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Design, Development, and Characterization of Novel Antimicrobial Peptides for Pharmaceutical Applications

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by

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ABSTRACT

Candida species are the fourth leading cause of nosocomial infection. The increased incidence of drug-resistant *Candida* species has emphasized the need for new antifungal drugs. Histatin 5 is a naturally occurring human salivary antifungal peptide and the first line of defense against infections of the oral cavity. This research has focused on understanding the activity of histatin 5, and subsequently designing novel peptides that may serve as models for the further development of therapeutics to treat fungal infection. This objective has been achieved in three steps: studying the structural requirement of histatin 5 involved in antifungal activity, the identification of a short peptide sequence, referred to as KM motif, important for fungicidal activity, and finally, the development of a novel antifungal peptide with potent activity. In the initial phase of this work it was demonstrated that reversing the sequence of histatin 5 C-16 peptide to create a retro peptide did not interfere with the fungicidal activity or secondary structure of the peptide. This suggested that the spatial arrangement of amino acid residues was more relevant for fungicidal activity than the actual peptide sequence. In the second phase of the work, we identified and characterized a five amino acid sequence, termed the KM motif, within histatin 5 that maintained fungicidal properties. Although this short peptide was less active than histatin 5, the data suggested it was killing fungi via a mechanism similar to histatin 5. In the final phase, a novel antimicrobial peptide, termed KM-12, was generated containing two KM motifs dimerized via disulfide bonds. The activity of KM-12 on *C. albicans* was approximately fifteen times more potent than the monomeric peptide and ten times more active than the native histatin 5. KM-12 was shown to have antifungal activity with several *Candida* species, including fluconazole-resistant species. In conclusion, KM-12 is promising antifungal peptide that will serve as a lead candidate for the development of antifungals peptide for pharmaceutical applications.

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DEDICATION

I dedicate this dissertation to my family, especially my wife who has been fathering my three riotous kids along five years. Their constant love, support, and encouragement have sustained me throughout my career and life.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1-*Candida* species

The *Candida* genus represents a group of eukaryotic microorganisms belonging to the kingdom of Fungi - phylum *Ascomycota*. The genus *Candida* contains about 150 species and approximately 30 have been associated with human diseases [1, 2]. Fungi differ from other eukaryotes by the presence of a rigid cell wall and the presence of ergosterol instead of cholesterol in the cell membrane [3]. Normally, *Candida* species live in a commensalism relationship with the host, including humans. However, this genus is also the most common fungal pathogen of humans and the cause of several diseases including mucosal and bloodstream infections [4]. Vaginal candidiasis, for example, alone affects approximately 75 % of women around the world [5, 6]. Furthermore, *Candida* infections can lead to significant mortality and extended hospital treatment [7]. In the United States, C*andida* species are the fourth leading cause of nosocomial blood stream infection with up to 40% mortality [8]. *Candida* infections are dramatically increased in patients suffering from immunodeficiencies as several forms of *Candida* can become invasive and cause systemic disease. For instance, research has shown 90% of HIV patients and 50% of AIDS patients had oral candidiasis. In terms of health care costs, systemic *Candida* infections in the United States alone cost about 1.8 billion dollars and that accounts for 70% of the costs of fungal infections [4]. *Candida* species can be subdivided into *Candida albicans* and non-*albicans* groups. The major non-*albicans* species include *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, *Candida lusitaniae*, *Candida kefyr* and *Candida dubliniensis* [9].

1.1.1 *Candida albicans*

Candida albicans is a dimorphic opportunistic fungal pathogen naturally found as a commensal in the gastrointestinal tract and oral cavity, even though it is the most common isolated fungus in

clinical microbiology laboratories [2, 10, 11]. *Candida albicans* has the ability to grow in two distinct forms: budding yeast and hyphae. In vitro transitions between these two morphological forms can be induced in response to several environmental factors such as pH, temperature, or compounds such as N-acetylglucosamine or serum [12]. Morphologically, *Candida albicans* can be found in one of three forms: yeast, pseudohyphae and true hyphae. Yeast forms as rounded cells and the daughter cells separate from the mother cell via budding. Pseudohyphae consist of a chain of cells, which have different degrees of elongation but still have a constriction between adjacent cellular compartments. True hyphae are tube-like as the sides are parallel along the entire length without any constriction [13, 14].

