CL][KP]C, ^M{0,1}G(C)..S[AKS]	*S*-palmitoylation motif	Hicks et al., 2011; Dinkel et al., 2012

(Continued)

Table 1 | Continued

Virulence factor	Motif	Function	References
ExoS, SopE	[FIV]..[FIV].[FIV]..[NC].[FIV]	Membrane localization motif (targets ExoS to the Golgi-endoplasmic reticulum)	Zhang and Barbieri, 2005
SopD2, SifA, SseJ, SspH2	**WEK[IM]..FF**	Translocation/late endocytic compartments targeting motif	Brown et al., 2006
ExoU	**KAWRN**	Plasma membrane localization/ubiquitinylation motif	Rabin and Hauser, 2005; Stirling et al., 2006
SopE, BopE	**GAG[AT]**	Catalytic loop motif essential for guanine nucleotide exchange	Schlumberger et al., 2003
AvrPphB	**GDK**	Autoproteolytic cleavage motif	Dowen et al., 2009
SopA, IpaH, SspH1	L. . . .TC, C.D	E3 ubiquitin ligase motif	Zhang et al., 2006; Rohde et al., 2007
VirF	LP. L	F-box domain motif, mediates protein–protein interactions	Tzfira et al., 2004
VopL, VopF	[^R]..((.[ILMVF])|([ILMVF].))[^P][^P][ILVM].{4,7}L(([KR].)|(NK))[VATIGS], [R]..[ILVMF][ILMVF][^P][ILVM].{4,7}L(([KR].)|(NK))[VATI]	WH2-domain motif	Liverman et al., 2007; Dinkel et al., 2012
SpvC, OspF, VirA	[KR]{0,2}[KR].{0,2}[KR].{2,4}[ILVM].[ILVF]	D motif, Docking motif required for specific binding to MAPKs	Zhu et al., 2007; Dinkel et al., 2012
IpaA	L..AA..VA..V..LI..A.	Vinculin binding domain motif	Hamiaux et al., 2006
ExoS, ExoT	**LLDALDLA**	FAS (14-3-3 protein) binding motif, mediates activation of the ADPRT domain	Sun et al., 2004; Dean, 2011
EspF	[RKY]..P..P, P..P.[KR],...[PV]..P, KP..[QK]. . .	SH3 binding motif	Alto et al., 2007; Dinkel et al., 2012
Map, NleH1, EspI (NleA)	...[ST].[ACVILF]$, . . . [VLIFY].[ACVILF]$,...[DE].[ACVILF]$ (EspI*)	C-terminal PDZ1 binding motif	Lee et al., 2008; Martinez et al., 2010; Dinkel et al., 2012
TOXIN			
Listeriolysin O (LLO)	PPASP*	PEST-motif, involved in phagosomal escape of bacteria in infected cells	Lety et al., 2001
OTHER			
VirD4, VirB11, VirB4, SecA	G. . . .GK[TS]*	Walker A motif, nucleotide-binding motif	Sato et al., 1996; Atmakuri et al., 2004
SecA	[RK]. . . .G. . . .L[VILFWYMC]{4,4}D	Walker B, nucleotide binding motif	Sato et al., 1996
MsbA, PiaA, PiuA	LSGGQ (PiaA*, PiuA*)	ABC-motif, ATP binding cassette transporter motif	Garmory and Titball, 2004; Buchaklian and Klug, 2006
EsxA, EsxB, esat6	**W.G**	W.G motif helps to create a shallow cleft structure and may represent a peptide recognition feature by which cargo proteins are acquired for transport	Burts et al., 2005; Sundaramoorthy et al., 2008

**Proven role in virulence. Bold, a non-eukaryotic motif. "^ " start of the protein or if in the middle of the motif sequence states which amino acids are excluded in the position, "$" end of the protein, "." any amino acid, {} defines the length of a range in the motif sequence, [] defines which amino acids can occur at a given motif position, () marks positions of specific interest e.g., covalent modification or is used to group parts of the expression. Motif table modified from Dean (2011), additional motifs added from the literature.*

Table 2 | Functional search terms used to retrieve and download protein sequences from virulence factor database MvirDBbrowser tool.

Virulence protein group	Number of sequences	Number of unrelated proteins
Adherence	749	181
Capsule	332	57
Chemotaxis	192	18
Effector	111	27
Endotoxin	66	27
Enzyme	647	121
Exotoxin	92	12
Lipoprotein	463	70
Motility	86	23
Siderophore	150	43
Type III secretion system	571	75
Type IV secretion system	181	38

Drawgram tree drawing program of Phylip package (version 3.695). Branches were colored according to the following scheme: Purple, High GC Gram+ bacteria; Blue, Firmicutes; Yellow, a-proteobacteria; Light Brown, b-proteobacteria; Dark Brown, e-proteobacteria; Green, g-proteobacteria (non-enterobacteria); Red, g-proteobacteria (enterobacteria); Black, others (CFB).

RESULTS

Our objective was to discover novel SLiMs in non-homologous bacterial proteins with similar roles in virulence that may have functional importance in pathogenesis, and thus have potential to be developed into antimicrobial peptides or drugs. Our analysis returned both previously characterized and novel motifs in several different functional categories indicating the suitability of SLiMFinder for the analysis of bacterial sequence data as well as eukaryotic data. We focused on 12 groups of bacterial proteins with predefined roles in pathogenicity (**Table 2**). SLiMFinder identified numerous motifs among these proteins. **Table 3** lists those with a *p*-value (Sig) less than 0.05. Bonferroni correction for significance with 12 search datasets would suggest that motifs with a Sig value of less than 0.004 are significant. Since pathogenesis proteins from bacteria often interact with host protein components, we examined whether any of the identified motifs showed similarity to known eukaryotic linear motifs, using the Comparimotif tool. However, we did not find any convincing similarities, in spite of the known occurrence of eukaryotic motifs in bacterial effector proteins. We also investigated if any of the motifs were known prokaryotic motifs identified in the literature.

KNOWN MOTIFS

Three of the motifs highlighted by SLiMFinder were previously known bacterial motifs. The most significant of these was the well-characterized prokaryotic N-terminal lipid modification [LVI][ASTVI][GAS]C motif that has been previously shown to be essential for the anchoring of bacterial proteins to the membrane surface (Braun and Rehn, 1969; Babu et al., 2006). The square brackets enclose alternative amino acids which are possible at that position in the motif. This motif is present in a wide range of proteins across Gram-positive and Gram-negative bacteria and is a clear example of a motif that has convergently evolved in many unrelated proteins. It was found in numerous configurations in the lipoprotein dataset of which seven are listed in **Table 3**. This "lipobox" motif sequence is located at the C-terminal end of the signal peptide and the lipid-modifiable cysteine (+1 position) is invariant (Juncker et al., 2003). Lipid modification of this cysteine residue (*N*-acyl-*S*-diacylglyceryl-Cys) has been found to be an essential, ubiquitous, and unique bacterial post-translational modification. Such a modification allows anchoring of even highly hydrophilic proteins to the membrane surface leaving the rest of the protein to carry out a variety of relevant functions in the aqueous or aqueous-membrane interface (Juncker et al., 2003; Babu et al., 2006). Bacterial lipoproteins affect a wide range of mechanisms in virulence. They have been shown to play key roles in adhesion to host cells and in translocation of virulence factors into host cells (Kovacs-Simon et al., 2011). Furthermore, they are potent inducers of host inflammatory responses.

The second known motif identified was an N-terminal ^MK.{0,2}K motif present in several search categories in varying configurations (**Table 3**, Adherence, Capsule, Enzyme, Lipoprotein, Siderophore, and Type IV secretion system). This motif representation indicates that the second K (lysine) may lay 0, 1, or 2 residues after the K that follows the initiator methionine. The "^" symbol indicates the start of the protein, which is treated as a distinct character in motif discovery. SLiMFinder omits the M from the returned motif resulting in ^.K.{0,2}K representation, since initiator methionines were deliberately masked out to avoid returning motifs reliant simply on the strong enrichment of M at the start of proteins. The ^MK.{0,2}K motif is commonly found in bacterial signal peptides both in proteins that are targeted to the membrane and in secreted proteins (Juncker et al., 2003; Bagos et al., 2008). Both of the known motifs are presented as regular expressions in **Figure 1**, which provides some information on additional contextual preferences beyond the simple motif description. Signal peptides in bacteria are mainly divided into the secretory signal peptides that are cleaved by Signal Peptidase I and those cleaved by Signal Peptidase II which characterize the membrane-bound lipoproteins (Juncker et al., 2003; Bagos et al., 2008). The signal peptides in both classes of proteins in Gram-positive and Gram-negative bacteria are quite similar, sharing the N-terminal region which is characterized by presence of the positive amino acids at the start of the protein, as well as the preference for hydrophobic residues further along the signal peptide.

The third previously characterized bacterial motif returned in our analysis is the C-terminal KK$ motif (where $ indicates the end of the protein, and is treated as a distinct character in motif discovery) found in adherence and lipoprotein datasets (**Table 3**; **Figure 1**). This motif has been shown to play a role in plasminogen binding in *S. pyogenes* and *S. pneumoniae* α-enolase (Bergmann et al., 2003; Derbise et al., 2004; Itzek et al., 2010). Binding of plasminogen by α-enolase and its subsequent activation has been demonstrated to promote invasion of pathogenic bacteria and therefore represents an important determinant of virulence in invasive infection (Bergmann et al., 2003). Moreover, KK motifs close to the C-terminus are present in a family of

Table 3 | Significant motifs returned by SLiMFinder in each dataset (where probability <0.05).

Virulence protein group	Number of sequences	Motif pattern	Motif name	Information content	Occurrences	Unrelated proteins	Probability (Sig value)	References
(A) PREVIOUSLY DESCRIBED MOTIFS								
Adherence	749	^.K.{0,2}K	Signal peptide motif	3	151	47	0.00E+00	Juncker et al., 2003; Bagos et al., 2008
		^.{1,2}K.{1,2}L	Signal peptide motif	3	102	35	1.15E–10	Juncker et al., 2003; Bagos et al., 2008
		^.{1,2}K.{0,2}I	Signal peptide motif	3	91	34	1.41E–08	Juncker et al., 2003; Bagos et al., 2008
		^.K.[IL]	Signal peptide motif	2.77	58	21	5.80E–07	Juncker et al., 2003; Bagos et al., 2008
		^.K..S	Signal peptide motif	3	24	12	0.002	Juncker et al., 2003; Bagos et al., 2008
		KK$	C-terminal KK	3	23	11	0.034	Bergmann et al., 2003; Itzek et al., 2010
Capsule	332	^.{1,2}K.{0,1}I	Signal peptide motif	3	55	18	6.96E–06	Juncker et al., 2003; Bagos et al., 2008
		^.{1,2}K.{0,1}I..V	Signal peptide motif	4	16	7	0.002	Juncker et al., 2003; Bagos et al., 2008
		^.{1,2}K.{0,2}I	Signal peptide motif	3	59	18	0.003	Juncker et al., 2003; Bagos et al., 2008
		^.K.[ILV]	Signal peptide motif	2.63	39	13	0.003	Juncker et al., 2003; Bagos et al., 2008
		^.{1,2}K.{0,2}K	Signal peptide motif	3	52	17	0.006	Juncker et al., 2003; Bagos et al., 2008
Chemotaxis	192	–		–	–	–	–	–
Effector	111	–		–	–	–	–	–
Endotoxin	66	–		–	–	–	–	–
Enzyme	647	^.{1,2}KK	Signal peptide motif	3	27	14	9.52E–05	Juncker et al., 2003; Bagos et al., 2008
		^..K.{0,2}I	Signal peptide motif	3	30	13	0.042	Juncker et al., 2003; Bagos et al., 2008
Exotoxin	92	–		–	–	–	–	–
Lipoprotein	463	L.[AG]C[AGS]	Lipobox	3.4	78	30	0.00E+00	Braun and Rehn, 1969; Babu et al., 2006
		[FLV].L.[AG]C	Lipobox	3.4	136	24	0.00E+00	Braun and Rehn, 1969; Babu et al., 2006
		[ILV].[AGS]C	Lipobox	2.27	370	53	0.00E+00	Braun and Rehn, 1969; Babu et al., 2006
		[AGS]C[AGS]	Lipobox	2.27	285	50	7.22E–15	Braun and Rehn, 1969; Babu et al., 2006
		^.{1,2}K.{0,2}K	Signal peptide motif	3	109	27	1.27E–09	Juncker et al., 2003; Bagos et al., 2008

(Continued)

Table 3 | Continued

Virulence protein group	Number of sequences	Motif pattern	Motif name	Information content	Occurrences	Unrelated proteins	Probability (Sig value)	References
		L.{1,2}GC.{0,1}A	Lipobox	4	41	15	1.65E–09	Braun and Rehn, 1969; Babu et al., 2006
		A.{0,2}L..C.{0,2}S	Lipobox	4	68	19	3.02E–09	Braun and Rehn, 1969; Babu et al., 2006
		^.K..[ILV]	Signal peptide motif	2.63	66	19	4.36E–09	Juncker et al., 2003; Bagos et al., 2008
		^..K..[FLV]	Signal peptide motif	2.63	65	17	1.07E–07	Juncker et al., 2003; Bagos et al., 2008
		[ILV]..C.[AGS]	Lipobox	2.27	217	36	4.11E–06	Braun and Rehn, 1969; Babu et al., 2006
		KK$	C-terminal KK	3	22	9	0.016	Bergmann et al., 2003; Itzek et al., 2010
Motility	86	–		–	–	–	–	–
Siderophore	150	^.[KR]I	Signal peptide motif	2.77	12	7	0.024	Juncker et al., 2003; Bagos et al., 2008
Type III secretion	571	–		–	–	–	–	–
Type IV secretion	181	^.K[KR]	Signal peptide motif	2.77	29	9	0.003	Juncker et al., 2003; Bagos et al., 2008
		^.K..[FIL]	Signal peptide motif	2.63	27	10	0.025	Juncker et al., 2003; Bagos et al., 2008
(B) NOVEL MOTIFS								
Adherence	749	LP.G.Y		4	37	12	0.012	
Capsule	332	G.S..M.L		4	15	7	0.029	
Chemotaxis	192	E..Q.I[AG].I		4.77	22	5	0.004	
		E..Q.[IV]..I		3.77	24	7	0.02	
Effector	111	^..I.{0,1}N		3	21	6	0.012	
		[LV].PY		2.77	46	11	0.042	
		^..I[ST]		2.77	17	6	0.049	
Endotoxin	66	–		–	–	–	–	
Enzyme	647	A.I.P.VL		5	14	7	0.019	
		VSIL.S		5	11	7	0.049	
Exotoxin	92	–		–	–	–	–	
Lipoprotein	463	ML..C		3	14	7	0.017	
Motility	86	–		–	–	–	–	
Siderophore	150	I.K..G		3	28	17	0.044	
		GYP..TP		5	5	4	*0.052*	

(Continued)

Table 3 | Continued

Virulence protein group	Number of sequences	Motif pattern	Motif name	Information content	Occurrences	Unrelated proteins	Probability (Sig value)	References
Type III secretion	571	–		–	–	–	–	
Type IV secretion	181	G[AG].$		2.77	19	9	2.64E–04	

Where very similar motifs are returned for a protein group, only a representative motif is shown.

Information content (Edwards et al., 2007), "^ " start of the protein, "$" end of the protein, ". " any amino acid, {} defines the range of a repeat in the motif sequence, [] defines which amino acids can occur at a given motif position.

Italic font is used when Probability (Sig-value) is higher than the 0.05 confidence level.

Shigella flexneri glucosyl transferases (Gtr) that are integral membrane proteins embedded within the cytoplasmic membrane. These glucosyl transferases contribute to the altering of the structure of the bacterial surface lipopolysaccharide (LPS) O-antigen along with O-acetyltransferase (Lehane et al., 2005; Ramiscal et al., 2010). The KK motif has been shown to be essential for the activity of Gtrs. However, Ramiscal et al. showed that the KK motif in a recently identified GtrIc is not critical for its activity (Ramiscal et al., 2010). We hypothesize that the KK$ motif instances identified here in diverse proteins may play an adhesive role similar to the plasminogen binding instances in α-enolase. We note that plants have a KK$ variant (Gidda et al., 2009) of a known eukaryotic cytoplasmically exposed endoplasmic reticulum (ER) localization motif KKxx$ found in mammals, yeast and plants (Nilsson et al., 1989; Jackson et al., 1990; Contreras et al., 2004). It is therefore conceivable that the bacterial KK$ motif could in some proteins direct invading proteins to certain parts of the eukaryotic host cell. However, we do not think this is very plausible, since the enrichment of KK$ motifs spans many known bacterial lipoproteins (**Table 3**) which seem unlikely to migrate to this host cell location.

NOVEL MOTIFS

The most significant novel motif (p-value 0.0003) discovered is a C-terminal G[AG].$ motif in the type IV secretion system dataset. The full list of unrelated proteins containing the G[AG].$ motif is represented in **Table 4**. The MEME regular expression pattern of the motif in these proteins is described in **Figure 1**. Four of the nine unrelated proteins containing this motif appear to be identified equivalents of the type IV secretion system components in the well-studied *Agrobacterium tumefaciens*: VirB4, VirB8, VirB11, and VirB7 [TrwH has 59% identity with VirB7 family (Patey et al., 2006)]. VirB4 and VirB11 are known energetic components of the type IV secretion system in *A. tumefaciens*. Both of these proteins are membrane associated NTPases on the inner membrane (Tegtmeyer et al., 2011). VirB8 on the other hand, is an essential inner membrane component of type IV secretion systems that is believed to form a homodimer and has been shown to be of importance for complex stability in *A. tumefaciens* (Sivanesan and Baron, 2011). The VirB7 is an outer membrane lipoprotein that localizes exocellularly and associates with the type IV secretion system pilus. Both VirB7 lipid modification and disulfide cross-linking have been shown to be important for pilus assembly (Sagulenko et al., 2001). The *Helicobacter pylori* protein Cag7 that is among the proteins containing the C-terminal G[AG].$ motif has previously been proposed to be a transmembrane protein that is associated with the pilus (Rohde et al., 2003; Tegtmeyer et al., 2011). At least five of the nine unrelated proteins containing the G[AG].$ motif seem to be associated with the bacterial membranes and it is thus possible that this motif would be involved in the targeting and/or attachment of these proteins into the bacterial membranes. However, since the motif has been specifically identified within type IV secretion proteins, it is more likely that the motif facilitates interaction with a component of the type IV secretion system itself. We inspected the distribution of the motif across effector proteins (**Figure 2**) and noted that there are typically one or none per species, suggesting that the motif is not itself enriched strongly among effector proteins themselves.