The virulence of *Candida albicans* has been shown to be associated with their ability to switch between the yeast and hyphal morphologies [15]. Several observations have supported the role of hyphae in virulence: the *C. albicans* hyphal form is usually found at sites of infection [14], hyphae and pseudohyphae have been shown to be important for tissue invasion [16], and finally other *Candida* species that do not form true hyphae are much less frequently isolated from humans [17]. The suggested virulence mechanism is that hyphal cells express cell-wall proteins which may facilitate the adhesion to the human tissues, as well as the escape from phagocytosis [18]. So while the hyphae and pseudohyphae forms can promote tissue penetration at the early stages of infection, the yeast form might be more appropriate for spreading in the bloodstream [16, 19, 20].

As mentioned previously, *Candida albicans* is the fourth most common hospital acquired infection in the United States, the treatment of which is estimated to cost more than $1 billion annually in the United States[4]. *Candida albicans* represents one of the major causes of mucosal infection and systemic infection. Furthermore, it causes a range of complications including superficial infections such as vaginitis in healthy women, severe mucosal infection in mouth and

esophagus of HIV patients (chronic mucocutaneous candidiasis), and systematic infections among post chemotherapy and organ or bone marrow transplant patients, such as myocarditis and *Candida* septicemia [12, 16, 21].

1.1.2 Epidemiology and risk factors

The major risk factors for *Candida* infections have changed dramatically in last 20 years since it was prominently associated with patients having malignancy or neutropenia [22] . Currently, the risk factors have changed with the growing number of immunocompromised individuals. New risk factors include the suppression of the immune response, uncontrolled use of broad-spectrum antibiotics and the exposure to pathogens and *Candida* [9]. The suppression of immunity is not limited to HIV and patients with malignancies, it also includes premature infants, patients over 70 years of age, post-chemotherapy patients, post-transplantation patients or those on steroid medication, renal failure patients, patients with malnutrition or with another severe disease[23, 24]. The uncontrolled usage of broad antibiotics promotes fungal colonization and encourages development of antibiotic resistance bacterial strains. For example, research has shown that post-surgery patients that received multiple different antimicrobial therapies were 800 times more susceptible to *Candida* derived wound infections than other patients [8]. Exposure to *Candida* can happen through direct contact with patients via sexual intercourse or being in the hospital for a prolonged stay. Moreover, direct contact with body fluids is another way to expose patients to *Candida*, such as the bloodstream during catheterization during surgery and hemodialysis, peritoneal fluid during peritoneal dialysis and in the lung during mechanical ventilation [20].

1.1.3 *Candida* and human immunity

The human immune response is a combination of innate and adaptive immunity. Innate immunity is the first to be activated [25]. Skin and mucosal membranes are considered as the first line of innate immunity by forming a physical barrier and prevent microorganisms from passing through. After the yeast passed the first barrier it will be detected by germ line-encoded receptors (pattern recognition receptors) that recognize conserved molecular patterns common in microorganisms (pathogen-associated molecular patterns)[26]. This recognition will activate phagocytosis and initiate the production of chemotactic factors. The involved phagocytic cells vary depending on yeast type. For example, the primary effector cells during *Candida albicans* infections are neutrophils while macrophages are the primary cells that respond to *Cryptococcus* infection [27]. Chemotactic factors play an important role in human immunity as they are the link between innate and adaptive immunity. Moreover, they attract more leukocytes to the site of infection and activate several biological procedures including antigen presentation, Th cell differentiation, and production of defense peptides. Activation of the adaptive immunity means the activation of B-cells, T-cells and the general inflammatory responses [28]. Some yeast has succeeded in escaping from the recognition and later from the host defense. For example, *Candida albicans* not only escapes recognition, it also suppresses host immunity by inducing the production of TLR2-mediated anti-inflammatory cytokine (IL-10).

Antimicrobial peptides (AMPs) play important roles in host immunity. These peptides are able to attack the yeast cells and inactivate them by direct contact via several mechanisms of action [29]. Moreover, the AMP killing activity does not always associate with the specific activation of the host immune response, since some AMPs are produced constantly under normal conditions. For example, the histatins , anti-fungal peptides produced by salivary glands, are constantly secreted

in human saliva at an average level of 3.33 microgram/ml, despite the absence of fungal infection [30]. Antimicrobial peptides also play an important role in activation and mediation of adaptive immunity in response to inflammation [31]. Their roles can be summarized as being chemotactic for leukocytes and T-cells, and they activate the production of several immune factors such as interleukin-1, Interferon-gamma and tumor necrosis factors [32].