Other novel motifs discovered are summarized in **Table 3** and in **Figure 1**. Their significance is in the range between that for the nominal significance level ($p < 0.05$) and the Bonferroni adjusted significance level ($p < 0.004$). While it is likely that a number of these motifs are genuine, a few may be false positives. The LP.G.Y motif found in the adherence dataset superficially resembles a Gram-positive bacteria cell wall anchoring LP.TG motif. Cleavage between the Thr and Gly by sortase or a related enzyme leads to covalent anchoring of the new C-terminal Thr to the cell wall (Navarre and Schneewind, 1994; Gaspar et al., 2005). Cell wall-anchored surface proteins of Gram-positive pathogens play important roles during the establishment of many infectious diseases. While it could be hypothesized that the LP.G.Y motif is similarly involved in the anchoring of bacterial proteins to the cell surface, there are two lines of evidence that argue against this. Firstly, there is no enrichment for T or similar amino acids between P and G in the instances of the motif returned (**Figure 1**). Secondly, this motif is present both in Gram-positive and Gram-negative bacterial proteins in our study. Accordingly, we consider LP.G.Y a potential novel motif involved in bacterial adhesion through an unidentified mechanism.

REPEATED MOTIFS

While SLiMFinder looks for motifs which recur one or more times in a number of independent proteins, it is of biological

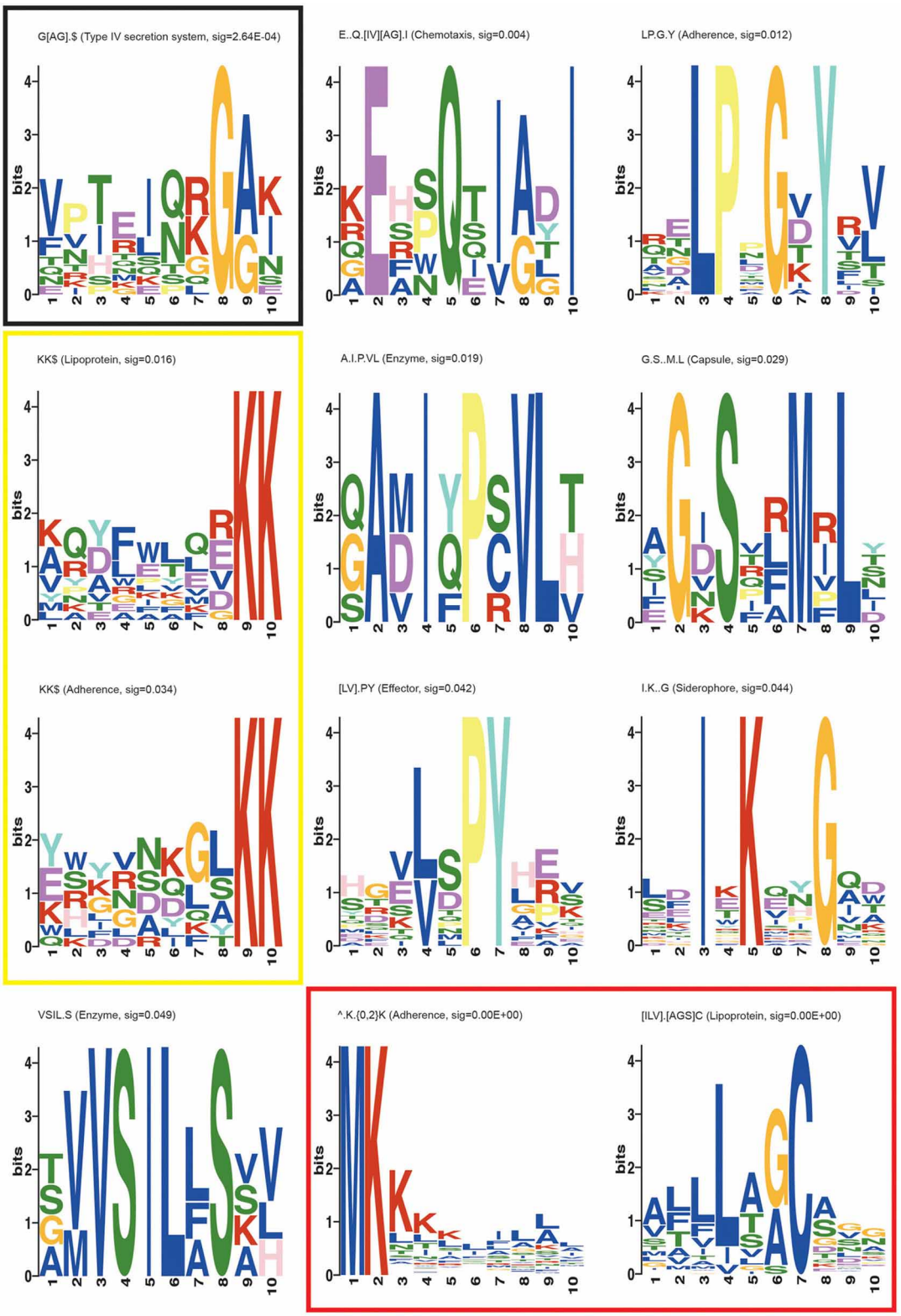

FIGURE 1 | MEME suite motif logos of the novel and known motifs returned in the SLiMFinder analysis. Each position in the motif is represented as a stack of letters. The total height of the stack is the "information content" of that position in the motif in bits. The height of the individual letters in a stack is the probability of the letter at that position multiplied by the total information content of the stack. Black box: the most significant novel motif G[AG].$, Yellow box: KK$ motifs found in Adherence and Lipoprotein datasets, Red box: Known bacterial motifs ^.K.{0,2}K and [ILV].[AGS]C.

Table 4 | List of proteins containing G[AG].$ and KK$ motifs.

Pattern	Protein group	*P*-value (Sig)	Match	No. unrelated proteins	Description
G[AG].$	Type IV secretion	2.64E–04	GGN	9	virB11 protein homolog|9992|YP_034060|49476019|8040|[*Bartonella henselae* str. Houston-1]
			GAK		virB4|10558|NP_863348|32469876|8343|VirB4 [*Campylobacter jejuni* subsp. *jejuni* 81-176]
			GAK		virB8|10560|NP_863298|32469826|8344|VirB8 [*Campylobacter jejuni* subsp. *jejuni* 81-176]
			GAE		cag pathogenicity island protein (cag11)|10866|NP_207327|15645157|8497|[*Helicobacter pylori* 26695]
			GGK		trwF protein|9938|YP_034270|49476229|8013|[*Bartonella henselae* str. Houston-1]
			GAI		trwH2 hypothetical protein BH15720|9944|YP_034268|49476227|8016 [*Bartonella henselae* str. Houston-1]
			GAS		cag pathogenicity island protein (cag25)|10894|NP_207342|15645172|8511|[*Helicobacter pylori* 26695]
			GGN		Putative type IV secretion system protein|41299|NP_790379|NP_790379.1|18355|[*Pseudomonas syringae* pv. tomato str. DC3000]
			GAI		trwH1 hypothetical protein BH15690|9942|YP_034265|49476224|8015|[*Bartonella henselae* str. Houston-1]
			GGN		cag pathogenicity island protein (cag7)|10904|NP_207323|15645153|8516|[*Helicobacter pylori* 26695]
KK$	Adherence	0.034	KK	11	hmw2C putative accessory processing protein [*Haemophilus influenzae*]|2847|AAA20526|482843|2554|
			KK		kpsC polysaccharide modification protein [*Campylobacter jejuni* subsp. *jejuni* NCTC 11168]|10454|NP_282555|15792732|8291|
			KK		ica operon transcriptional regulator [*Staphylococcus aureus* subsp. aureus MW2]|3137|NP_647402|21284314|2699|
			KK		pavA adherence and virulence protein A [*Streptococcus agalactiae* 2603V/R]|9719|NP_688199|22537348|7896|
			KK		Type 4 fimbrial biogenesis protein PilO [*Pseudomonas aeruginosa* PAO1]|8738|NP_253729|15600235|7426|
			KK		Type 4 fimbrial biogenesis protein PilN [*Pseudomonas aeruginosa* PAO1]|8740|NP_253730|15600236|7427|
			KK		Putative collagen binding protein [*Streptococcus pyogenes* MGAS315] SpyM3_0098|9709|NP_663902|21909634|7891|
			KK		oapA opacity associated protein [*Haemophilus influenzae* Rd KW20]|2865|NP_438494|16272282|2563|
			KK		neuC1 putative N-acetylglucosamine-6-phosphate 2-epimerase/N-acetylglucosamine-6-phosphatase [*Campylobacter jejuni* subsp. *jejuni* NCTC 11168]|10349|NP_282290|15792467|8218|
			KK		waaE D,D-heptose 1-phosphate adenosyltransferase/7-phosphate kinase [*Campylobacter jejuni* subsp. *jejuni* NCTC 11168]|10396|NP_282297|15792474|8262|
			KK		hmw1C putative accessory processing protein [*Haemophilus influenzae*]|2841|AAA20529|475773|2551|
			KK		cytotoxin [*Escherichia coli* O157:H7]|11176|AAC70163|3822209|8652|
KK$	Lipoprotein	0.016	KK	9	Multidrug resistance outer membrane efflux protein mdtP; Flags: Precursor|58083|Q8CVH8|24068|*Escherichia coli*
			KK		ylpB/yscJ needle complex inner membrane lipoprotein [*Yersinia pestis* CO92]|20866|NP_395193|16082747|13354|
			KK		Yop proteins translocation lipoprotein J OS = *Yersinia enterocolitica* GN = yscJ PE = 2 SV = 1|18600|AltName: Full = Lipoprotein ylpB; Flags: Precursor; |Q01251|11895|

(Continued)

Table 4 | Continued

Pattern	Protein group	*P*-value (Sig)	Match	No. unrelated proteins	Description
			KK		Lipoprotein [*Salmonella enterica* subsp. *enterica* serovar Typhi str. CT18]|44580|NP_455466|NP_455466.1|19996|
			KK		LPP20 lipoprotein OS = *Helicobacter pylori* GN = lpp20 PE = 1 SV = 1|17433|P0A0V0|10728|
			KK		Outer membrane factor of efflux pump [*Escherichia coli* str. K-12 substr. MG1655]58055|NP_418504|NP_418504|24053|
			KK		Lipoprotein, putative [*Enterococcus faecalis* V583]9889|NP_816134|29376980|7982|
			KK		Iron transport lipoprotein SirF [*Staphylococcus aureus* subsp. *aureus* Mu50]|8986|NP_371657|15924123|7550|
			KK		Export protein prsA cytoplasmic membrane protein, protein folding|3480|GBAA2336 prsA-3 |GBAA2336|2870|
			KK		LPP20 lipoprotein OS = *Helicobacter pylori* J99 GN = lpp20 PE = 3 SV = 1|17432|P0A0V1|
			KK		Yop proteins translocation lipoprotein J OS = *Yersinia pseudotuberculosis* GN = yscJ PE = 2 SV = 1|18863|AltName: Lipoprotein ylpB |P69973|12158|
			KK		YaeC family lipoprotein [*Enterococcus faecalis* V583] [*Enterococcus faecalis* V583]|39473|NP_815743|NP_815743.1|17442|
			KK		Major outer membrane lipoprotein OS = *Yersinia pestis* GN = lpp PE = 3 SV = 1|24369||Q8ZDZ6|15171|
			KK		Putative lipoprotein [*Salmonella enterica* subsp. *enterica* serovar Typhi str. Ty2]|44956|NP_805720|NP_805720.1|20184|

interest when those motifs are themselves repeated within the proteins, for example, representing multiple adhesion sites. Accordingly, we investigated the frequency of repeats of the identified motifs. Duplicated motifs were found with between two and four copies in proteins. The lipoprotein lipid anchoring motif was found repeated three times in the protein HrpB3 of *Xanthomonas euvesicatoria* (instances LAGC, LALC, and LSAC). Among these motif instances, LAGC and LSAC are known lipid anchoring motifs (Klein et al., 2005; Konkel et al., 2010). The third instance may represent a true positive anchoring motif, a degenerate motif that is no longer functional or a false positive sequence that fulfills some other functional role in the protein. However, it is clear that the repetition of this well-known motif is in some cases biologically important for function. Thus, for novel motifs, repetition within as well as between proteins may be a potential further indication of important function. An example would be the threefold repetition of the "LP.G.Y" motif in the surface-anchored fimbrial subunit protein SpaG of *Corynebacterium diptherae*. This motif has a known structure in the collagen binding domain of *Staphylococcus aureus* (PDB entry 1D2P) (Deivanayagam et al., 2000). Collagen is itself a repetitive structure, occurring in many dense repeats in the host extracellular matrix. The repetition of this bacterial motif in this particular protein may indicate its potential role in making multiple contacts with collagen. However, other instances of the motif detected by SLiMFinder only occurred once in each protein, suggesting that a single copy may be sufficient.

DISTRIBUTION OF SHORT LINEAR MOTIFS ACROSS EFFECTOR PROTEINS OF DIFFERENT SPECIES

We visualized the cross-species distribution of the SLiMFinder identified novel motifs (see **Table 3B**) among the annotated effector proteins of other species. The species were chosen to include those present in the MvirDB database that contributed motifs to the discovery, in order to display a varied set of species that could be visualized with ease. It is likely that they also exist in other organisms, although distinguishing true and false positives is not possible computationally. The visualization is normalized to correct for the fact that some species have very few proteins and that some motifs have very few instances. The total number of UPCs are indicated in brackets before each bacterial species as well as the total incidences of a motif in UPCs across all bacterial species indicated before each motif regular expression. The novel SLiMFinder identified effector protein motifs ^..I.{0,1}N, [LV].PY and ^..I[ST] are found among the effector proteins of many species, but are absent in those of many other species, including those with a reasonable number of annotated effector proteins (**Figure 2**).

We also looked at the distribution of known motifs (see **Tables 1**, **3A**) across species (**Figure 3**). While some effector motifs (see second section of **Table 1**) show a wide phylogenetic distribution, others are restricted to only a few species, such as the G.LR…T motif involved in Rho GAP function. The nuclear localization signals (at the bottom of **Figure 3**) show a relatively restricted distribution. The WEK[IM]..FF late endocytic compartment localization motif is restricted to the genus

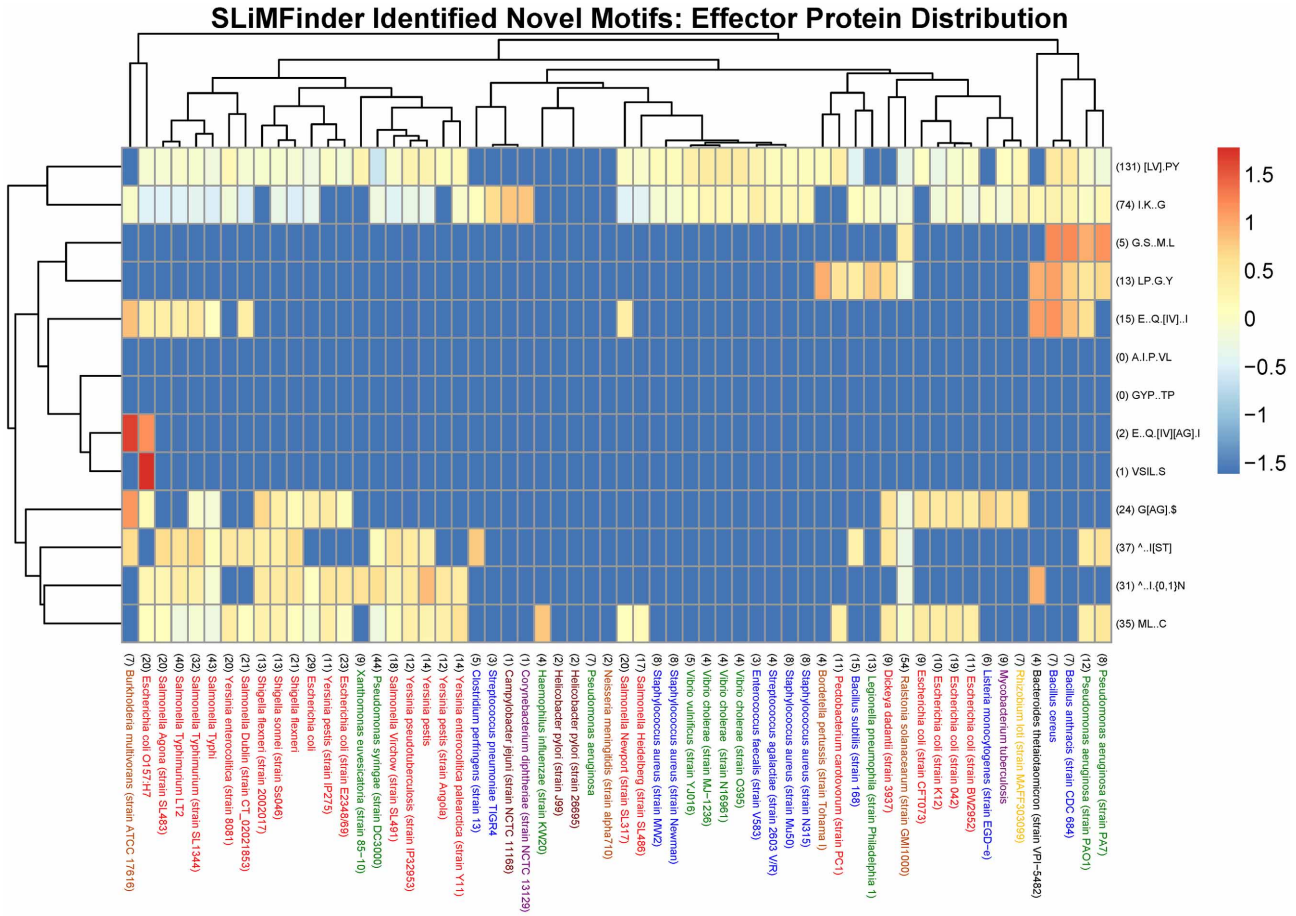

FIGURE 2 | Heat map visualization of the distribution of novel SliMFinder identified motifs amongst effector proteins from a selection of 60 organisms represented in the MvirDB. Columns: The bacterial species name with the total number of UPCs indicated in brackets at the start of the description. Purple, High GC Gram+ bacteria; Blue, Firmicutes; Yellow, a-proteobacteria; Light Brown, b-proteobacteria; Dark Brown, e-proteobacteria; Green, g-proteobacteria (non-enterobacteria); Red, g-proteobacteria (enterobacteria); Black, others (CFB). Rows: The motif regular expression with the total number of incidences in UPCs across all 60 organisms indicated in brackets at the start of the description. Color scale: The logarithm of the normalized N_UPC returned from the SLiMSearch results.

Salmonella. While the ubiquitin ligase motifs L.…TC and C.D are found in more than 71 and 199 instances respectively across the dataset whereas a number of species lack one or both of these motifs. The two SH3 binding motifs [RKY]..P..P and P..P.[KR] also show a restricted distribution. Similarly, the two PDZ binding motifs,...[ST].[ACVILF]$ and … [VLIFY].[ACVILF]$ show a restricted distribution. Bacterial effector proteins may under certain circumstances be under negative selection to avoid motifs that bind to common domains in the host such as PDZ and SH3 domains.

It can be seen that strains of a species often have very similar motif distributions (**Figures 2–4**). There is a weak but not convincing trend (**Figure 3**) for the known motif distribution among effector proteins of the Firmicutes (Blue) to group together, relative the gamma-proteobacteria (Red and Green). While the Group 2 Bacillus species, anthracis and cereus, cluster together (**Figure 3**), many sets of closely related species (**Figure 4**) do not show particularly close relationships in terms of motif distribution. This may result from two factors: firstly, motifs are highly dynamic during evolution, and secondly, factors that play a role in pathogenicity also evolve very fast. It is also difficult to compare rare vs. common motifs, since rare ones may be missed simply because of variation among proteins in the definition of effector proteins, while common motifs may be dominated by false positives that obscure the biologically relevant signals.

DISCUSSION

We believe that SLiMs are one potential class of new antimicrobial substances for the development of antimicrobial peptides and drugs. While they may lack the potency of antimicrobial peptides that damage the bacterial membrane, they may have other benefits. In particular, those that mimic peptide components of uniquely prokaryotic motifs are likely to have less off-target effects. The value of developing such therapeutic approaches depends on the range of species likely to be affected by the peptide therapeutic. While targeting eukaryotic peptides mimicked by prokaryote effector proteins provides a potential line of

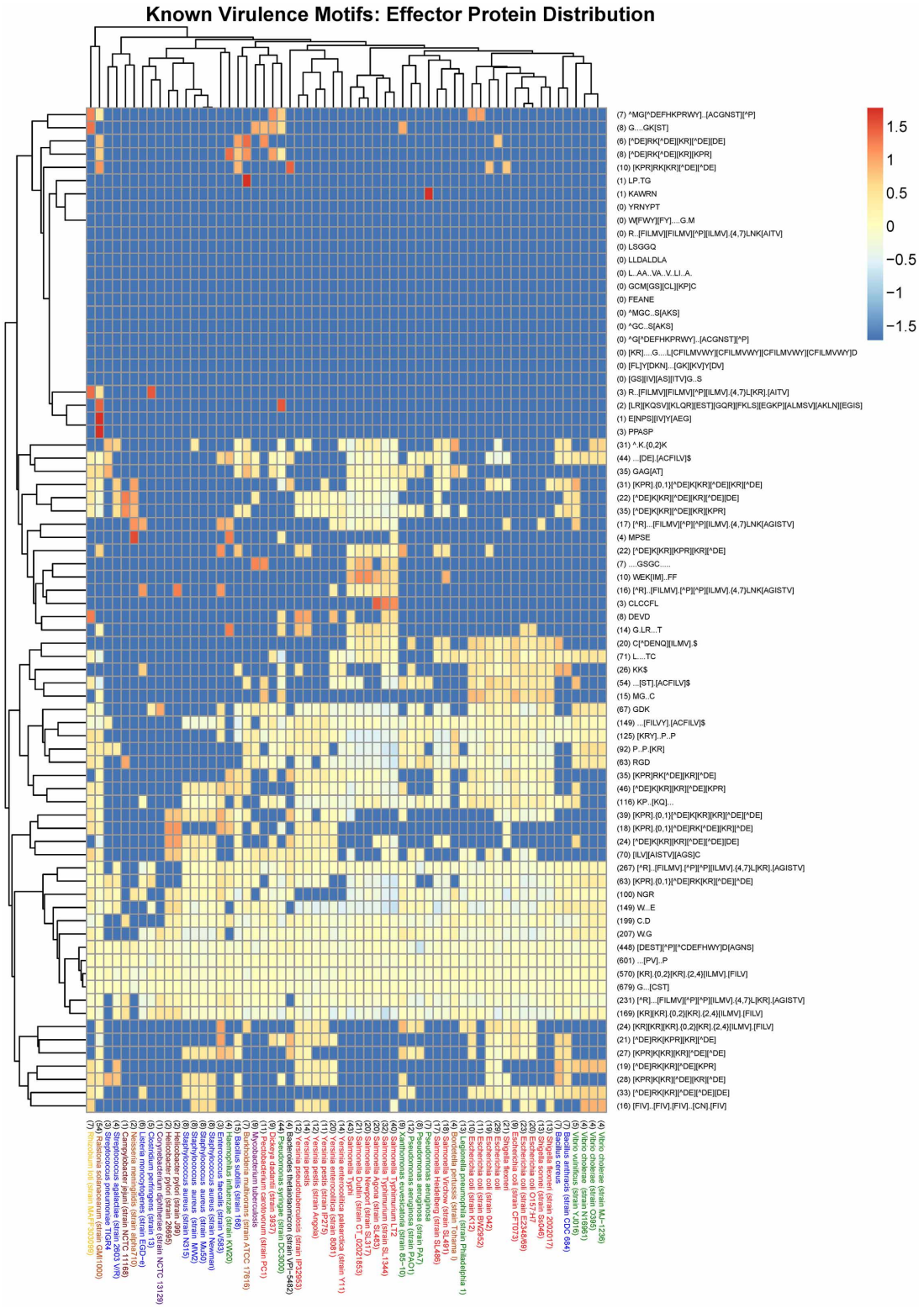

FIGURE 3 | Heat map visualization of the distribution of known virulence motifs amongst effector proteins from a selection of 60 organisms represented in the MvirDB. Columns: The bacterial species name with the total number of UPCs indicated in brackets at the start of the description. Purple, High GC Gram+ bacteria; Blue, Firmicutes; Yellow, a-proteobacteria; Light Brown, b-proteobacteria; Dark Brown, e-proteobacteria; Green, g-proteobacteria (non-enterobacteria); Red, g-proteobacteria (enterobacteria); Black, others (CFB). Rows: The motif regular expression with the total number of incidences in UPCs across all 60 organisms indicated in brackets at the start of the description. Color scale: The logarithm of the normalized N_UPC returned from the SLiMSearch results.

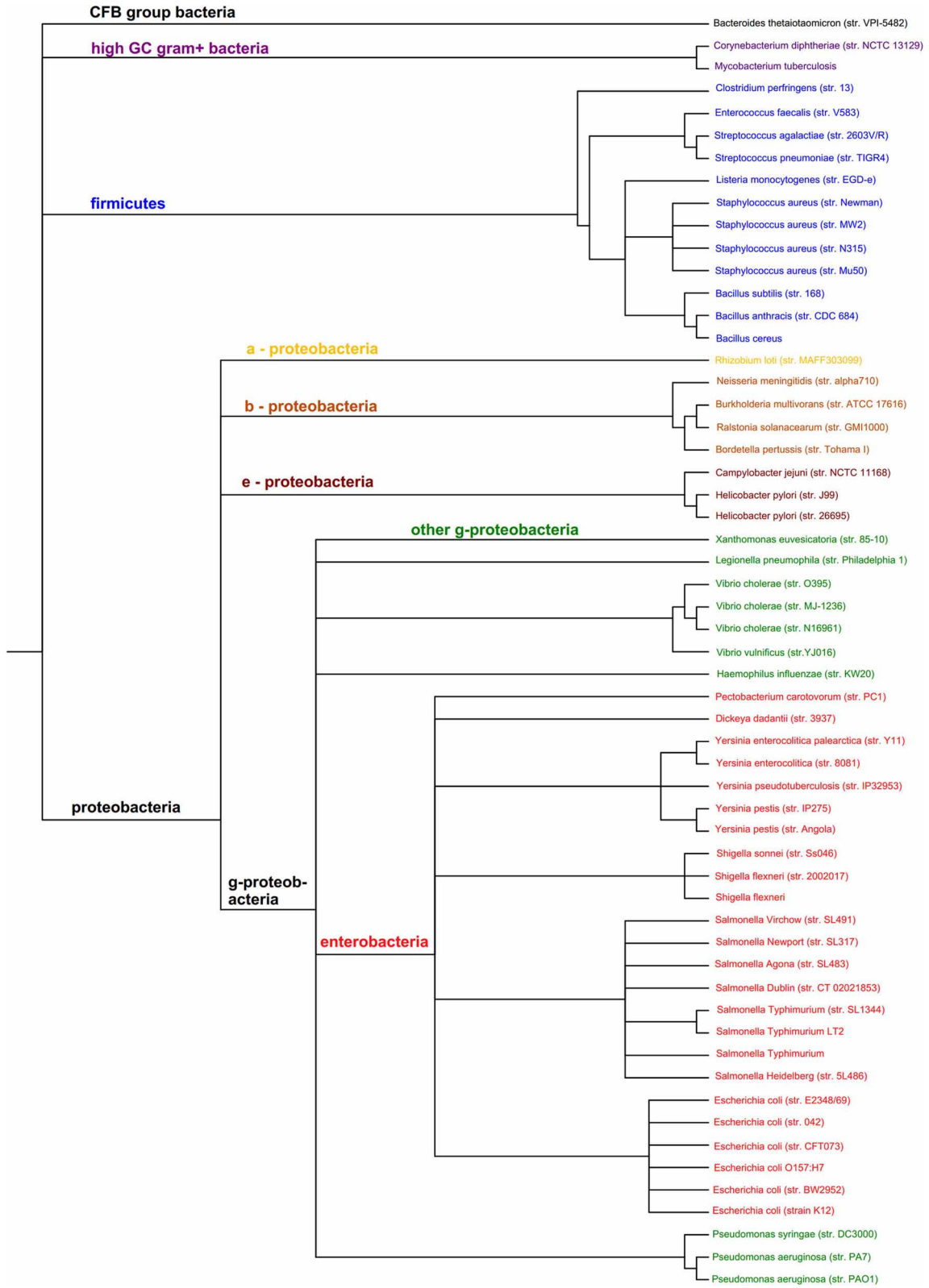

FIGURE 4 | Phylogenetic tree of the 60 organisms used to assess the distribution of prokaryotic protein motifs in Figures 2, 3. Purple, High GC Gram+ bacteria; Blue, Firmicutes; Yellow, a-proteobacteria; Light Brown, b-proteobacteria; Dark Brown, e-proteobacteria; Green, g-proteobacteria (non-enterobacteria); Red, g-proteobacteria (enterobacteria); Black, others (CFB).

attack, the evolutionary plasticity of such motifs in both bacteria (**Figure 3**) and in hosts (Neduva and Russell, 2005) suggest that bacteria can rapidly evolve alternative effector strategies to replace one targeted host component with another. Nevertheless, where such drugs are developed for other indications in treating non-infectious disease, they may also have an impact on bacterial pathogenesis and would certainly be worth investigating. This problem of evolutionary evasion by pathogens is also relevant, however, to many adhesion motifs. In order for peptide therapeutics to be more robust in the face of rapid evolution of pathogen resistance, they may need to target fundamental components of bacterial biology. Targeting aspects of the central machinery of bacterial Type IV secretion systems may be a good compromise between targeting a component that is central to pathogenicity, while not affecting the biology of advantageous bacteria in the host. In this respect, the G[AG].$ motif identified in this study is a potential candidate worthy of further investigation. Some clues as to the function of this motif may be provided by the pattern of evolution. Presumably this motif has evolved in multiple components of the Type IV secretion system because of a selection pressure for these proteins to interact with some common factor. Identifying the common interaction partners of these proteins may help in pinpointing its potential functional role. In targeting such pathogenicity systems, the benefit of focusing on recurrent motifs is that they may be small enough interaction surfaces to be feasibly targeted by peptidomimetics, and important enough that it is difficult for the bacterial system to evolve resistance (Baron and Coombes, 2007; Paschos et al., 2011).

The shortlist of predicted motifs that we have generated provides a resource for researchers interested in the mechanisms of action of virulence factor proteins across a diverse range of bacterial species. The limitations of the list are well-illustrated by the fact that the motif discovery failed to rediscover the many mimicked eukaryotic motifs. This reflects not only the fact that some motifs have not evolved multiple times in unrelated proteins, but also the limitations in the datasets provided to the SLiMFinder approach. Ideally, datasets should have less than a 100 proteins which have clearly identified similar functions. The challenge is to group proteins according to function efficiently, since the annotation of protein function is highly variable, and frequently relies on computational predictions arising from homology rather than from direct experimentation. The bigger challenge is how to test and manipulate these motifs to provide insights into the mechanisms of action and to determine potential means of interrupting pathogenic processes. While mutagenesis studies can identify the key features of motif function, targeting of a motif may also be progressed by experimental use of bioactive peptides. However, identification of more potent peptidomimetic compounds that resemble such motifs will ideally need 3D models of the peptide regions in complex with their target interactors.

What, then, is the contribution that computational screening of novel motifs may play in the discovery of novel antimicrobial peptides? Firstly, it clearly will not identify all known motifs, since patterns of recurrent evolution or of strong sequence conservation are not seen for all antimicrobial peptides. Computational screens will also have some "false positives" in two senses: firstly, statistical false positives where the motif arose simply by chance; and secondly, biological false positives where the motif that functions effectively within its biological context of a larger protein and that protein's complexes, but it will not function as a standalone synthetic peptide. This could reflect a lack of strong affinity for its targets or it could reflect an inability to be delivered to the appropriate context in the first place. Nevertheless, computational screens have the advantage that they can be performed on high throughput sequencing of organisms about which little else is known and for which biological screening by mutagenesis is painstaking or impossible. The advantage of computational prioritization is that it identifies a subset of peptides which are enriched for biologically active peptides. Clearly, the strategy we adopted here is only detecting a small fraction of known motifs, in part because of the stringent correction for statistical mismatches that could be false positives, but also because many motifs do not recur in known unrelated proteins that fall into the same functional class. Discovery for bioactive peptides could follow other strategies, including searches for evolutionary conservation (Davey et al., 2012a). However, pathogenicity factors frequently evolve rapidly, and so conservation may not be an effective signal. Bioactivity predictors based on biophysical properties within the peptide sequences are an alternative strategy (Dosztanyi et al., 2009; Thomas et al., 2010; Mooney et al., 2012, 2013). These have the disadvantage that there is no straightforward statistical approach available to determine likely false discovery rates, but are very valuable in prioritizing a list of peptides for further experimental characterization. Other computational approaches focus more on particular classes of antimicrobial peptides with a strong therapeutic potential, including ribosomal and non-ribosomal cyclic peptides (Prieto et al., 2012; Kedarisetti et al., 2014). While their computational screening methods have the benefit that they focus more strongly on peptides in classes of known therapeutic benefit, we believe that the computational screening approach we identified here complements their approaches, and widens the diversity of peptides for experimental investigation and validation.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00004/abstract

Table S1 | Protein sequence identifiers for each functional category downloaded from the MvirDB.

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JcTI-I: a novel trypsin inhibitor from *Jatropha curcas* seed cake with potential for bacterial infection treatment

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Jatropha curcas seed cake is a low-value by-product resulting from biodiesel production. The seed cake is highly toxic, but it has great potential for biotechnology applications as it is a repository of biomolecules that could be important in agriculture, medicine, and industry. To explore this potential, a novel trypsin inhibitor called JcTI-I was purified by fractionation of the crude extract with trichloroacetic acid (2.5%, v/v) followed by affinity chromatography (Trypsin-Sepharose 4B) and molecular exclusion (Sephacryl S-200). Non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration showed that JcTI-I has approximately 20.0 kDa. Mass spectrometry analysis revealed that the intact molecular mass of JcTI-I is 10.252 kDa. Moreover, JcTI-I is a glycoprotein with 6.4% (m/m) carbohydrates, p*I* of 6.6, N-terminal sequence similarity around 60% to plant albumins and high stability to heat, pH, and salinity. JcTI-I presented antibacterial activity against the human pathogenic bacteria *Salmonella enterica* subspecies *enterica* serovar choleraesuis and *Staphylococcus aureus*, with minimum inhibitory concentration less than 5 μg/mL. Furthermore, JcTI-I did have inhibitory activity against the serine proteases from the tested bacteria. Otherwise, no hemolytic activity of human erythrocytes and signs of acute toxicity to mice were observed for JcTI-I. The results demonstrate the benefits of *J. curcas* seed cake as a source of trypsin inhibitor with potential for biotechnological application as a new antimicrobial agent against human pathogenic bacteria.

Keywords: *Jatropha curcas*, seed cake, serine proteinase inhibitor, trypsin inhibitor, bacterial infections, antimicrobial agent

INTRODUCTION

Hospital-acquired infection is the major cause of death in critically ill patients, both in developing and developed countries (Ramsamy et al., 2013). These infections are a challenge to patient safety because the causal agents have developed antibiotic-resistance, thus limiting treatment options (Mulvey and Simor, 2009; Becker et al., 2012). For example, both the methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Salmonella enterica* are the principal multidrug resistant bacterial pathogens that cause serious community and hospital-acquired infections, responsible for high annual health care costs and psychological stress associated with social stigma (Cosgrove et al., 2003; Brydon et al., 2009; Broughton et al., 2010). Therefore effective new therapeutic agents with novel mechanisms of action for treatment of infections caused by multidrug resistant bacteria are urgently needed (Hughes et al., 2012). In this context, as plants have numerous therapeutic compounds they constitute natural targets from which new antibacterial drugs with high efficacy and less toxicity can be developed to treat infectious diseases (Ngo et al., 2013). Indeed, biologically active compounds from plant resources have been extracted from different species (Savoia, 2012).

Jatropha curcas, also known as physic nut, is a shrub belonging to the Euphorbiaceae family. This species is highly adaptable to adverse conditions and is resistant to many pests and pathogens (Debnath and Bisen, 2008; Sabandar et al., 2013). In addition, *J. curcas* seeds constitute an oil-rich plant source from which biodiesel is produced (Rashid et al., 2010). After oil extraction by screw press, the remaining seed cake is highly toxic to a number of animal species, probably due to the presence of phorbol esters and curcin, a type-I ribosome inactivating protein (Goel et al., 2007; Zhao et al., 2012). Protease inhibitor, lectin, and phytate are also present in high amounts (Saetae and Suntornsuk, 2011). Nevertheless, these compounds could be isolated and characterized to exploit their possible medicinal applications, as it was suggested that the *J. curcas* seed cake could be utilized as a source of antibacterial and antifungal agents (Sundari and Selvaraj, 2011).