1.1.4 Treatment of fungal infections

1.1.4.1 Azoles

The azole type of anti-fungal agents has been used to treat various fungal infections for more than 30 years. The azoles can be divided into two groups, the imidazoles and the triazoles. Imidazoles (i.e. miconazole and ketoconazole) have a two-nitrogen azole ring, while triazoles (i.e. fluconazole, itraconazole, voriconazole and posaconazole) have 3 nitrogen azole rings. Both the triazoles and imidazoles share a common mechanism of action. Imidazoles are mostly used as topical agents to treat fungal infections and they have been essentially replaced by the triazoles for systemic infection due to their pharmacokinetics and safety issues [33]. Azoles inhibit the cytochrome P450 enzyme-dependent 14-α-sterol-demethylase which is required for the conversion of lanosterol to ergosterol. Thus, ergosterol is depleted and the sterol intermediate (14α-methyl-3,6-diol) accumulates within the cell membrane, that leads to growth arrest [34]. Azoles are in general considered fungistatic; however, there have been some exceptions reported with itraconazole and voriconazole, that have shown some fungicidal activity against *Aspergillus spp* [35].

Different azole compounds have different affinities for the 14-α-sterol-demethylase; consequently, they have various antifungal activities, side effects and drug-drug interactions. Imidazoles have a low affinity to the demethylase enzyme and as a result, a higher dosage is required. Due to toxicity problems, imidazoles has been restricted to topical applications such as creams, lotions, shampoos, vaginal suppositories, lozenges, and solutions for epidermal candidiasis. In contrast, the triazoles can be found in several formulations that can be administrated topically, orally and intravenously.

Several side-effects have been reported with azoles including events as simple as skin dryness associated with fluconazole to as serious as hepatic failure with itraconazole [34, 36]. Liver toxicity is the main adverse reaction associated with all azoles [37]. Azoles have several significant drug interactions in humans due to the inhibition of liver oxidative metabolism via the binding to cytochrome P450 enzymes. The inhibition of these enzymes interferes with liver metabolism, and subsequently increases drug concentrations in blood resulting in drug toxicity. Azole treatment can increase the overall blood levels of cyclosporine, calcium channel blockers and warfarin [34, 38] due to negative drug interactions when taken together. Nevertheless, the azoles remain the safest available anti-fungal agent. Aqueous solubility has been a significant barrier to the development of new azole-based drugs and the lack of solubility has limited their systematic use outside of a hospital environment.

The extensive use of azoles has resulted in an appearance of drug resistant *Candida* strains and their incidence has increased dramatically since last decade [40-43]. Moreover, a report has shown AIDS patients may harbor azole resistant *Candida* in their oral cavities [40]. However , the rate of azole resistance among the most common *Candida* species remain low with the exception of *C. glabrata* [44].

Four different mechanisms of azole resistance have been described in *Candida spp*. The first mechanism decreases the azole concentration near the target enzyme via efflux pumps. Two gene families that encoded efflux pumps have been defined, *Candida* drug resistance gene (*CDR*), which encodes an ATP-binding cassette (ABC)-type transporter, and multi-drug resistance gene (*MDR*),which encodes a major facilitator transporter [45]. These genes have been identified in different *Candida spp.*: *C.albicans* (*MDR1, CDR1, CDR2*) [45, 46], *C. glabrata* (*CDR1, CDR2*)[47, 48], and *C. dubliniensis* (*MDR1 , CDR1*)[49]. The induction of the *CDR* genes generates resistance to all azole drugs, while the *MDR* genes are selective for fluconazole [43].

The second mechanism of resistance involves the alteration of the target enzyme. It has been shown that mutations in *ERG11*, the gene encoding for the target enzyme (C14α-demethylase) , reduces or halts the azole-binding capability of the enzyme[50]. Furthermore, the mutations can range from a single point mutation [51] to multiple mutation within the same gene [41, 52].

The third mechanism involves the overexpression of azole target enzyme. Increasing the concentration of the target enzyme means more drugs are needed and the common therapeutic concentration is no longer effective [41]. The fourth mechanism of azole resistance in *Candida* species involves the development of bypass pathways [43] . The alternative pathway prevents the accumulation of steroid intermediates via replacing the ergosterol with a latter product. This replacement keeps the membrane functionality and negates the action of azole on the ergosterol biosynthesis pathway. Mutations in the *ERG3* gene, an example of this class, avoids the formation of 14α-methyl-3,6-diol from 14α-methylfecosterol [53]. Finally, more than one mechanism of resistance can be occurred within one strain and their effect will be additive.

1.1.4.2 Polyenes

Polyenes are a group of antifungal drugs originally derived from *Streptomyces* species and they have been widely used to treat systemic and oral fungal infections [54]. Some examples of the polyene drug family include amphotericin B (AmB), nystatin and natamycin. They are macrocyclic molecules with multiple conjugated double bonds, in which a heavily hydroxylated region of the ring is facing the conjugated system [55].