Among these various molecules present in *J. curcas* seed cake, the protease inhibitors could be a potentially novel class of antimicrobial agents, as they specifically inhibit the catalytic action of enzymes by formation of stoichiometric complex with the target enzymes, blocking or altering its active site (Kim et al., 2009; Volpicella et al., 2011). In fact, protease inhibitors are found to be involved in various important physiological functions like regulators of endogenous proteinases and defense mechanism (Bhattacharjee et al., 2012). However, protease inhibitors also have received new interest due to their biological properties with

potential for use as clinical agents. Of importance in the context of seeking plant protease inhibitors as novel therapeutic agents is that Xb-KTI, a Kunitz trypsin inhibitor present in *Xanthosoma blandum* corms with bactericidal activity (Lima et al., 2011). Similarly, the fistulin, a naturally occurring inhibitor of serine protease present in *Cassia fistula* leaves, showed to be very active against several pathogenic bacterial strains, namely, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, and *Klebsiella pneumoniae*, and its efficacy was comparable to the standard drug, streptomycin sulfate (Arulpandi and Sangeetha, 2012).

To the best of our knowledge, a trypsin inhibitor from *J. curcas* seed cake has never been isolated neither its antibacterial activity tested yet. Thus, the present paper describes the purification and physicochemical characterization of a novel trypsin inhibitor from *J. curcas* seed cake, designated JcTI-I. Additionally, to gain better insights on the biological activity of this protein and to devise future use as a new therapeutic drugs, its inhibitory activity against the growth of the human pathogen bacteria *Staphylococcus aureus* and *Salmonella enterica*, as well as the ability of JcTI-I to inhibit the bacterial proteases were evaluated.

MATERIALS AND METHODS

MATERIALS

Jatropha curcas seed cake was obtained from Instituto Fazenda Tamanduá (Paraíba, Brazil), grounded in a coffee grinder and passed through a 1-mm-mesh screen. The resulting flour was treated with *n*-hexane (1:5, m/v) to remove the remaining oil left after biodiesel extraction. Defatted flour was stored in air-tight containers at 4°C until analysis. The bacteria *Salmonella enterica* subspecies *enterica* serovar choleraesuis (ATCC 10708), *Bacillus subtilis* subspecies *spizizenii* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 25619), and *Staphylococcus aureus* (ATCC 25923) were obtained from the Department of Biology (UFC), Fortaleza, Brazil. Swiss mice (*Mus musculus*), 20–25 g, were from the animal house at UFC. Azocasein, bovine pancreatic trypsin, bovine pancreatic chymotrypsin, bovine serum albumin (BSA), soybean trypsin inhibitor (SBTI), *N*α-benzoyl-D,L-arginina-*p*-naftilamida (BANA), 4-(dimethylamino)cinnamaldehyde (DMACA), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), L-cysteine and *N*α-benzoyl-D,L-arginine 4-nitroanilide hydrochloride (BAPNA), sodium dodecyl sulfate (SDS), molar mass markers, acrylamide, bis-acrylamide, dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chromatographic matrixes were from GE Healthcare. All other chemicals and reagents used were of analytical grade.

PROTEIN DETERMINATION

The method described by Bradford (1976) was used with BSA as standard. Absorbance at 280 nm was also used to detect the presence of protein in the chromatographic eluates.

TRYPSIN INHIBITION ASSAY

Trypsin inhibitory activity against bovine trypsin (EC 3.4.21.4) was performed as described by Erlanger et al. (1961) using the chromogenic substrate BAPNA. The enzyme concentration used was established based on a dependent concentration assay and was that producing an absorbance between 0.25 and 0.30 in the enzymatic assay and reaction rate corresponding to $\frac{1}{2}V_{max}$. The inhibitory assay mixture consisted of 100 μL of crude extract (4.74 mg/mL) or the purified inhibitor (0.03 mg/mL) dissolved in distilled water, incubated with 10 μL of trypsin (0.3 mg/mL in 10^{-3} M HCl) and 690 μL of 0.05 M Tris–HCl/0.02 M $CaCl_2$, pH 7.5, at 37°C for 10 min. Next, 500 μL of 1.25 × 10^{-3} M BAPNA dissolved in the above buffer were added and the mixture further incubated for 15 min, 37°C. Reaction was stopped by addition of 120 μL of 30% (v/v) acetic acid. The enzymatic activities in the absence and presence of the inhibitor were evaluated by *p*-nitroanilide release from BAPNA measured at 410 nm using a Biochrom Libra S-12 spectrophotometer. One trypsin inhibitory activity unit (TIU) was defined as the decrease in 0.01 U of absorbance per 15 min assay, at 37°C. Appropriate blanks for the enzyme, inhibitor and the substrate were also included in the assay along with the test samples.

PREPARATION OF THE PROTEIN EXTRACT

Defatted flour was extracted with 0.1 M borate buffer, pH 10.0, in a proportion of 1.0 g of meal to 10.0 mL of buffer for 2 h under sonication, at 4°C. Next the suspension was maintained under constant stirring for 2 h at 4°C and filtered through cheesecloth. The filtrate was centrifuged at 10,000 × *g*, 4°C, 30 min and the clear supernatant, denoted crude extract, was dialyzed (cut-off 12 kDa) against 0.050 M sodium phosphate buffer, pH 7.5. The protein content and trypsin inhibitory activity of this dialyzed extract were determined and it was further used for purification of the trypsin inhibitor as described below.

PURIFICATION OF *J. curcas* TRYPSIN INHIBITOR

The crude extract prepared as described in Section "protein Determination" was fractionated by precipitation with 2.5% (v/v) trichloroacetic acid (TCA) final concentration, at 4°C and centrifuged at 14,000 × *g*, 4°C, 30 min. The clear supernatant obtained was dialyzed exhaustively against water (Milli-Q grade), lyophilized, and assayed for antitrypsin activity. The TCA fraction (30 mg) was dissolved in 0.050 M sodium phosphate buffer/0.2 M NaCl, pH 7.5 and applied to a trypsin-Sepharose 4B column (11.5 cm × 2.2 cm) equilibrated with the above buffer. After complete removal of the non-retained proteins with the equilibrating buffer, the proteins bound to the immobilized trypsin were eluted with 0.1 M HCl, dialyzed exhaustively against water (Milli-Q grade) and lyophilized. This material (2 mg) was loaded on a Sephacryl S-200 column connected to an ÄKTA-Prime System (GE Healthcare) previously equilibrated and eluted with 0.050 M sodium phosphate buffer/0.2 M NaCl, pH 7.5. Fractions (1 mL) were eluted at the flow rate of 0.5 mL/min and the protein fractions obtained evaluated for trypsin inhibitory activity as described before. The purified trypsin inhibitor was named JcTI-I (*J. curcas* Trypsin Inhibitor I).

CHARACTERIZATION OF JcTI-I

Molecular mass determination

The apparent molecular mass of JcTI-I was determined by denaturing electrophoresis [SDS-polyacrylamide gel electrophoresis

(SDS-PAGE); Laemmli, 1970], in 12.5% (m/v) polyacrylamide gels (10 cm × 8 cm). Samples were prepared in 0.5 M Tris–HCl buffer, pH 6.8, containing 1% SDS, in the presence or absence of 1% β-mercaptoethanol and boiled at 98°C, for 5 min, before electrophoresis, which was performed at 20 mA. Protein bands were stained with Coomassie Brilliant Blue G-250 (Candiano et al., 2004). The native molecular mass of JcTI-I (3 mg) was determined by gel filtration on Sephacryl S-200 column, coupled to an ÄKTA-Prime System (GE Healthcare) and equilibrated with 0.050 M sodium phosphate buffer/0.2 M NaCl, pH 7.5. Chromatography was carried out at a constant flow rate of 0.5 mL/min and 1 mL fractions were collected. Before loading on the column, the sample was centrifuged at 14,000 × *g*, 4°C, for 5 min and the supernatant filtered through a Pro-X TM filter unit (0.22 mm hydrophilic cellulose acetate membrane). The column was previously calibrated with proteins of known molecular mass (BSA, 66 kDa; egg albumin, 45 kDa; chymotrypsinogen, 25 kDa; ribonuclease, 13.7 kDa, and aprotinin, 6.5 kDa).

In addition, native mass was obtained by mass spectrometry analysis of JcTI-I (0.1 mg/mL) dissolved in water/acetonitrile (1:1, v/v). The intact mass spectra was acquired in a Synapt G1 HDMS Acquity UPLC instrument (Waters Co., Milford, MA, USA) programmed with a RF *offset* (MS profile) adjusted such that the LC/MS data were effectively acquired from *m*/*z* 400 to 3,000, which allowed to obtain multiply charged mass ions. Analysis was performed using a nanoelectrospray ionization in positive ion mode (ESI+) and a NanoLockSpray source. For all measurements, the mass spectrometer was operated in the "V" mode with a resolving power of at least 10,000 *m*/*z*. The data collection was performed using MassLynx 4.1 software (Waters Co., Milford, MA, USA) and charge distribution spectra were then deconvoluted by the Maximum Entropy Technique (MaxEnt). To assess whether JcTI-I is composed of subunits covalently linked by disulphide bridges it was previously treated with 0.1 M DTT followed by 0.3 M iodoacetamide and trypsin digestion to be analyzed by capillary liquid chromatography/nanoelectrospray ionization tandem mass spectrometry (ESI–LC–MS/MS), using the same equipment as described above, coupled with a nano-high-performance liquid chromatography (UPLC) unit (Waters Co., Milford, MA, USA). The reduced and alkylated JcTI-I was injected using the nanoAcquity UPLC sample manager and the chromatographic separation was performed using an UPLC C18 column (75 μm × 10 cm) with a flow of 0.35 μL/min. The mass spectra were acquired using a data-dependent acquisition (DDA) methodology, where the three top peaks were subjected to MS/MS. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. Column was equilibrated with 3% solution A and the gradient conditions used were as follows: 3–40% B for 20 min, 40–70% B for 40 min, 70% for further 50 min and 70-3% of B for 10 min. The data were processed using the Protein Lynx Global Server (Waters Co., Milford, MA, USA) software and the searches made with the assumption that there was a maximum of one missed trypsin cleavage, peptides were mono-isotopic, methionine residues were partially oxidized and cysteine completely carbamidomethylated.

Carbohydrate content

The neutral sugar content of JcTI-I-I was estimated by the method of Dubois et al. (1956) using galactose as standard. To 250 μL of JcTI-I-I (0.20 mg/mL), 250 μL of phenol 5% (v/v) and 1.25 mL of concentrated sulfuric acid were added. The mixture was stirred and left at 25°C for 30 min. Readings were taken at 490 nm. The carbohydrate content was estimated based on a calibration curve built with different concentrations of galactose. The value is expressed in percentage (%) of carbohydrate per protein mass. To confirm the presence of covalently bound carbohydrate in JcTI-I a specific staining of the protein bands after SDS-PAGE was carried out (Zacharius et al., 1969). Briefly, after the electrophoresis run the gel was fixed in a solution of 7.5% (v/v) acetic acid for 2 h, followed by incubation in a solution of 0.2% (v/v) periodic acid, at 4°C for 45 min and incubation with the Schiff reagent at 4°C for further 45 min. The glycoprotein nature of the band was revealed by immersion of the gel in a solution of 0.5% (m/v) potassium metabisulfite in 0.05 M HCl.

N-terminal sequence analysis

This was established in a Shimadzu PPSQ-10 Automated Protein Sequencer performing Edman degradation. Phenylthiohydantoin (PTH) amino acids were detected at 269 nm after separation on a reversed phase C_{18} column (4.6 mm × 2.5 mm) under isocratic conditions, according to the manufacturer's instructions. Percentage sequence identity with trypsin inhibitors was searched for automatic alignment, performed using the NCBI-BLAST search system (Altschul et al., 1990).

Effect of temperature, pH, and salt

This was evaluated according to Klomklao et al. (2011). JcTI-I (0.035 mg/mL) was diluted with distilled water to obtain 60–70% trypsin inhibition. For thermal stability assay, JcTI-I aliquots were incubated at 90°C for 0, 10, 20, 30, 40, 50, 60, 90, and 120 min and then cooled in ice-water. The trypsin inhibitory activity was determined and reported as the percentage of the residual activity compared to 70% trypsin inhibition. The effect of pH on JcTI-I stability was evaluated by measuring the residual activity after incubation at various pH values for 30 min at 25°C. The buffers used were: 0.05 M glycine-HCl, pH 2.2; 0.05 M sodium acetate, pH 5.2; and 0.1 M sodium borate, pH 10.0. The stability of JcTI-I to salinity was tested by incubating the trypsin inhibitor in NaCl solutions ranging from 0% up to 3% (m/v) concentration, at 25°C for 30 min. After treatment the inhibitory activity against trypsin was determined as previously described and the residual inhibitory activity reported.

Enzyme specificity

The papain inhibitory assay was performed as described by Abe et al. (1992), using BANA as substrate. The enzyme concentration used was established based on a dependent concentration assay and was that producing an absorbance between 0.25 and 0.30 in the enzymatic assay and reaction rate corresponding to $½V_{max}$. To 60 μL of papain (0.02 mg/mL in 0.25 M sodium phosphate buffer, pH 6.0), 40 μL of the activation solution (2×10^{-3} M EDTA + 3×10^{-3} M DTT, pH 6.0), 200 μL of JcTI-I, and 200 μL of 0.25 M sodium phosphate buffer, pH 6.0, were added. The

mixture was incubated for 10 min at 37°C. The reaction was initiated by addition of 200 μL 0.001 × 10^{-3} M BANA, prepared in 1% (v/v) dimethyl sulfoxide (DMSO) and 0.025 M sodium phosphate buffer, pH 6.0. After 20 min at 37°C, the reaction was stopped by addition of 500 μL 2% (v/v) HCl in 95% (v/v) ethanol. The color product was developed by adding 500 μL of 0.06% (m/v) DMACA in ethanol and the absorbance was measured at 540 nm. The chymotrypsin inhibitory activity was measured by the method of Erlanger et al. (1961), using azocasein as substrate. Twenty microliters of chymotrypsin (0.1 mg/mL in 2.5 × 10^{-3} M HCl) was pre-incubated with JcTI-I (0.2 mg/mL) and 380 μL of 0.05 M Tris–HCl pH 7.5, for 15 min, 37°C. Next, 1% (m/v) azocasein was added to the mixture and incubated for 30 min. Reaction was stopped by addition of 300 μL 20% (v/v) TCA. After centrifugation (10,000 × *g*, 10 min, 25°C), aliquots were withdrawn from the supernatants and added to 2 M NaOH, in an appropriate proportion, and the absorbance taken at 440 nm.

IC$_{50}$ *value and kinetic studies*

The amount of JcTI-I needed for 50% inhibition of trypsin activity was determined as previously described in Section "Trypsin Inhibition Assay," but using the inhibitor in the concentration range of 1.0 × 10^{-7} to 2.0 × 10^{-7} M. Kinetic studies of protease inhibition by JcTI-I were conducted according to Bijina et al. (2011), with minor modifications, using bovine trypsin (molecular weight 23.3 kDa) and different concentrations of BAPNA (8.0 × 10^{-5} to 1.6 × 10^{-3} M). To calculate the kinetic parameters, JcTI-I at 5 × 10^{-7} and 10 × 10^{-7} M concentrations and 8.0 × 10^{-6} M trypsin were previously incubated in 0.05 M Tris–HCl/0.02 M $CaCl_2$, pH 7.5, for 10 min, at 37°C, to reach the assay temperature. Then, 100 μL fractions of JcTI-I were added to 10 μL of the trypsin solution and further incubated for 10 min, at 37°C. Next, both the trypsin solution and trypsin + JcTI-I mixture were added separately to the BAPNA solutions and incubated at 37°C, for 15 min. Reaction was stopped by addition of 120 μL of 30% (v/v) acetic acid. The release of *p*-nitroanilide by the action of trypsin on BAPNA was measured at 410 nm as before. The velocity of enzyme reaction (v) was determined from progress curves using different BAPNA concentrations on the base of product concentrations measured at 410 nm at a fixed time of a reaction. To calculate K_m (Michaelis–Menten constant) and V_{max} (maximal rate) of the reaction and to study the pattern of inhibition, data were plotted as a function of BAPNA concentration using the usual non-linear curve fitting of Michaelis–Menten and linear Lineweaver–Burk, respectively. A secondary plot of $1/K_m$ versus JcTI-I concentrations was also drawn and the X-intercept used to calculate the dissociation constant (K_i; Cornish-Bowden, 1995).

ANTIMICROBIAL ACTIVITY

In vitro antibacterial activity

The antibacterial activity (Hancock, 2000) of JcTI-I was tested *in vitro* against *Salmonella enterica*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*. The pathogenic bacteria were grown to mid-logarithm phase in 5 mL of Mueller–Hinton broth medium, at 37°C. JcTI-I, at a 500 μg/mL final concentration, was prepared in 0.25 M sodium phosphate buffer, pH 7.5, and sterilized using a 0.22 μm membrane, was incubated in 96-well flat microplates (Nunc) with 100 μL of each bacterial suspension (10^5–10^6 CFU/mL) dissolved in the growth broth for 4 h at 37°C. Bacterial growth was monitored at 630 nm, every hour within the incubation period, using an automated microplate reader (Bio-TekElx800). The cell growth of both bacteria in the absence of JcTI-I was monitored as a blank control. Experiments were run in triplicate. To determine the minimum inhibitory concentration (MIC), JcTI-I, at 5–500 μg/mL concentration, was dissolved in 0.25 M sodium phosphate buffer, pH 7.5 and 100 μL incubated with equal volume of the bacterial cell suspension (10^5–10^6 CFU) in wells of a 96-well polypropylene plate. The plates were kept for 4 h at 37°C and absorbance readings recorded at 630 nm every hour.