The mechanism of action for this class of drugs begins with the hydrophobic moiety binding to ergosterol within the fungal cell membrane and producing an aggregate. This subsequently leads to pore formation in cell membrane and leakage of vital cytoplasmic components, such as K+, and ultimately inducing cell death [56]. Molecular modeling studies have suggested that AmB creates aqueous pores consisting of eight molecules linked hydrophobically to ergosterol embedded in yeast membrane phospholipids [57]. In addition, AmB causes a direct membrane damage in *Candida albicans* by the generation of oxidative reactions [58].

AmB is fungicidal against the majority of *Candida* species [59]. Though AmB is the most effective antifungal drug available, its narrow therapeutic index continues to limit its clinical utility. AmB has the ability to bind cholesterol of mammalian cell membranes, which is responsible for a major aspect of human toxicity[60]; however , studies have shown that AmB exhibits a greater affinity for ergosterol-containing membranes versus cholesterol-containing membranes[60]. Renal failure and nephrotoxicity are common toxic consequences of AmB treatment, as several studies have reported the rate of acute renal failure in patients on AmB range from 49% and 65% [61]. To overcome the toxicity, several AmB formulations have been evaluated and the best formulation was found to be AmB in liposomes as it permits higher doses

with the lowest rate of nephrotoxicity [62]. Currently, the lipid formulations have been used as second-line of therapy since the liposome formulations cannot completely solve the mammalian cell toxicity and the liposome formulations are considerably more expensive than other anti-fungal drugs [63].

Although polyene resistance has not been a major clinical problem to date, multiple cases have been reported in *Candida* species, including *C.albicans*[64], *C. krusei*[65], *C. lusitaniae*[66], *C. glabrata*[67] and *C. tropicalis*[68]. The exact mechanism of resistance is not clear but most of the resistant strains shares a common feature, their ergosterol content was low compared to susceptible strains [41]. Proposed mechanisms of polyene resistance include alterations in membrane ergosterol content via the accumulation of other sterols, alterations in the sterol to phospholipid ratio or changing the ergosterol structure by reorienting or masking[69]. It is worth mentioning that cross-resistance to azole and polyene classes of drugs have been reported in *Candida* species [70]. Furthermore, the efflux pump mechanism that functions with azole compounds has not been identified, so far, in polyene resistance strains [71, 72].

1.1.4.3 Echinocandins

Echinocandins are the most recently discovered class of antifungal drugs that has been introduced for clinical use. There are now three echinocandins approved for clinical use: caspofungin, micafungin, and anidulafungin [73]. Echinocandins are derived from semisynthetic modifications of fungal lipoproteins: caspofungin from pneumocandin B0 from *Glarea lozoyensis*[74], micafungin from FR901370 (hexapeptide) from *Coleophoma empedra*[75], and anidulafungin from echinocandin B0 from *A. nidulans*[76]. Moreover, they are cyclic hexapeptides N-linked to long acyl lipid side chains with a molecular weight of approximately

1,200 [77]. The enchinocandins have a unique mode of action in which they act as noncompetitive inhibitors of the enzyme 1, 3-β- and 1, 6-β-D-glucan synthase. 1,3-β-D-glucan is an essential carbohydrate component of all fungal cell walls which covers 30%–60% of the fungal cell wall in *Candida* species. Inhibition of enzymatic activity leads to changes in the cell wall components that result in osmotic instability and cell lysis [77]. Compared to other antifungal drugs, the echinocandins possess low human toxicities due to the fact that human cells lacks 1,3-β-D-glucan[78]. The most common side-effects are urticarial, pruritus and elevation in transaminase levels [79].

Echinocandins are fungicidal against a wide range of species and they are effective against biofilms [80]. In some cases, echinocandins are fungistatic. For example, caspofungin is considered fungistatic against *Aspergillus* whereby they block hyphal tip growth [80]. Since the glucan composition varies between yeast species the echinocandins activity against these species will vary. These agents are not recommended as the first line of treatment for fungal infections [76, 80]. Echinocandin resistance has been identified in *C. albicans*[81], *C. krusi* [82], *C. glabrata* [83], C. *lusitaniae* [84] and *C. parapsilosis* [85]. The echinocandins resistance is mediated by point mutations of *FKS1* gene encoding a component of the β-1,3-D-glucan synthase complex [86] .