Activity of JcTI-I against the Salmonella enterica and Staphylococcus aureus proteases

The capacity of JcTI-I to inhibit the secreted and endogenous bacterial proteases was done following the methodology described by Lima et al. (2011) with minor modifications. *Salmonella enterica* and *Staphylococcus aureus* were cultured in 5 mL of Mueller-Hinton broth overnight, at 37°C, and subcultured in 300 mL of Mueller-Hinton broth, at 37°C and 240 rpm, until the log phase was reached, as measured at 630 nm (Bio-TekElx800 reader). The bacterial cell cultures were collected at the late exponential growth phase by centrifugation at 4,000 × *g*, for 15 min, at 4°C and the pellet resuspended in the protease extraction solution [HCl 0.1% (v/v) + 5.0 × 10^{-3} M Tris + 5.0 × 10^{-3} M $CaCl_2$ + 0.1% (v/v) Triton X-100, pH 7.5] and incubated in ice bath for 15 min. Freezing and thawing took place three times, followed by centrifugation at 4,000 × *g*, for 30 min, at 4°C. The supernatants obtained, containing the protease from *Salmonella enterica* and *Staphylococcus aureus*, were pooled separately for every bacterium species and the serine protease activity as well as the inhibitory activity of JcTI-I assayed, according to the method previously described, using azocasein as substrate, bovine trypsin and PMSF as serine protease control inhibitor.

HEMOLYTIC ACTIVITY ASSAY

The hemolytic assay, performed in triplicate, was carried out as previously described (Kim et al., 2006), with minor modifications. Human red blood cells were collected in the presence of heparin, washed three times with phosphate-buffered saline (PBS; 3.5 × 10^{-2} M phosphate buffer containing 0.15 M NaCl, pH 7.0) and recovered by centrifugation in the same buffer. The hemolytic activity of JcTI-I at 5–500 μg/mL concentration was evaluated by measuring the release of hemoglobin from fresh human erythrocytes. Aliquots (25 μL) of a 2.5% suspension of red blood cells were transferred to 96-well plates and incubated with 25 μL of JcTI-I for 30 min, at 37°C followed by centrifugation at 5,000 × *g*, for 5 min. Hemolysis (%) was determined by measuring the supernatant absorbance at 540 nm. PBS (0.05 M, pH 7.4) and 0.1% (v/v) Triton X-100 were used, respectively, as negative (0% hemolysis) and positive (100% hemolysis) controls.

TOXICITY ASSAY

The toxicity assay was reviewed and approved by the Animal Ethics Committee (CEPA) of UFC, Brazil, and realized according to the methodology described by Vasconcelos et al. (1994). Toxic activity was defined as mortality observed in Swiss mice within 24 h after intraperitoneal injections of JcTI-I at varied concentrations.

STATISTICAL ANALYSIS

The results are expressed as the mean ± SEM. The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. The results were considered to be significant at $P < 0.05$.

RESULTS

PURIFICATION OF JcTI-I

Purification of JcTI-I encompassed TCA precipitation of the *J. curcas* protein extract followed by two additional chromatography steps. TCA, at 2.5% (v/v) final concentration, precipitated the proteins with a recovery of up to 35.6% and 29.0-fold purification of the trypsin inhibitor compared to the protein extract (**Table 1**). This TCA precipitated fraction was chromatographed on a trypsin-Sepharose 4B affinity column and the adsorbed proteins, eluted with 0.1 M HCl, concentrated the inhibitory activity that was specific for trypsin (**Figure 1A**), indicating that this chromatography step was important to exclude other protease inhibitors also present in the TCA precipitated fraction, as for papain (data not shown). At this stage, the trypsin inhibitor was purified to 331.5-fold with a protein yield of 1.8% and specific activity of 6.63×10^{-3} TIU/mg protein (**Table 1**). The fraction obtained from the trypsin-Sepharose 4B affinity chromatography displaying trypsin inhibitory activity was composed of multiple protein bands as evaluated by SDS-PAGE (**Figure 1A**; insert: lane 3). Therefore, this fraction was further purified on a Sephacryl S-200 column (**Figure 1B**). The eluted protein present in the major peak showed a specific activity of 28.35×10^{-3} TIU/mg protein, 1417.5-fold purification, but a low protein yield of 1.2%. Nevertheless, it was homogeneous by SDS-PAGE as it presented a unique protein band with a relative molecular mass around of 20.0 kDa (**Figure 1B**, insert: lane 2). This purified protein is a trypsin inhibitor of *J. curcas* seed cake that was denominated JcTI-I thereafter.

CHARACTERIZATION OF JcTI-I

Molecular mass determination, pI, carbohydrate content, and N-terminal sequence

Native molecular mass of JcTI-I was assessed by gel filtration chromatography on Sephacryl S-200 column. A molecular mass of 20.2 kDa was calculated for the inhibitor dissolved in 0.05 M

Table 1 | Purification steps of a trypsin inhibitor from *J. curcas* cake.

Steps	Total protein[a] (mg)	Total activity[b] (TIU × 10^{-3})	Specificity activity (TIU/mg protein × 10^{-3})	Yield[c] (%)	Purification index[d]
Crude extract	165.6	4.1	0.02	100	1.0
$F_{2.5}$ (TCA)	59.0	34.4	0.58	35.6	29.0
Trypsin-Sepharose 4B	3.0	19.9	6.63	1.8	331.5
Sephacryl S-200	2.0	56.7	28.35	1.2	1417.5

Results are presented as mean values of three replications.
[a] *The total amount of protein recovered from 1 g of defatted flour from J. curcas seed cake.*
[b] *One trypsin inhibitory activity unit (TIU) was defined as the decrease in 0.01 U of absorbance per 15 min assay, at 37° C.*
[c] *The recovery of protein at each purification step (crude extract, 100%).*
[d] *Purification index is calculated as the ratio between the specificity activity obtained at each purification step and that of the crude extract taken as 1.0.*

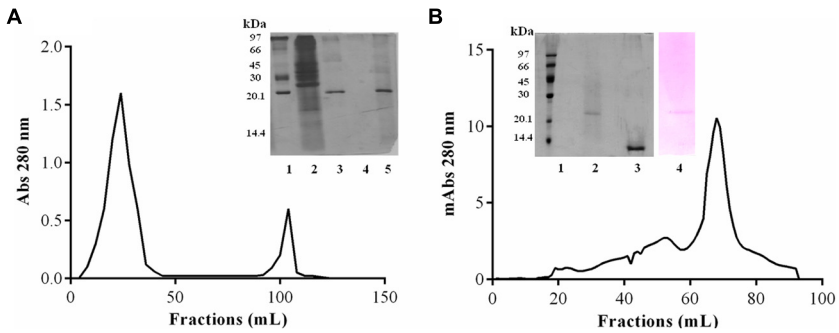

FIGURE 1 | JcTI-I purification. (A) Affinity-chromatography (Trypsin-Sepharose 4B column). Inset: SDS-PAGE: (1) molar mass markers; (2) crude extract; (3) 2.5% TCA fraction; (4) unbound fractions; (5) bound fractions. **(B)** Size exclusion chromatography (Sephacryl S-200 column) of JcTI-I purified after chromatography on Trypsin-Sepharose 4B column. Inset: SDS-PAGE: (1) molar mass markers; (2 and 3) JcTI-I (5 μg) in the absence and presence of 1% β-mercaptoethanol, respectively; (4) JcTI-I (5 μg) stained with Schiff's reagent.

sodium phosphate buffer, pH 7.5, containing 0.2 M NaCl. This value is very similar compared to the molecular mass obtained by SDS-PAGE in the absence of reducing agents (**Figure 1B**, insert: lane 2). However, ESI mass spectrometry under native conditions, with JcTI-I dissolved in water/acetonitrile (1:1, v/v), revealed a 10.252 kDa polypeptide (**Figure 2A**) which is very close to the molecular mass of JcTI-I found under denaturing condition in the presence of β-mercaptoethanol (**Figure 1B**; insert: lane 3) and after two-dimensional (2D) gel electrophoresis that also showed a p*I* of 6.6 (data not shown) for the purified inhibitor. DTT reduced and alkylated JcTI-I produced two derived peptides of 7.133 and 3.124 kDa, after MS/MS analysis, suggesting that these polypeptides are linked by disulfide bond (**Figures 2B,C**, respectively). Moreover, JcTI-I has 6.4% covalently linked carbohydrate (**Figure 1B**; insert: lane 4) and an N-terminal sequence of VRDICKKEAERRDLSSCENYITQRRGY (**Table 2**). This sequence was confirmed after mass spectrometry analysis of JcTI-I (**Table 3**). Alignment of this N-terminal sequence with known sequences in the NCBI non-redundant database showed 68 and 58% similarity with an allergenic 2S albumin from *J. curcas* seeds and with an albumin 2S from *Ricinus communis*, respectively. In addition, other peptide sequences were obtained similar to 2S albumins, particularly from *R. communis* (**Table 3**). However, no similarity was found between the N-terminal sequence of JcTI-I generated by Edman degradation and the peptide sequences obtained by MS/MS analysis with known members of the protease inhibitor family.

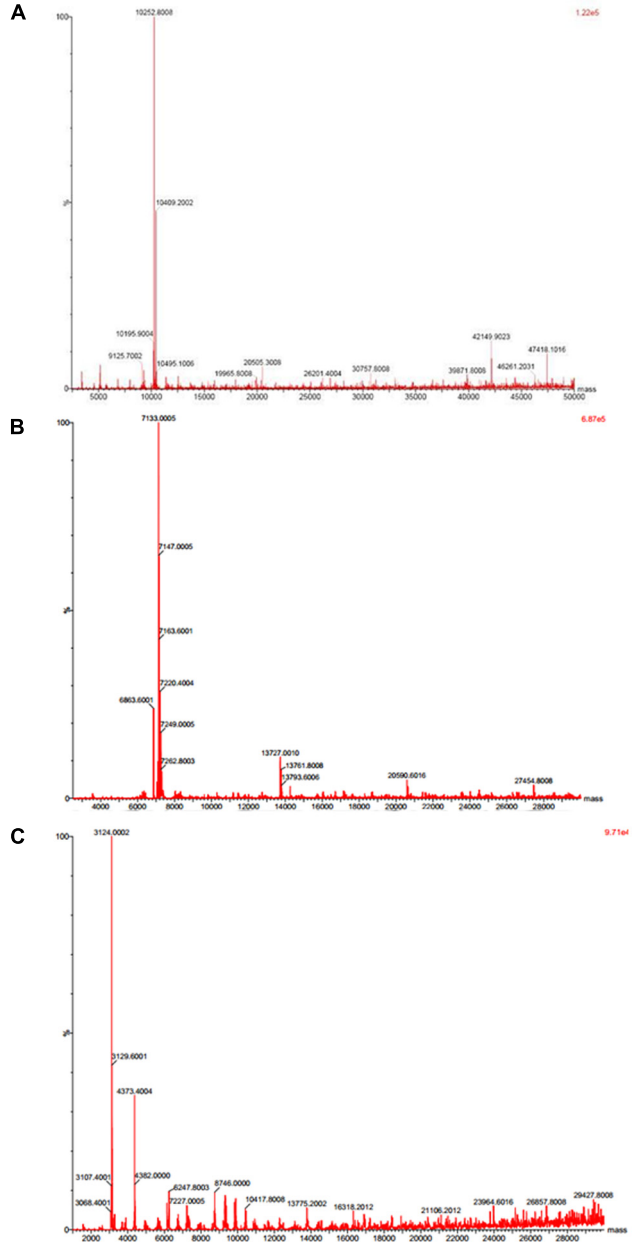

FIGURE 2 | Mass spectrometry analysis of JcTI-I. (A) Native JcTI-I; **(B,C)** JcTI-I previously reduced and alkylated with DTT and iodoacetamide, respectively.

Thermal and pH stability

Heating of JcTI-I at 90°C for up to 20 min induced a slight increased in the inhibitory activity upon trypsin as compared to 70% trypsin inhibition of the native inhibitor (**Figure 3A**). Afterward the heat treatment for up to 120 min had little consequence on the inhibitory effect of JcTI-I as it was reduced only to around 4–5% of that of the untreated inhibitor. Incubation of JcTI-I at the pH range of 2.0–10.0 (**Figure 3B**) had overall a slight inducing effect (10%) on inhibition (**Figure 3B**), except at pH 5.0 that maintained 70% trypsin inhibition. Incubation of JcTI-I with different concentrations of salt (0–3%) showed that the protein retained approximately its original activity up to 2.0% NaCl concentration (**Figure 3C**). However, at 2.5% NaCl concentration an 11.6% reduction in the inhibitory activity was noticed (**Figure 3C**), whereas at 3.0% the trypsin inhibition was 10% higher than the 70% inhibition induced by the untreated inhibitor.

Enzyme specificity

JcTI-I was specific for inhibiting bovine trypsin, but did not inhibit significantly chymotrypsin and papain.

IC_{50} value and kinetic parameters

The data obtained for the studies conducted on protease–protease inhibitor interaction is depicted in **Figure 4**. The amount of inhibitor needed for 50% trypsin inhibition (IC_{50}) was 1.25×10^{-6} M. Moreover, it is calculated that the stoichiometry of trypsin–protease inhibitor interaction is 1:1.7. Kinetic studies of inhibition of trypsin by JcTI-I showed that plots of a fixed concentration of trypsin (8.0×10^{-6} M) preincubated with the buffer alone (control) and with 0.5 and 1.0×10^{-6} M of JcTI-I produced different slopes of $1/v$ versus $1/[s]$ (**Figure 5**). These data indicated a non-competitive mechanism of action of JcTI-I as lines are converging to the same point on the $1/[S]$ axis where K_m is unchanged, whereas V_{max} is reduced. Furthermore, calculated K_i (**Figure 5**, insert) was on average 0.24×10^{-6} M, indicating that inhibition of the trypsin hydrolysis by JcTI-I occurred at very low concentration characterizing a very potent inhibitor.

Table 2 | Comparison of N-terminal sequence of JcTI-I with similar protein sequences.

Protein	Species	N-terminal sequence	Identity (%)	Access number[a]
JcTI-I	*Jatropha curcas*	VRDICKKEAERRDLSSCENYITQRRGY		C0HJF7[b]
Jat c 1[c]	*Jatropha curcas*	VRDKCGEEAERRTLXGCENYISQRR	68	
Precursor albumin 2S	*Ricinus communis*	CRQEIQRKDLSSCEQYIRQSSSRR	58	XP_002522851
Glycine-rich protein	*Arabidopsis thaliana*	DIAKKKTKKKDLSSSDNYFTKR	55	NP_683354
Short chain of napin-like peptide	*Ricinus communis*	CRQEVQRKDLSSCERYLRQSSSRR	54	AAB50869
Short chain of napin-like peptide	*Momordica charantia*	REQLRSCESFLRQSRGY	53	AAB50872

[a] *Results of a BLAST search.*
[b] *UniProt accession number of JcTI-I.*
[c] *Deduced amino acid sequence of Jat c 1[b], a allergenic 2S albumin (Maciel et al., 2009).*

Table 3 | Peptide sequences of JcTI-I identified after data-dependent acquisition (DDA) analysis.

m/z	Charge	Delta (ppm)	Sequence	Modifications
821.3901	2	4.091931	(R)RDLSSCENYITQR(R)	Carbamidomethyl C (6)
743.3438	2	10.359877	(R)DLSSCENYITQR(R)	Carbamidomethyl C (5)
424.7177	2	−2.8809798	(K)ELSAICR(C)	Carbamidomethyl C (6)
646.3268	2	7.850316	(R)CESIHYLLEK(Q)	Carbamidomethyl C (1)
774.3472	2	2.6834285	(K)QLEEGEVGSEDEAR(R)	

ANTIMICROBIAL ACTIVITY OF JcTI-I

JcTI-I did not affect the growth of *Bacillus subtilis* and *Pseudomonas aeruginosa*, even at a concentration of 500 μg/mL (data not shown). However, the protein inhibited the growth of *Staphylococcus aureus* and *Salmonella enterica* at all concentrations tested when compared to controls. The MIC was calculated as 5 μg/mL for both bacteria (**Figures 6A,B**). Moreover, JcTI-I caused 84.6% and almost 100% inhibition of the proteases extracted from *Staphylococcus aureus* and *Salmonella enterica*, respectively, values similar to that found for protein sparing modified fast (PSMF; **Figure 7**).

ASSESSMENT OF HEMOLYTIC ACTIVITY OF JcTI-I

JcTI-I did not promote hemolysis of human erythrocyte under the experimental conditions tested (**Figure 8**).

ASSESSMENT OF TOXIC ACTIVITY OF JcTI-I

JcTI-I did not display toxic activity to mice, even at a concentration 100 times greater than the MIC for *Staphylococcus aureus* and *Salmonella enterica*.

DISCUSSION

Purification of a novel trypsin inhibitor (JcTI-I) from *J. curcas* seeds cake was achieved by TCA precipitation of the protein extract followed by two chromatographic steps. JcTI-I is a 10.252 kDa neutral glycosylated protein with a p*I* of 6.6 and 6.4% of carbohydrate. Both the molecular mass and p*I* are similar to other seed trypsin inhibitors previously characterized as, for example, the 10.0 kDa trypsin inhibitor from *Zea mays* (Baker et al., 2009) and that purified from *Phaseolus limensis* seeds, with p*I* of 7.6 (Wang and Rao, 2010).

Analyzing the results of both SDS-PAGE and MS/MS, it appears that the 10.252 kDa JcTI-I molecule is composed of a 7.133 and 3.124 kDa polypeptides jointed together by at least one disulphide bridge and that DTT + iodoacetamide treatments, but not β-mercaptoethanol alone, were able of irreversibly splitting the molecule in the small and large subunits. The results of size exclusion chromatography of JcTI-I on Sephacryl S-200 gave a 20.2 kDa molecule. It is plausible that under such mild conditions in which JcTI-I was dissolved in 0.050 M sodium phosphate buffer containing 0.2 M NaCl, pH 7.5, the 10.252 kDa monomer associated to form a 20.2 kDa dimer, which does not occur in the presence of acetonitrile in the MS/MS analysis.

Alignments of JcTI-I sequence with other protein sequences showed that the highest similarity was verified with Jat c 1, a *J. curcas* allergen that belongs to the 2S albumin family (Maciel et al., 2009), but not with known protease inhibitor sequences. Several trypsin inhibitors show sequence similarities with the 2S albumin-like proteins, as two barley Bowman–Birk type trypsin inhibitor isoforms and the trypsin inhibitor of *Brassica juncea* seeds (Terras et al., 1993; Mandal et al., 2002). Therefore, JcTI-I might be a novel trypsin inhibitor that belongs to the 2S albumin family.

Protease inhibitors exhibit a considerable stability to high temperatures and to large pH variations (Bhattacharyya et al., 2006; Bijina et al., 2011). JcTI-I has also these common properties as it was active and stable after incubation at 90°C for 60 min and in the pH range 2.0–10.0, in agreement with other plant protease inhibitors. Moreover, JcTI-I was stable to high salt concentrations as it retained full activity after treatment with up to 2% NaCl. The trypsin inhibitor purified from adzuki bean (*Vigna angularis*) seeds was also stable when

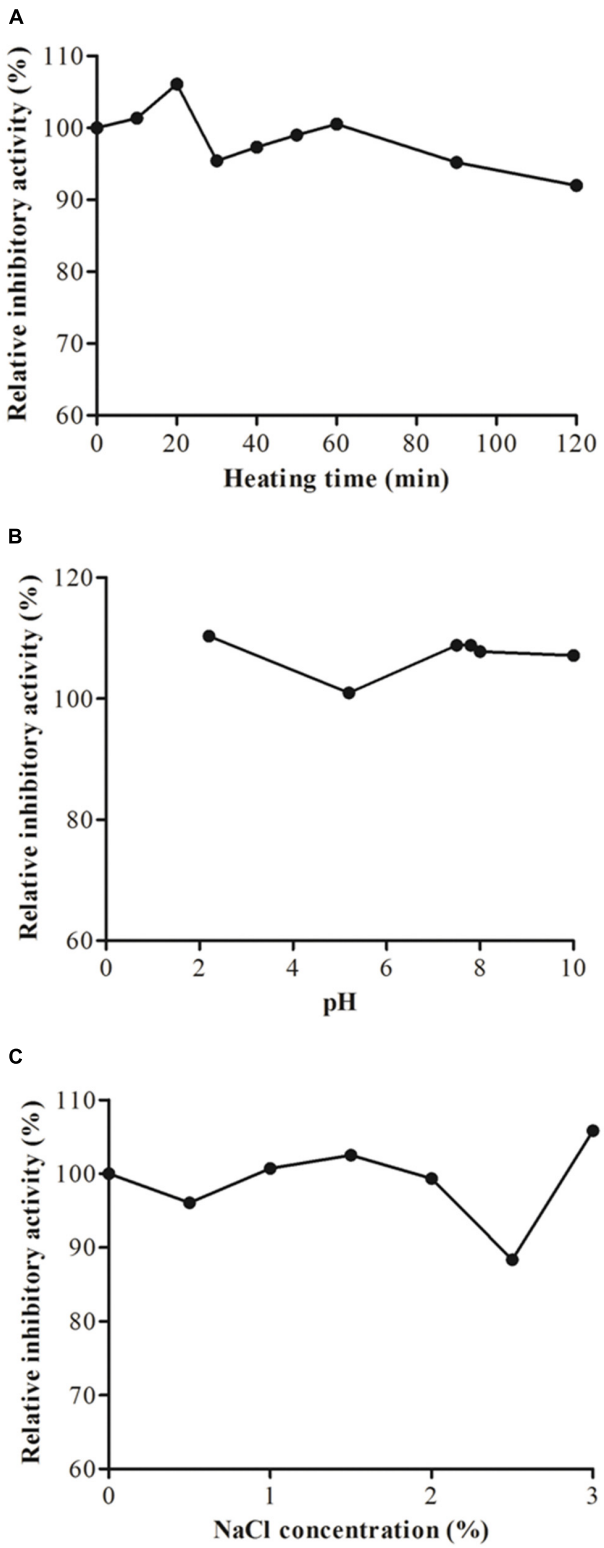

FIGURE 3 | Trypsin inhibitory activity assay of JcTI-I at pH 7.5, at room temperature (around 25°C) after: (A) heat-treatment; (B) incubation at pH 2–10 for 30 min; (C) incubation with 0–3 M NaCl concentration. Each point represents the mean of three estimates. The standard deviation was less than 10%.

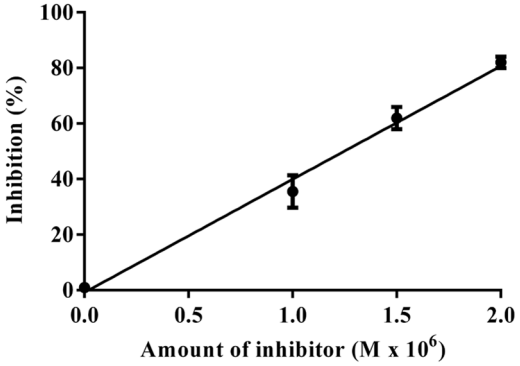

FIGURE 4 | Stoichiometry of the JcTI-I-protease interaction. Bar indicates standard deviation from triplicate determinations.

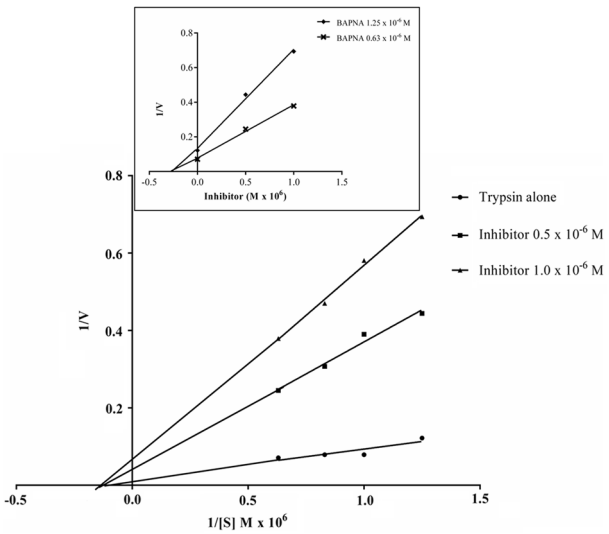

FIGURE 5 | Lineweaver–Burk plot ($1/v \times 1/[S]$) of the inhibition of trypsin by JcTI-I, at two different inhibitor concentrations, in conjunction with the Dixon plot ($1/v \times [I]$, insert) for identification of K_m, K_i and the likely mechanism of enzyme inhibition. Each point represents the mean of three estimates. The standard deviation was less than 10%.

incubated with NaCl up to 3% concentration (Klomklao et al., 2011). Such stability was associated to disulphide bridges that are presumably responsible for the functional stability of Kunitz type protease inhibitors in the presence of physical and chemical denaturants such as temperature, pH, and reducing agents (Kridric et al., 2002).

JcTI-I is a non-competitive trypsin inhibitor similar to other trypsin inhibitors, such as AETI, APTI, and TTI from the seeds of *Archidendron ellipticum* (Bhattacharyya et al., 2006), *Adenanthera pavonina* (Macedo et al., 2004), and *Tamarindus indica* (Araújo et al., 2005), respectively. The IC_{50} of JcTI-I for trypsin was 1.25×10^{-6} M, indicating that it was more potent than EvTI, the trypsin inhibitor from *Erythrina velutina* seeds ($IC_{50} = 2.2 \times 10^{-6}$ M; Machado et al., 2013) and less than SSTI2, the trypsin inhibitor from *Sapindus saponaria* seeds

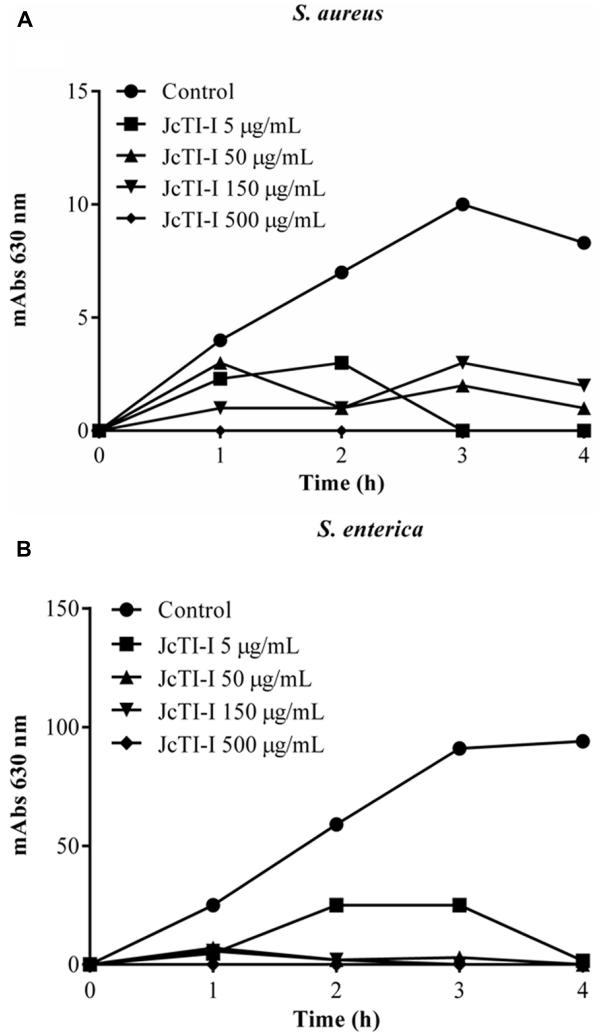

FIGURE 6 | Effect of JcTI-I at different concentrations on the growth of *Staphylococcus aureus* (A), and *Salmonella enterica* (B). Control: 0.05 M sodium phosphate buffer (pH 7.5). The minimal inhibitory concentration (MIC) of JcTI-I was determined based on these data. Assay was done in triplicate for every inhibitor concentration. The standard deviation was less than 10%.

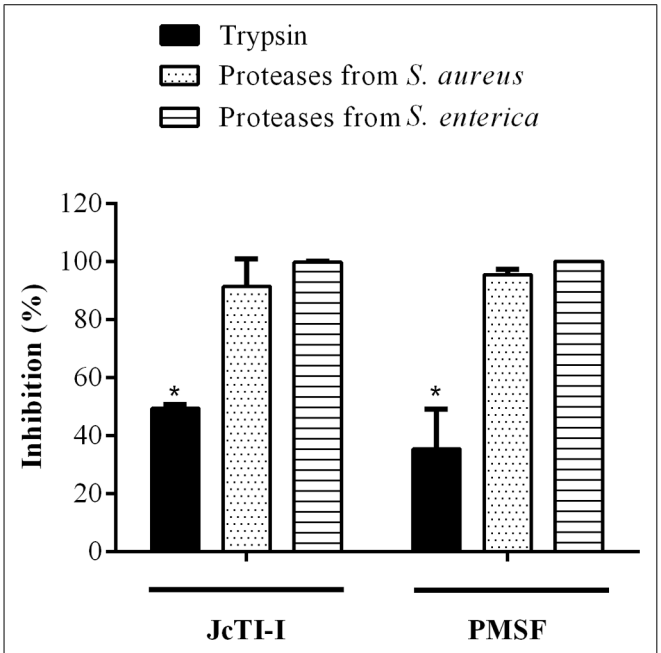

FIGURE 7 | Inhibitory effect (%) of JcTI-I on the proteases extracted from *Staphylococcus aureus* and *Salmonella enterica*. Asterisks represent significant difference ($P < 0.05$) in comparison to control (0.25 M sodium phosphate buffer, pH 7.5). The experiments were run in triplicate and each value is presented as mean ± standard deviation.

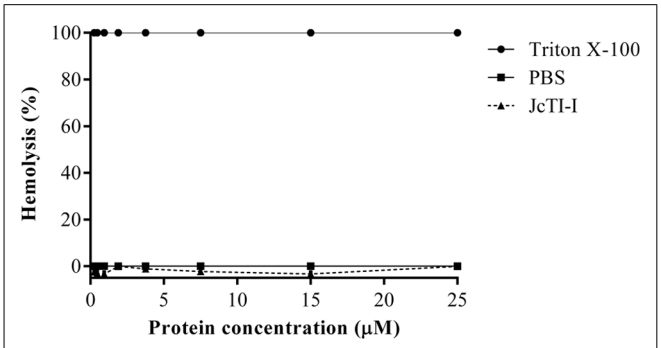

FIGURE 8 | Assessment of the hemolytic effect of JcTI-I on human erythrocytes. A suspension of washed human erythrocytes was incubated with various concentrations of JcTI-I (-▲-). Values (OD_{540}) were normalized against 100% lysed erythrocytes (0.1% Triton X-100) (-●-). Control was the red cells incubated with 0.05 M PBS, pH 7.4 (-■-). Each point represents the mean of three estimates. The standard deviation was less than 10%.

($IC_{50} = 8.3 \times 10^{-8}$ M; Macedo et al., 2011). JcTI-I interaction with trypsin occurred at the 1:1.7 molar ratio, indicating a 1:2 stoichiometry, with two equivalent binding sites. Similar result was found for a protease inhibitor from *Moringa oleifera* leaves (Bijina et al., 2011). The inhibition constant (K_i) for JcTI-I was 2×10^{-11} M under the assay conditions. Low K_i values were also found for the trypsin inhibitors CBTI-2 from *Caesalpinia bonduc* (Bhattacharyya et al., 2007) and AETI (Bhattacharyya et al., 2006) with K_i of 2.4 and 2.7×10^{-10} M, respectively.

Resistance of some strains of Gram-positive and Gram-negative bacteria to conventional antibiotics has increased dramatically caused or induced by the widespread misuse and overuse of antibiotics and represents a serious threat to public health worldwide. Therefore, discovery and/or development of alternative, non-conventional drugs with activity against most resistant bacteria for infection control are of paramount importance (Lima et al., 2011). *J. curcas* is a plant traditionally used for medicinal purposes and its antimicrobial potential has been previously reported. Indeed, extracts from leaves, root barks, latex, and various fruit parts of *J. curcas* showed inhibitory activity against *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* (Arekemase et al., 2011; Nyembo et al., 2012; Rachana et al., 2012). However, few reports are related to the antimicrobial activity of compounds present in the seed cake of *J. curcas*, although its potential use as a source of antibacterial agents has

been suggested (Sriprang et al., 2010; Sundari and Selvaraj, 2011). The seed cake of *J. curcas* represents an attractive raw material to extract novel antibacterial drugs because it is produced in large scale as a waste material after oil extraction to produce biodiesel. It is estimated that 1-tn per day of *J. curcas* extracted oil generates, approximately, 3 tn of seed cake (Srividhya et al., 2010).

The use of protease inhibitors as novel therapeutics has previously been proposed (Bhattacharjee et al., 2012; Machado et al., 2013). Therefore, take into consideration the huge amount of *J. curcas* seed cake generated, from which JcTI-I was purified, the hypothesis that it could behave as a natural drug against human pathogens was also tested. This was experimentally confirmed as JcTI-I behaved as a potent inhibitor of *Staphylococcus aureus* and *Salmonella enterica* growth. The MIC of JcTI-I for both bacteria was 5 μg/mL. This concentration is sixfold lower compared with that found for a potato inhibitor effective against various bacteria, including *Staphylococcus aureus* with a MIC around 30 μg/mL (Kim et al., 2006), about 50-fold lower than that of Xb-KTI, a kunitz proteinase inhibitor from *X. blandum* corms, active against *Salmonella typhimurium*, with a MIC of about 256 μg/mL (Lima et al., 2011) and about 25-fold lower than that of cloxacillin, a β-lactam antibiotic used as therapeutic drug against MRSA with a MIC around 128 μg/mL (Islam et al., 2008). According to Hancock (2000), the best protein/peptide candidates for antibacterial drugs have MICs ranging from 1 to 8 μg/mL. Based on these results, JcTI-I can be considered as a potent inhibitor of bacterial growth and could be explored as an antibiotic protein to help bring down the 10–30% mortality caused by *Staphylococcus aureus* (van Hal et al., 2012).

Serine proteases have been implicated in the virulence of some bacterial strains (Speranskaya et al., 2006; Tripathi et al., 2011). Therefore, substances that can interfere with the proteolytic activity of these enzymes, like protease inhibitors, could be an effective strategy to combat bacterial infections (González-Lamothe et al., 2009). JcTI-I did have inhibitory activity against the serine proteases from the tested bacteria. It caused about 85 and 100% inhibition of the proteases extracted from *Staphylococcus aureus* and *Salmonella enterica*, respectively, values compared to those achieved by PSMF, a synthetic inhibitor. These results are similar to that reported by Lima et al. (2011) who found that Xb-KTI caused about 80% inhibition of the serine proteases from *Salmonella typhimurium*.

To assess whether JcTI-I promotes lysis of eukaryotic cells, this protease inhibitor was incubated with human erythrocytes up to the concentration of 500 μg/mL, about 100-fold higher than the MIC of JcTI-I (5 μg/mL) for both bacteria. Even at such concentration, JcTI-I does not lysis the red cells. This result suggests that the mode of action of JcTI-I is not by disrupting cell membranes and it does not have toxic effects toward mammalian cells. In addition, JcTI-I was not toxic to mice at a concentration very much higher than the MIC for *Staphylococcus aureus* and *Salmonella enterica*.

In summary, we have isolated, purified, and characterized a novel trypsin inhibitor from *J. curcas* seed cake, named JcTI-I, which possesses a potent activity against the human pathogenic bacteria *Staphylococcus aureus* and *Salmonella enterica*. The lack of hemolytic activity against human erythrocytes and toxic activity to mice together with resistance to heat treatment, pH, high salt concentrations, and putative resistance to proteases make JcTI-I a pharmacologically interesting and valuable drug for the design of a novel antibiotic medicament.

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Helminth defence molecules—immunomodulators designed by parasites!

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HELMINTH DEFENCE MOLECULES (HDMs)—ANTIMICROBIALS OR IMMUNOMODULATORS?

Parasitic helminths (worms) are one of the most successful animal groups in nature. They are large multicellular organisms and therefore cannot penetrate host cells but must reside inside tissue or organs. They infect over 1 billion people globally, mostly in tropical/sub-tropical regions, taking an enormous toll on animal and human health (Hotez et al., 2008). Although the main evolutionary driving force for parasitism may have been the ease of access to food this brought other challenges, most importantly the need to overcome expulsion by the immune responses of the host. Accordingly, helminths have evolved elaborate mechanisms to manage, suppress or manipulate the mammalian immune system.

It is generally thought that worms influence the host immune response by secreting factors into their environment—the host parasite interface. Over the past 10 years the application of proteomics techniques has allowed us to identify molecules secreted by helminths. Although the exact complement of secretory molecules differs between species, most helminths release proteolytic enzymes with endo- and exo-peptidase activities (Robinson et al., 2008a,b), antioxidants such as glutathione-*S* transferase and peroxiredoxin (Jefferies et al., 2001; Donnelly et al., 2008; Robinson et al., 2009) and other molecules with a range of biochemical activities including protease inhibitors and metabolic enzymes. Whilst the biological function of a few of these helminth molecules are well defined, this is generally restricted to those that possess evolutionarily conserved catalytic domains/active site residues that are easily identified using bioinformatics search tools (e.g., *Fasciola* cysteine proteases function in fluke nutrition; Lowther et al., 2009). However, many helminths also secrete a range of molecules whose primary sequences offer no clue as to their biochemical activity or biological function.