1.1.4.4 Antimetabolites

Flucytosine (5-FC), also known as 5-fluorocytosine, is a fluorinated analog of cytosine and one of the oldest antifungal agents. Furthermore, it is the only antifungal agent that functions as an antimetabolite. Flucytosine is considered a fungistatic agent and its activity comes from the rapid conversion of 5-FC to 5-Fluorouracil (5-FU); therefore, 5-FC by itself has no antifungal activity. 5-FC gets into the cell via a cytosine permease, which are also transports cytosine,

hypoxanthine and adenine. Inside the fungal cell, 5-FC undergoes a deamination process via cytosine deaminase to generate 5-FU [87].

There are two potential pathways for 5-FU fungistatic activity [88]. The first pathway is 5-FU undergoes further metabolism by uridine monophosphate pyrophosphorylase to generate 5-fluorodeoxyuridine monophosphate (FdUMP). Then, FdUMP, an inhibitor to thymidylate synthetase, halts thymidine biosynthesis, and accordingly DNA synthesis. The second pathway starts with the transformation of 5-FU into 5-fluorouridine triphosphate (FUTP), then FUTP replaces uracil in fungal RNA and later inhibits protein synthesis. 5-FC is highly selective against fungi because mammalian cells lack the cytosine deaminase. Due to a high rate of resistance, the use of 5-FU as a monotherapy is restricted. The compound is typically used in combination with other antifungal agents such as amphotericin B [59].

The mechanisms of resistance to 5-FC in *Candida* is mediated by enzymatic modifications that either impair 5-FC cellular uptake via a mutation in cytosine permease [89] or a defect in 5-FC metabolism which can be achieved by mutations in the gene encoding either the cytosine deaminase or uracil phosphoribosyl transferase [41, 90] .

1. 2 Antifungal peptides (APFs)

The major reservoir for generating new antimicrobial peptides (AMPs) is natural sources that might be any living organism such as vertebrates, invertebrates, plants and bacteria [91]. Besides their roles in innate immunity, AMPs serve as promising candidates for new therapeutic compounds. AMPs are attractive models because they possess unique features, such as broad activity, rapid action, low microbial resistance and high selectivity. Thus, several synthetic and semi-synthetic peptides have been synthesized for this purpose. Therapeutic peptides are

designed mainly after careful studies of the biophysical characteristics and structure-activity relationship of naturally occurring AMPs [92].

The broad activity of AMPs makes it difficult to exclusively classify them as either antifungal or antibacterial. There are relatively few examples where a peptide retains only antifungal or antibacterial activities. In some cases, AMPs could be antibacterial; however, their antifungal activities have not been tested yet, and Vice versa. This section will discuss antimicrobial peptides that exhibit anti-fungal activities and they will be referred to as antifungal peptides (AFPs).

1.2.1 Classification

The antimicrobial (AMPs) and antifungal peptides (AFPs) represent a diverse array of sequences and there could be no perfect way to classify them. Several reviews have classified the AMPs using different criteria: secondary structures [93, 94], source and the mechanism of action [94-96], cells that produce the peptide [97, 98,99], post-translation modification [100] and the species (eukaryotic and prokaryotic) [101, 102]. In general, there are around 700 AMPs with antifungal activity found in the antimicrobial peptide database (http://aps.unmc.edu /AP/ main.php)[103] . Peptide net charge is an alternative way to categorize them. Consequently, the peptides could be divided into three groups: anionic, neutral and cationic.

1.2.1.1 Cationic AFPs:

The vast majority of the AFPs are cationic and they display a positive net charge (Figure 1). Cationic AFPs are gene-encoded peptides mainly derived from larger precursors via proteolytic processing [104].

1.2.1.1.1 Biophysical properties of cationic AFPs.

This section will discuss the main biophysical characteristics of AFPs in general and the cationic peptides more specifically. The characteristics are: stereospecificity, conformation, charge, amphipathicity, and hydrophobicity. It is important to note that some of these characteristics are interdependent; therefore, modification of one character could lead to alterations in the other.

Stereospecificity. Stereospecificity means certain biological processes or chemical reactions are specified for only one of several possible stereoisomers. Within peptides the focus is on enantiomers or optical isomers. Generally, stereospecificity in binding processes is an essential requirement for peptide or protein-target interaction and lacking this characteristic might suggest a lack of overall specificity[105]. In AMPs, binding to the target microorganism is required to achieve an activity; however, the majority of the AMPs are not stereospecific with some exceptions [106]. Several publications have shown that all-D-amino acid peptides demonstrated similar antimicrobial activities as their all-L-enantiomers [107-110]. As a subgroup of AMPs, AFPs exhibited the same characteristic [107, 111] and multiple examples were published demonstrating the stereoisomeric AFPs was equal in activity[107, 111]. For example, all-D variants of the histatin peptide fragments (including P-113) are equally internalized and they exhibited the same activity. Therefore, the involvement of a stereospecific receptor was excluded [112, 113] . However, in some case the stereospecificity may interfere with the AFPs activity but at a lesser extent than other AMPs. For example , the Bac7 peptide, a cathelicidin derived peptide, has shown a stereospecific binding to *C. neoformans* membrane at concentrations near the MIC values; however, this interaction became non-stereoselective at higher concentrations [114]. The thanatin peptide is another example of a stereospecific peptide, where the activity against Gram-positive