During our on-going analysis of the secretory proteome (i.e., the secretome) of the helminth *Fasciola hepatica*, a parasite that infects its host via the intestine following ingestion and then migrates to the liver, we identified a novel and abundant 8 kDa protein. The protein contained a secretory signal peptide but BLAST analysis of its primary sequence failed to infer a function. However, structural studies revealed that a 37 amino acid C-terminal region adopted a similar secondary structure (amphipathic α-helix) to a number of peptides with known antimicrobial and/or immunomodulatory functions, most particularly mammalian LL-37 (Robinson et al., 2011). Phylogenetic analysis discovered that these secretory proteins are conserved across the major trematode species that collectively infect >1 billion humans, including the liver flukes *Clonorchis sinensis*, *Paragonimus westermani*, and *Opisthorchis viverrini* and the blood flukes *Schistosoma mansoni* and *S. japonicum* (Robinson et al., 2011). Accordingly, by analogy with the mammalian antimicrobial and/or immunomodulators we hypothesized that these helminth molecules may play a critical role in the parasites interaction with its host and thus named them helminth defence molecules (HDMs). This hypothesis, however, posed the question that is central to understanding how they may perform this function, namely, are HDMs antimicrobial or immunomodulatory peptides, or both?

HDMs DO NOT POSSESS ANTIMICROBIAL OR HAEMOLYTIC ACTIVITY

To begin to address this question, we compared the ability of helminth-derived HDMs with several well-known mammalian host-derived antimicrobial peptides [AMPs; also termed host defence peptides (HDPs) where they display immunomodulatory activities] to directly kill clinically-relevant microorganisms (Thivierge et al., 2013). The antimicrobial effect of AMPs/HDPs is attributed to the ability of their amphipathic helices to bind, and thus disrupt, negatively charged bacterial cell membranes (Hancock and Chapple, 1999). Despite having similar biochemical/biophysical properties to the host-derived AMPs/HDPs including LL-37, CRAMP, SMAP-29, and BMAP-28 which variously showed activity against both gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*) and gram-positive (*Staphylococcus epidermis* and *Staphylococcus aureus*) bacteria, none of the HDMs tested demonstrated

bactericidal activity against any species of bacteria at the concentrations tested (<0.25–128 μg/ml; Thivierge et al., 2013).

Previous studies had also shown that AMPs/HDPs are cytotoxic to kinetoplastid protozoan parasites such as *Leishmania major* (Lynn et al., 2011), *Trypanosoma cruzi* (Haines et al., 2009), and the apicomplexan protozoan parasites *Cryptosporidium* sp. (Carryn et al., 2012). In our studies, whilst we confirmed that several vertebrate AMPs/HDPs killed the apicomplexan protozoan parasite *Cryptosporidium parvum* and *C. hominis in vitro* at concentrations as low as 0.025 μM none of the HDMs tested displayed any parasiticidal activity even at 2.5 μM (Thivierge et al., 2013).

The predominant mechanism of AMP/HDP bactericidal activity is the formation of pores in the membrane lipid bilayer, destroying its integrity and causing cell death (Oren and Shai, 1998). However, this effect is not specific to bacterial cells since eukaryotic cells can also be lysed via this mechanism (Ciornei et al., 2005). Whilst the mammalian AMPs/HDPs rapidly lysed red blood cells in a concentration-dependent manner (from 8 to 256 μg/ml), the HDMs did not induce significant lysis at equivalent concentrations (Thivierge et al., 2013). Using a fluorescent membrane-impermeant dye we demonstrated that the mammalian peptides LL-37, CRAMP, SMAP-29, and BMAP-28 (50 μM) induced the formation of pores in a murine macrophage cell line and in general, were cytotoxic at concentrations of >25 μM. However, at the same concentrations none of the helminth peptides exhibited these lytic or cytotoxic effects (Thivierge et al., 2013).

The contrasting effects of the mammalian AMPs/HDPs and the helminth HDMs may be due to the targeting of specific membrane components by HDMs necessary for internalization into host cells rather than non-specific "carpet" binding to the phospholipid bilayer that may occur with the AMPs/HDPs (Brender et al., 2012). We have shown that *F. hepatica* HDM (FhHDM-1) binds to host macrophage plasma membrane lipid rafts, possibly via selective interaction with cholesterol, before being internalized by endocytosis (Robinson et al., 2012). In contrast, human LL-37 cannot bind to cholesterol; indeed its presence strongly reduces the ability of LL-37 to interact with phospholipid membranes (Sood and Kinnunen, 2008). Whilst the precise mechanism(s) governing the selectivity of the HDMs vs. AMPs/HDPs is not fully understood, our observations show that, rather than simply destroying host cells by lysis, HDMs have evolved specifically to interact with host cell membranes without causing their disruption.

HDMs DISPLAY A VARIETY OF IMMUNOMODULATORY ACTIVITIES

Parasitic helminths secrete a range of soluble effector molecules that modulate host immune responses in a myriad of ways to establish an environment that facilitates their survival and a prolonged reproductive phase (reviewed by Harnett and Harnett, 2010). We have previously shown that *F. hepatica* and *S. mansoni* secrete molecules with specific immunomodulatory functions: peroxiredoxin (Prx) promotes the development of host Th2 responses via the induction of M2 macrophages (Donnelly et al., 2005, 2008) and cathepsin L1 (FhCL1) inhibits the macrophage MyD88-independant, TRIF-dependant signaling pathway via cleavage of toll-like receptor (TLR) 3 within the endosome (Donnelly et al., 2010). However, our recent studies have shown that HDMs are "utility players" in the host-parasite interaction and exert multiple effects on host immune cells.

In order to infect their mammalian host, the infective stage of *F. hepatica,* termed newly excysted juveniles (NEJs), secrete an array of cysteine peptidases including cathepsins B and cathepsins L that digest a path through the intestinal wall (McGonigle et al., 2008; Robinson et al., 2009). Despite this loss of barrier function of the intestinal epithelium and consequent translocation of luminal antigens (bacteria and their toxins) into the circulation, potent host responses such as septicaemia are not common events during helminth infections. We found that FhHDM-1 binds directly to *E. coli* lipopolysaccharide (LPS) preventing its interaction with the TLR4/MD2/CD14 complex on the macrophage surface (Robinson et al., 2011). FhHDM-1 also exhibited a striking ability to protect mice against LPS-induced inflammation by preventing the release of inflammatory mediators (TNF and IL-1β) from macrophages (Robinson et al., 2011).

IFNγ is one of the key cytokines in the innate immune response to intracellular pathogens, and augments cellular responses to TLR ligands such as bacterial LPS (Held et al., 1999; Schroder et al., 2006). We also found that, like the mammalian peptides LL-37, CRAMP, SMAP-29, and BMAP-28, HDMs significantly inhibited macrophage TNF production in response to combined stimulation with LPS and IFNγ (Thivierge et al., 2013). Both AMPs/HDPs and HDMs can seemingly also inhibit inflammatory macrophages using mechanisms that are independent of direct binding to LPS (Brown et al., 2011; Thivierge et al., 2013). Thus, the secretion of HDMs by the parasite may protect the host against excessive bacterial-induced inflammation that would otherwise occur during migration of the parasite through the host intestinal wall due to concurrent translocation of luminal bacteria. By offering this protection the parasite enhances the survival of its host and, accordingly, its own longevity.

Confocal microscopy, using fluorescently labeled peptides, has shown that after initial interaction with lipid rafts on the macrophage surface, FhHDM-1 enters the cell via the endolysosomal pathway (Robinson et al., 2012). FhHDM-1 is cleaved by endogenous (host) cathepsin L to specifically release a C-terminal peptide (containing the conserved HDM amphipathic helix) which then prevents the acidification of the endolysosomal compartments by inhibiting vacuolar (v) ATPase activity. Uncoupling endolysosomal acidification impedes macrophage antigen processing by proteases, such as cathepsin L, preventing the presentation of peptides at the cell surface in conjunction with MHC class II to CD4 + T cells (Robinson et al., 2012). By suppressing the antigen presenting function of host macrophages, HDM indirectly impairs the subsequent development of adaptive immune responses

against the parasite. However, by altering the secretion of immunoglobulins from activated B cells, HDMs are also capable of directly influencing the host adaptive response. HDMs enhanced the IL-4 induced production of IgG1 and suppressed the release of IgG2a from murine B cells in response to IFNγ (Thivierge et al., 2013). This is consistent with a wound healing scenario (Nishio et al., 2009) which, again, may serve to protect the host from helminth-induced tissue damage.

PARASITE-DESIGNED HDMs MAY HAVE THERAPEUTIC POTENTIAL

The immune-modulatory properties of mammalian HDPs, and in particular their ability to prevent excessive immunopathology associated with bacterial sepsis, has attracted interest in exploiting these as anti-infectives and immunotherapeutic agents (Easton et al., 2009). However, their clinical development has been hampered by the occurrence of toxic off-target effects and cell lysis. Efforts to improve delivery of HDPs to their desired site of action (e.g., by conjugation with targeting moieties) are on-going with the aim of enhancing efficacy whilst reducing deleterious side effects (Devocelle, 2012). However, helminth-derived HDMs may represent a more attractive therapeutic option: they show all the potent immunomodulatory effects of the HDPs without the cytotoxic and cytolytic effects (Thivierge et al., 2013). We are currently determining the translatability of the immune-modulatory effect of HDMs from murine to human cells rather than screening an array of animal models of disease (Robinson et al., 2013) and we are hopeful that the therapeutic potential of these parasite-designed molecules can be realised (Donnelly et al., 2011).

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Purification, biochemical characterization and self-assembled structure of a fengycin-like antifungal peptide from *Bacillus thuringiensis* strain SM1

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An antifungal lipopeptide fengycin, producing strain SM1 was isolated from farm land soil sample and identified as *Bacillus thuringiensis* strain SM1 by using 16S rDNA analysis. Fengycin detected in the culture extract was further purified using HPLC and showed a molecular mass of 1492.8 Da by MALDI-TOF-MS analysis. Purified fengycin was allowed to construct their self-assembled structure onto a hydrophobic surface showing a clear improvement of antibacterial activity. In self-assembly, fengycin adapts a spherical micelle core shell like structure. Self-assembled fengycin may be a successful antimicrobial compound modifying its action from confined antifungal function. Besides it can open up a new area of research in supramolecular lipopeptide based compound making. This can revealed the mode of action of this unique self-assembled structure to fully evaluate its potential for use as an antimicrobial drug to control the emergence of bacterial infection.

Keywords: antimicrobial activity, *Bacillus thuringiensis*, fengycin, self-assembled structure

INTRODUCTION

The increasing tendency of microbial infections, rapid emergence of drug-resistant to recent antibiotics and quick evolution through mutation are of great threats to control of microbial infection (Samanta et al., 2013). Infectious disease has become the biggest killer among children and young adults, and has now become a burden to global economics and public health (Jones et al., 2008). This situation is becoming uncontrollable due to unique gained properties of genetically altered pathogens assigned with their unusual clinical symptoms (Gilsdorf and Zilinskas, 2005). Impediment in usual diagnostic and clinical evaluations or preventive strategies forces the modern research to overcome this fatal situation. Research strategies like applying of naturally occurring antimicrobial peptides (AMPs), combined administration of antibiotic agents (Anantharaman et al., 2010), and structural modification of antibiotics are extensively studied (Roy et al., 2013). Furthermore, structural modification of AMPs by self-assembly mechanism is reported to enhance the spectrum of the AMPs (Fernandez-Lopez et al., 2001).

Fengycin, a cyclic lipodecapeptide produced by *Bacillus subtilis* strain, containing a β-hydroxy fatty acid with a side-chain length of 16–19 carbon atoms offers an efficient antifungal activity. Like most the natural AMPs, fengycin seems to acts by improving the plasma membrane permeability of the target cell (Vanittanakom et al., 1986). This AMP is known to exhibit strong fungitoxic activity specifically against filamentous fungi, inhibiting enzymes phospholipase A2 and aromatase (Loeffler et al., 1986; Steller and Vater, 2000). Otherwise this peptide also cause hemolytic activity 40-fold lower than that of surfactin, showing a clear disadvantage (Hbid, 1996; Schneider et al., 1999; Hathout et al., 2000). In this study, an antifungal peptide fengycin was purified from an *B. thuringiensis* strain SM1 soil isolate. This lipopeptide has been characterized and checked for its antifungal activity against *Candida albicans*. Additionally, self-aggregation and arrangement of the fengycin molecule were also studied to check whether interfacial modification can enhance the spectrum of the microbial action or not.

MATERIAL AND METHODS

IDENTIFICATION OF THE STRAIN

One gram of farm land soil sample, collected from agricultural farm of IIT-Kharagpur campus, India, was suspended in 9 ml of sterile distilled water. Then 100 μl of this was serially diluted and amplified on sterilized LB agar plates with the following composition (g/l): peptic digest of animal tissue, 5.0; beef extract, 1.5; yeast extract, 1.5; sodium chloride, 5.0; agar 15.0 (pH adjusted to 7.2). Colonies with inhibition zone in their surroundings were selected for study and streaked on to fresh nutrient agar (NA, HiMedia, India) medium plates. Upon testing their purity all isolates were preserved at −70°C in culture medium containing 50% (v/v) glycerol until further use. The tested strain SM1 was grown on tryptone soya agar (TSA) medium. Phenotypic properties including morphology, physiology and biochemical characteristics of the isolate were done using standard procedures (Baindara et al., 2013). The identity of strain SM1 was also confirmed by using 16S rRNA gene sequence (Korpole et al., 2006) blast search analysis. CLUSTAL-W program of MEGA version 5 was used to align the all 16S rRNA gene sequences of the nearest type strains (Tamura et al., 2011)

downloaded from the NCBI database. Manual correction of alignment was done using BioEdit sequence alignment editor (Hall, 1999). Pair-wise evolutionary distances were calculated with the Kimura two-parameter (Kimura, 1980) and a neighbor-joining phylogenetic tree was constructed using the MEGA version 5.0. 1000 replicates were taken to find the stability of phylogenetic tree.

EXTRACTION OF LIPOPEPTIDE

A combination of acid and solvent extraction procedure (Vater et al., 2002) was followed to isolate lipopeptide produced from the strain. Centrifugation (13,000 × *g*) for 15 min of the culture broth at 4°C resulted cells to pellet down. pH of the supernatant was adjusted to 2.0 by addition of concentrated HCl. Supernatant of pH 2 was allowed to stand at 4°C for 16 h and resulted in precipitation. The sample was then centrifuged (13,000 × *g*) for 20 min at 4°C. Precipitate was collected and further extracted with methanol by stirring for 2 h. The lipopeptide containing methanol was collected after filtration and vacuum-dried.

PURIFICATION OF LIPOPEPTIDES

Purification of lipopeptides was carried out as mentioned in Mandal et al. (2013). Extracted lipopeptides was dissolved in methanol. Then we fractionate it by reverse phase- HPLC (Agilent 1100 series, CA, USA) with a ZORBAX 300-SB18 column (4.6 mm × 250 mm, particle size 5 μm), at a flow rate of 1 ml/min. The mobile phase components were (A) 0.1% TFA in water and (B) 0.1% TFA in 70% acetonitrile solution. The gradient of solvent B was used to run the column were : 0–60% for 0–45 min, 60–80% for 45–55 min and 80–100% for 55–60 min. Eluted peptide from the column were monitored at 215 nm in a diode array detector. Fractions according to peaks obtained during HPLC were collected using a fraction collector (GILSON, France) coupled with the system. Speed vacuum concentration was used to concentrate the sample and it was then tested for antimicrobial activity. The fractions or peaks that showed antibacterial activity were re-chromatographed in the same column under similar conditions, except solvent B was used as 100% acetonitrile with a gradient of 0–10% for 30 min. The peptide concentration was determined using the RP-HPLC conditions and calibrated with surfactin (Sigma-Aldrich, St. Louis, USA).

MALDI-TOF-MS AND SEQUENCING

Molecular mass and MS/MS sequencing of the purified and active lipopeptides were performed using a Voyager time-of-flight mass spectrometer (Applied Biosystems, CA, USA). Peptides were incubated with 10% NaOH in methanol at room temperature for 16 h. Lactone ring present in lipopeptide in MS/MS sequencing was cleaved. Cleaved peptide was liophilizated and extracted with methanol, and allowed it for mass spectrometry analysis. Spectra were recorded in the post-source decay (PSD) ion mode as an average of 100 laser shots with a grid voltage of 75%. The reflector voltage was reduced in 25% steps and guide wire was reduced 0.02–0.01% with an extraction delay time of 100 ns.

FATTY ACID ANALYSIS BY GC-MS

Fatty acid content associated with the lipopeptides was assessed by incubating the peptides (5 mg of each) with 0.5 ml of 6 M HCl at 90°C for 18 h in sealed tubes for acid hydrolysis. Fatty acids were then extracted with ether and treated with 0.95 ml methanol and 0.05 ml of 98% H_2SO_4 at 65°C for 6 h. n-hexane extraction was then done to obtain fatty acid methyl esters. Then we analyzed it on GC-MS with a Clarus 500 GC (PerkinElmer, USA) using helium as carrier gas with a flow rate of 1.0 ml/min. The column temperature was then maintained at 120°C for 3 min and thereafter gradually increased (8°C/min) to 260°C.

STRATEGY TO FORM SELF-ASSEMBLED FENGYCIN

Chloroform solution of fengycin molecule was prepared at a concentration of 0.1 mg ml^{-1} at neutral pH. The solution was then rotated in chloroform solution for 12 h. and further characterized.

CIRCULAR DICHROISM AND FOURIER TRANSFORM INFRARED SPECTROSCOPY

Circular dichroism (CD) spectra were recorded using samples at 1% wt and pH 7 with a path length of 0.001 cm. Spectra were recorded at room temperature from 250 to 180 nm, with a 0.2 nm data pitch and a scan rate of 50 nm min^{-1} by Jasco-810 spectropolarimeter. Millidegrees of rotation were converted to molar residual ellipticity (MRE). For FTIR measurements, both the extract and the reduced colloidal solution were analyzed on a Perkin Elmer FTIR instrument in the diffused reflectance mode at a resolution of 4.0 cm^{-1}.