bacteria was inhibited in all-D enantiomers, but the activity is retained against fungi just like L-thanatin [115].

Conformation. Similar to other AMPs, there is no dominant conformation within the AFPs; they differ in sequence and secondary structure. The antifungal peptides could be defined according to their conformation into four categories: α-helix, β-hairpen or sheet, mixed α- helix /β-sheet, and amino acid rich peptides (Figure 2).

The α-helices are abundant within AFPs; however, they frequently exist as unstructured conformers. These peptides become helical only upon interaction with an amphipathic membrane such as a fungal plasma membrane [116]. The β-sheet containing AFPs are less abundant than α-helix and this category is highly diverse at the level of primary structure but they share common features, such as the amphipathic structure [117]. While studying the peptides within the antimicrobial database [118], it has been noted that AFPs with beta structure share a common feature: they have at least 2 cysteine residues which is not always true within the broader group of AMPs since beta structure peptides have been identified without a disulfide bridge [119-122] .

Most amino acid-rich AFPs could be further divided into one of the following groups: Gly-rich, Pro-rich, Arg-rich, His-rich, and Trp-rich. According to the antimicrobial peptide database, AFPs did not contain any Lys-rich peptides typically found in AMPs, such as the dermaseptin family [123] and GLK-19 [103]. AFPs that are enriched in particular amino acids exhibited different conformations and they can form unusual helices or sheets. For example, Tritrpticin, a tryptophan-arginine rich peptide, has retained multiple structures upon binding to a micelle, including a turn-turn and extended α-helix structure [124]. Another example is SP-B, a proline-rich

peptide that exhibited both antibacterial and antifungal activity. The secondary structure of this peptide is composed of polyproline-II helices, unordered and turn motifs [125].

Charge. The positive charge is an essential requirement for AFP activity whereby an electrostatic binding to the negatively charged membrane is initiated (Table 1). However, increasing the positive charge does not always improve the antifungal activity, depending on the peptide sequence and secondary structure [126]. The increase in positive net charge has shown different effects on the antifungal activity. For example, increasing the number of lysine residues within dF17-6K (compare with dF21-10K) improved the antifungal activity [127]. On the other hand, increasing the charge within MtDef4 peptide from +6 to +7 had an inhibitory effect on the activity against *F. graminearum* [128] . It has been demonstrated that increased peptide positive net charge beyond a threshold might lead to a strong interaction with the negatively charged membrane, resulting in an inhibition in peptide translocation into the cell [117]. In some peptides, the amount of the positive charge and the net charge was not as important as the location of the cationic residue [126]. Commonly, AFPs that exhibited positive charge achieve the activity via pore formation; however, some exceptions have been observed [129]. The remaining AFPs are neutral or anionic and they will be discussed later in this chapter.

Amphipathicity. Most of the AMPs as well as the AFPs form amphipathic structures upon interaction with target membranes. Amphipathicity is defined as the ability of a molecule to adopt a shape or structure in which clusters of hydrophobic and hydrophilic amino acids are spatially organized in discrete sectors [93]. Amphipathicity for a peptide is usually determined by the mean hydrophobicity (H) and the hydrophobic moment (μH) [117]. Mean hydrophobicity for a peptide is calculated by summing the hydropathy values of all the amino acids then dividing it by the number of residues. The hydropathy value for each amino acid has been determined and

tallied into different scales. The most common scales are the Kyte-Doolitle[130] and the Eisenberg scales [131]. The hydrophobic-moment (μH) is the hydrophobicity of a peptide measured for different angles of rotation per residue[132]. Moreover, it is calculated for all angles of rotation from 0 to 180 degrees [132, 133]. Measuring the μH for a peptide assist in recognizing amphipathic structures via determining when the residues on one side of the structure are more hydrophobic than on the other [134]. In general, increasing the hydrophobic moment leads to increased antifungal activity via promoting membrane permeabilization. However, it has been reported that high amphipathicity also leads to increased hemolytic activity. For the development of new peptides, especially with membrane lytic mechanisms, it has been suggested to keep μH less than for hemolytic peptides (μH less than 0.3) [92]. Several examples demonstrated the importance and the function of amphipathicity in antifungal activity. For instance, In P19(6/E) peptide research has shown reduction of the amphipathicity by scrambling the sequence was enough to reduce the antifungal activity against *C. albicans* and *C. neoformans* even though the amino acid composition, charge, mean hydrophobicity and helix forming propensity in 50% TFE were kept the same [135]. On the contrary, reduction of the YLK peptide amphipathicity by utilizing helix breaker residues enhanced the antifungal activity [92] .