SCANNING ELECTRON MICROSCOPY

Aliquots (100 μL) of each gel were prepared and placed in a 24 well plate. Overnight chloroform rotated samples were then affixed to SEM pucks using conductive carbon tape. The pucks were sputter-coated with gold using a CRC-150 sputter coater and imaged using an FEI Quanta 400 ESEM at 20.00 kV.

ANTIMICROBIAL ASSAYS AND MIC DETERMINATION

Minimum inhibitory concentration (MIC) values of fengycin for antifungal or antibacterial activities were determined as followed by Samanta et al. (2013). The bacterial strains *Staphylococcus epidermidis* NCIM 2493 and gram negative *Escherichia coli*, fungal strains *C. albicans* and *Aspergillus niger* were taken in the study. These strains were cultured according to their specifications. Both, fenzycin and self-assembled fengycin was used at a concentration range from 1 mg ml^{-1} to 1.95 μg $ml^{-1}$to evaluate the antifungal and antimicrobial activity. Microtiter plate dilution assay (Baindara et al., 2013) was done to study the MIC. MIC values were determined where no visible growth was observed. All independent experiments were repeated four times.

RESULTS AND DISCUSSION

CHARACTERIZATION OF BACTERIAL STRAIN

Based upon colony morphology and upon zone of clearance we have selected a strain designated as SM1 in our study (data not shown). The prime focus of our study was to assess the molecular basis of its antimicrobial activity. In this course we have tried to identify the strain by phenotypic characteristics. Phenotypic and biochemical results revealed that the strain SM1 was a

Gram-positive, rod shaped bacteria. It showed positive reaction for catalase activity and negative for oxidase activity and produced amylase. BLAST analysis of 16S rRNA gene sequence revealed significant identity (99.8%) with *B. subtilis* subsp. In aquosorum, a strain shown to produce fengycin-like lipopeptide. Neighbor joining phylogenetic tree which was constructed with 16S rRNA gene sequences of other members of the genus *Bacillus* gives confirmation of the strain *B. thuringiensis.* Result showed that a distinct cluster along with *B. thuringiensis* (**Figure 1**) is formed with a significant bootstrap value.

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF ANTIMICROBIAL PEPTIDE

Antimicrobial peptides were produced in conical flask for large scale preparation. The methanol extracts of lipopeptides obtained from the strain was screened for antifungal activity against *C. albicans* (data not shown) and subsequently purified using RP-HPLC. Methanol extract of sample showed multiple peaks during their HPLC analysis. Individual peaks fraction were collected and screened. Fraction 2 showed the highest antifungal activity. The fraction was further purified by a combination of chromatography techniques. The peptide obtained by affinity chromatography was purified by RP-HPLC (**Figure 2A**) and used to determine molecular mass by MALDI-TOF analysis. The peptide showed molecular mass of 1,492.84 (**Figure 2B**). The GC-MS analysis revealed the β-hydroxy fatty acid chain as C-18 long. Recently, Pathak et al. (2012) described details a series of fengycins from *Bacillus* species and also mentioned the production of new fengycin with molecular mass of 1492.8. The obtained fengycin (1492.84 Da) from SM1 strain is exactly identical with earlier reported fengycin from *B. subtilis* strain K1, as the amino acid composition of EOrnYTEVPEYV (Pathak et al., 2012).

FORMATION OF SELF-ASSEMBLED STRUCTURE

Fengycin (0.1 mg ml^{-1} at neutral pH) was dissolved in chloroform and was rotated at 1000 rpm for 12 h to form their self-assembled structure using hydrophobic interaction. The circular dichroism spectroscopy, Fourier transform infrared spectroscopy, scanning electron microscopic techniques were used to characterize the self-assembled structure of fengycin. The antifungal and antibacterial activity of the self-assembled structure along with the purified one was assessed.

CIRCULAR DICHROISM ANALYSIS

Circular dichroism is a sensitive method to the stereoisometry of amino acids constituting the peptide backbone. However, CD results of fengycin are useful, but they are not sufficient to draw a definitive conclusion about the conformation. As expected, this spectrum does not correspond to the conventional spectra of peptides which usually adopt α-helical or β-sheet conformation due to the cyclic structure of lipopeptide hindering that kind of conformation. This spectrum shows the broad positive band with peaks at the region of 218–227 nm and negative band centered at 205–211 nm (**Figure 3A**). It is expected that fengycin contains turns due to its closure ring structures of amino acids precluding the β-sheet or α-helical conformations. The presence of the positive band at 218 and 222 nm could be explained by the n-π^* transition occurred within D-amino acids (Vass et al., 2001). Moreover, this band might also be due to an unconventional turn conformation adopted by the peptide cycle of fengycin (Vass et al., 1998, 2001,

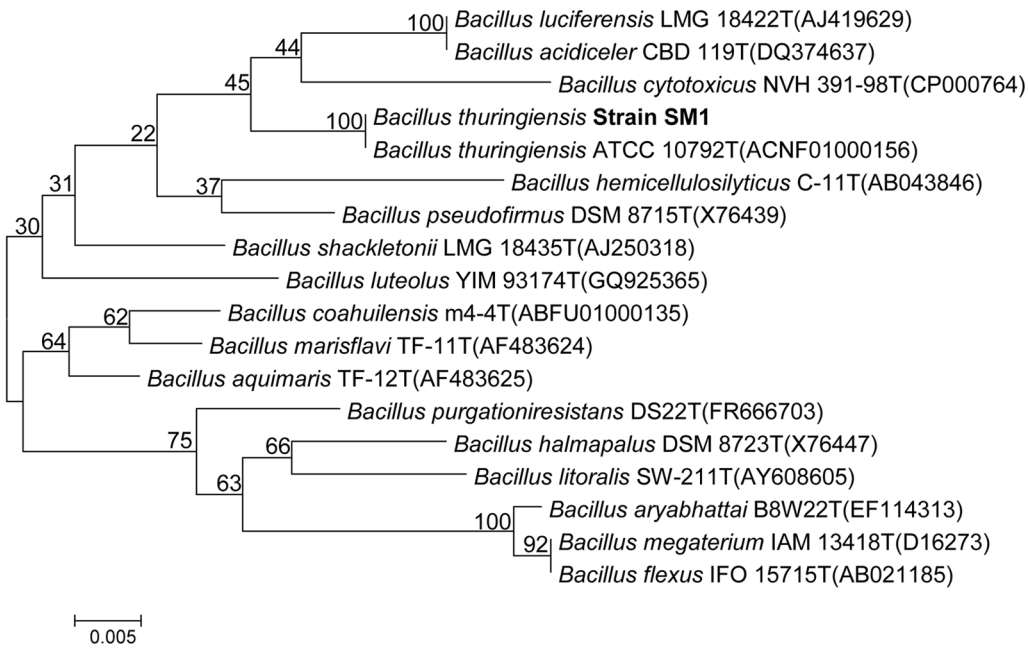

FIGURE 1 | Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic relationship between *Bacillus thuringiensis* strain SM1 and other members of the genus *Bacillus*. Bootstrap values (%) are given at the nodes.

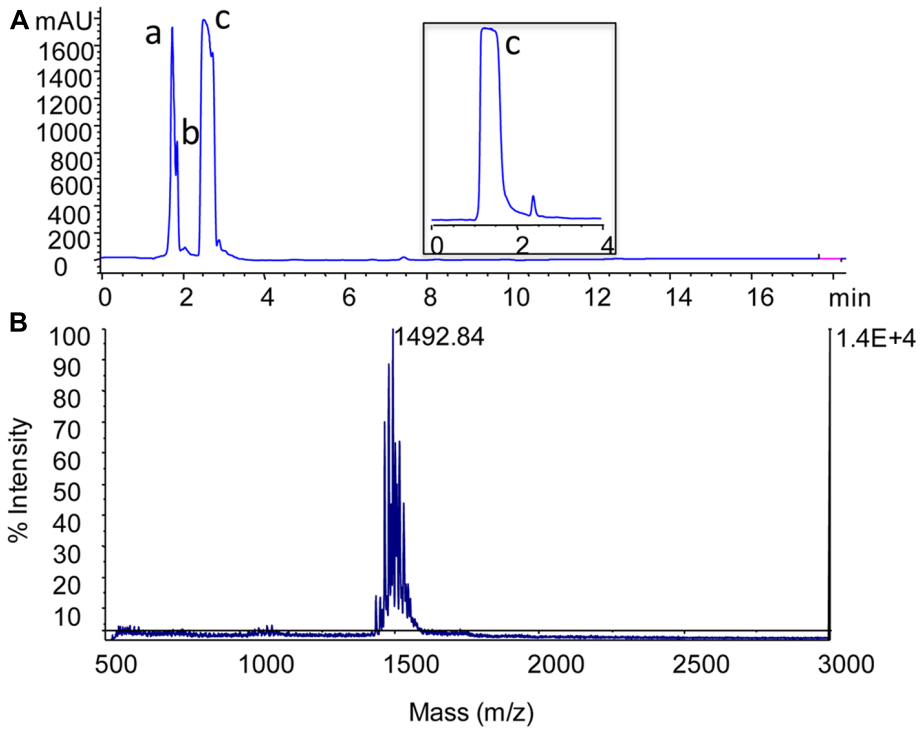

FIGURE 2 | Separation of the antimicrobial lipopeptide from acidic methanol extract by using reversed-phase HPLC. Chromatogram profile of acidic methanol extract showed three peaks (a, b, and c) and among these fractions, fraction c (lipopeptide) showed antimicrobial activity **(A)**. MALDI-TOF mass spectrum of fengycin-like peptide **(B)**. Spectrum was acquired in positive ion linear mode and reproducibility of the spectrum checked several times with different spots of same sample.

2003, 2010). Another characteristic of the fengycin CD spectrum is the presence of negative bands at 206 and 211 nm, might be corresponding to the π-π* transition occurring within peptide bonds and is compatible with the presence of β sheet conformations. There are no significant positive band shifting at 222 nm in self-assembled fengycin which suggest the turn conformation present in self-assembled cyclic peptide. Another negative band at the region at 194–206 nm shows major shift in self assemble formation may be indicates the formation of "β sheet like micelles" in the structure (Vass et al., 2001).

FTIR-ANALYSIS

Infrared spectrum of the revealed a broad stretching peak at 3441 cm^{-1}, characteristic of peptides and at 3157.22 cm^{-1} reflects hydroxyl and amine groups (**Figure 3B**). Absorption around 2955 cm^{-1} is assigned to the symmetric stretch (–C–H) of CH_2 and CH_3 groups of aliphatic chains. Moreover, an absorption band at 1660 cm^{-1} depicted stretching mode of the CO–N bond and an intense stretching peak around 1470 cm^{-1} indicated the presence of ester carbonyl groups (C = O in COOH) in peptide (**Figure 3B**). The ester carbonyl group was proved from the band at 1266.23 cm^{-1} which corresponds to C–O deformation vibrations. Absorption around 1402 cm^{-1} was also characterized as aromatic group. The amide peak at 1660 cm^{-1} is shifted to 1642 cm^{-1} which suggest the aggregation of lipopeptide by the hydrogen bonding in between amide group.

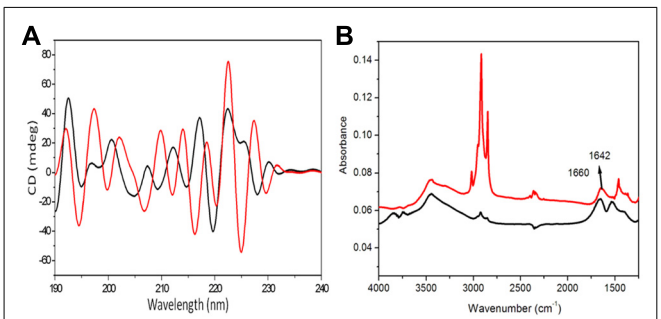

FIGURE 3 | Analytical confirmation of self-assembled structured of fengycin. The deconvoluted CD spectra of the fengycin-like peptides rendering self-assembled cyclic conformation **(A)**; the black and red line represents pure fengycin-like peptide and their self assembled structure, respectively. FTIR spectra of fengycin **(B)**, for both cases the line graph indicates only pure fengycin (black line), fengycin after 12 h rotation (red line).

SEM IMAGE AND ANTIMICROBIAL ACTIVITY

Self-assembled fengycin offered a globular micelle structure (**Figure 4A**). This self-assembled peptide showed same antifungal activity (MIC-15.62 μg ml^{-1}) against *A. niger* compared with pure fengycin, whereas, activity was increased one fold when tested against *C. albicans* **Table 1**. Like most the natural AMPs, fengycin seems to act by making the plasma membrane of the target cell

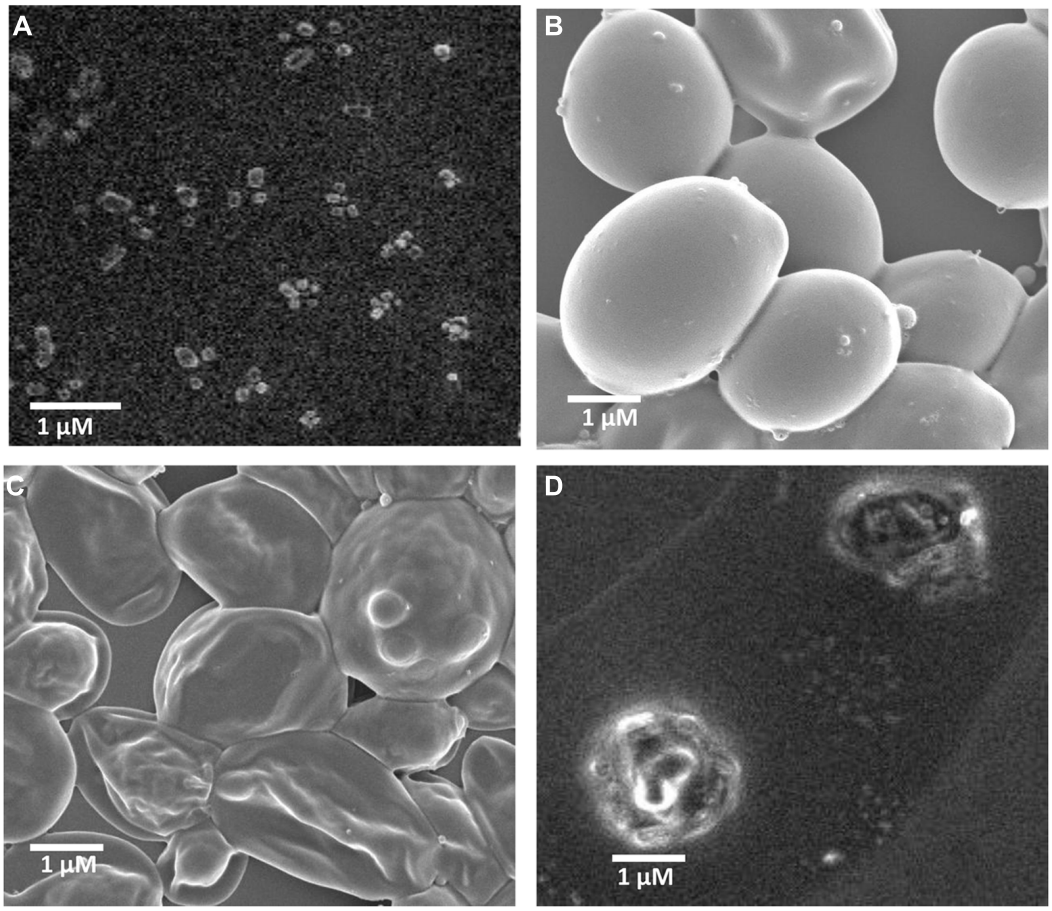

FIGURE 4 | Scanning electron micrograph of fengycin and activity against *C. albicans* cells. Self -assembled globular micelle structure of fengycin **(A)**, scanning electron micrograph of *C. albicans* cells without treatment of fengycin peptide **(B)**, scanning electron micrograph of *C. albicans* cells after treatment of fengycin peptide **(C)**, scanning electron micrograph of *C. albicans* cells after treatment of self-assembled fengycin peptide **(D)**.

more permeable. Fengycin offers a concentration-dependent perturbing effect on the structural and morphological characteristics of DPPC monolayers (Deleu et al., 2005). Antifungal mechanism of fengycin appears to be driven mainly by the physicochemical properties of lipopeptide, i.e., its amphiphilic character and affinity for lipid bilayers (Deleu et al., 2008). To confirm the action on membranolytic or not, SEM images were taken from *C. albicans* cells with and without treatment of drug molecules. **Figure 4B** clearly shows that the surface of control group (without fengycin treatment) of fungus was smooth in texture whereas the morphology of the treated cells (**Figures 4C,D**) displayed significant perturbations, rough with number of blebs. Bleb formation following fengycin treatment suggested breakage in the contact between cell wall and membrane. It is quite interesting that self-assembled fengycin affected worst to the fungal strain (**Figure 4D**). This might be due to change in amphiphilic character and affinity of the self-assembled molecule. The amphiphilic character and affinity can change via self-assembled interaction (Hüttl et al., 2013). Beside one another interesting finding needs attention of further study. Generally fengycin molecule did not show any significant anti bacterial effect. But after self-assembly, it offered a minimum inhibition concentration of 500 $\mu g\ ml^{-1}$ against both Gram-positive *S. epidermidis* and gram negative *E. coli*. This might be also due to shift of amphiphilic character and affinity resulting action against Gram-positive *S. epidermidis*.

Table 1 | Antimicrobial activity of both pure and self-assembled fengycin.

Compounds	MIC ($\mu g\ ml^{-1}$)			
	C. Albicans.	***A. niger***	***S. epidermidis***	***E. coli***
Self-assembled fengycin	7.81	15.62	125	125
Fengycin	15.62	15.62	1000	1000

In conclusion, many Gram-positive bacteria including *Bacillus* produce AMPs as defense molecule (Baindara et al., 2013). Here we are reporting a fengycin producing strain *B. thuringiensis*, isolated from soil sample. The strain showed highest similarity with *B. thuringiensis* strains related with fengycin isolation as *B. thuringiensis* CMB26 and BS8. The molecular mass of the lipopeptide is 1,493.84 suggested it is to be

fengycin-like peptide. The isolated peptide was subjected to self-assembly. This strategy yields modified fengycin molecule which is active against bacteria. In summary self-assembled fengycin may be a successful antimicrobial compound modifying its action from confined antifungal function. Besides it can open up a new area of research in supramolecular lipopeptide based compound making. This can revealed the mode of action of this unique self-assembled structure to fully evaluate its potential for use as an antimicrobial drug to control the emergence of bacterial infection.

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