Most of the α-helical peptides have amphipathic structures, yet it is not a requirement for the antifungal activity. For example, kaxins are a class of antifungal peptides that do not exhibit an amphipathic structure [127, 136]. This class has displayed antifungal activity with minimum hemolytic activity and that supported the correlation between peptide amphipathicity and hemolysis [127].

The amphipathicity isn't restricted for α-helix peptides; β-structures could also be amphipathic. Tachyplesin and polyphemusin are examples of amphipathic β-sheet [137] while Rhesus theta

defensin-1 (RTD-1) peptide is an example of non-amphipathic β-sheet [138]. In amino acid -rich peptides, the amphipathic structure was also observed such as in histatin 5(His-rich) [139] and Pac-525 (Trp-rich)[140] . It is also important to mention that all proline-arginine rich peptides (AMP or AFP) cannot form amphipathic structures, thus the proline abundance results in the formation of polyproline helical type-II structures [141].

Hydrophobicity. Peptide hydrophobicity is defined as the percentage of hydrophobic residues within a peptide length. The vast majority of antifungal peptides have hydrophobic values ranging between 30-60% (Figure 3). Hydrophobicity is an essential requirement for peptide membrane interactions as well as membrane permeabilization. Furthermore, the peptide partitioning into the phospholipids layer is also controlled by peptide hydrophobicity. Increasing peptide hydrophobicity might correlate with an increase in the activity, but it also has been linked to increased hemolysis activity [117, 142]. For example, increasing the hydrophobicity of the D1 peptide, to generate a peptide termed D4, led to the induction of increased hemolytic activity by approximately 286-fold. In the same example, the modification of the D1 peptide also interfered with the fungicidal activity; however, the change was observed for the target susceptibility rather than the peptide effectiveness. The D1 peptide was more active against *Zygomycota* fungi while the D4 peptide was more active against *Ascomycota* fungi [117, 142]. Lipopeptides is a class of antifungal peptide where lipophilic moieties are attached to cationic peptides. Although this class is very hydrophobic, they had a low hemolysis activity [143].
It should also be noted that hydrophobicity is not the only factor related to hemolysis, the presence of tryptophan was also linked to the hemolytic activity. A study has shown that replacing one asparagine residue with tryptophan in the NDGP peptide was enough to increase the hemolytic activity approximately 24 fold [92]. Further, it has been concluded that tryptophan-

tryptophan interactions and tryptophan-lipid interactions are responsible for the increase in hemolytic activity of melittin-tryptophan analogs rather than the hydrophobicity [92]. It has been published that tryptophan has a strong ability to insert into membranes as well as to interfere with lipid polymorphism [144]. The hemolytic activity associated with tryptophan-containing peptides has been inspected in multiple research studies and the results were varied. For instance, the Pac-525 peptide is a tryptophan-rich peptide that exhibits low hemolysis activity [140].

1.2.1.1.2 -Mechanisms of cationic AFPs action

AFPs achieved fungicidal or fungistatic activity via multistep mechanisms. First, the peptide is attracted to the negatively charged yeast membrane. The interaction with the membrane results in secondary structure changes that induce the required conformation for the activity, a required step for α-helical cationic peptides. The peptide then inserts into the cell membrane and interferes with the phospholipid bilayer integrity via pore formation or disruption of the whole membrane barrier. In some cases, the peptide is required to move into the cytoplasm and act on internal cellular targets.

For simplicity, the mechanism of action of AFPs could be split into two categories: pore-forming and non-pore forming mechanisms. In the pore forming mechanism, the peptide primarily works at the plasma membrane and no internal targets are required to achieve the activity. Furthermore, permeabilization and pore formation are the major cause of cell death. This mechanism has been reviewed on several occasions [145-149], and several models have been proposed to explain peptide action via this mechanism. In the first mechanism, peptides arrange perpendicular to the membrane to form either a barrel-stave or toroidal channels. The second model is achieved when peptides orient parallel to the surface of phospholipid bilayer and form

an extensive layer or carpet [149]. In the third mechanism, peptides aggregate in micelle-like complexes with lipids and span the membrane; however, no particular orientation has been observed[146]. In some cases, pore formation and membrane interruption are not enough to cause cell death unless they are combined with another mechanism as the peptide-generated pore could be the means by which the peptide reaches the cytoplasm. In some cases, the translocation across the membrane is energy-dependent, requiring a transporter protein and ATP[150] .

Several models have described the other mechanisms where the peptide target is actually inside the cell. The most common intracellular targets are; the binding to DNA[151] , the inhibition of protein synthesis [152], the inhibition of ATPase activity and protein refolding[153], the depolarization of mitochondria and depletion of ATP [129], the formation of reactive oxygen species and apoptosis[154] and the depolymerization of the actin cytoskeleton [155].

1.2.1.2 Anionic AFPs

The first anionic AMPs were described in 1984 [156] and after that several anionic peptides were identified in both eukaryotes and prokaryotes [157, 158]. It has been found that anionic AMPs might play an important role in innate immunity [159]. Out of 116 anionic peptides are described in the peptide database, only 11 peptides have shown antifungal activity.

The biophysical properties of the anionic AFPs could be summarized as: the net charge ranges from -1 to -8 at neutral pH, the size ranges from 11 to 90 amino acids, they exhibit an amphipathic structure and the secondary structure varies between α-helix and β-sheet (Table 2). This peptide category is more specialized than the cationic group and they displayed a narrow spectrum of activity, thus the majority of the peptides in this category are active only against fungi.

There is no definite antifungal mechanism for anionic peptides and almost every peptide has its own mechanism. Nevertheless, all of them share a common step, binding to the target membrane. In cationic peptides, the positive charge is essential for the initial interaction with the negatively charged membranes. Moreover, the ionic attraction between the opposite charges also plays a role in target selectivity. In contrast, the anionic peptides display a negative net charge that could lead to repulsion with the negatively charged membrane. It was found that the overall positive charge is not a prerequisite for the binding to the membrane and the key modulators of lipid bilayers-peptide interaction is the charge distribution and the secondary structure [160]. Consequently, the anionic peptides might be able to interact with the membrane because their basic amino acids are distributed in such a way as to give them the accessibility and limit the repulsive effect of negatively charged residues. This model does not fit all anionic peptides since some of these peptides do not have a single basic amino acid, such as Tn-AFP and EP-20 (Table 2). These peptides retain the capability of binding to the membrane and this binding is critical for the activity [159,161]. The other explanation is that the anionic peptides form cationic salt bridges with membrane negative charges via metal ion cofactors [158, 159, 161].

Various mechanisms are involved in anionic AFPs activities. The inhibition of cell wall biosynthesis, protein synthesis [162] as well as the inhibition of spore germination [163] have all been observed. Another proposed mechanism is the depletion of metal ion (Cu^{2+} or Fe^{2+}) supplementation and alteration of yeast respiratory profile [164, 165]. Furthermore, membrane permeabilization and the induction of reactive oxygen species as well as nitric oxide have also been observed[166] . Yet another mechanism reported is the alterations in cell morphogenesis and membrane disruption [160, 167].

1.2.1.3 Neutral AFP

As stated previously, the vast majority of AFP families are cationic; however, a few are neutral. For example, Aurein 1.1 is neutral; whereas, the other 11 Aurin peptides are cationic. The brevinin family of peptides is another example where brevinin-1- OR3, OR6, OR8, and OR9 are neutral peptides and the rest are cationic [168]. An exceptional case is GP-19 peptide, which is neutral, and GP-20 peptide is anionic [162].

The general biophysical properties of neutral AFPs are: the net charge is neutral at pH 7, the peptide size ranges from 13 to 50 amino acids, most of neutral AFPs form an amphipathic structure, the secondary structures for the know neutral AFPs are helical, except for Drosomycin-2 which is a mixture of α-helix and β-sheet (Table 3).

Most of these peptides have both antibacterial activity and antifungal activities with the exception of GHH20, Galleria defensin and Ha-DEF1, which lack the antibacterial activity. Surprisingly, Galleria defensin-like peptide, which shares 95% homology with Galleria defensin, possessed an antibacterial activity for gram-positive bacterium (*S. lutea*) along with the antifungal activity.

The mechanisms of action for the neutral AFPs are membrane permeabilization and disruption. It has been suggested that the positive charges within the neutral peptides are able to initiate electrostatic interactions with the negatively charged membrane prior the membrane disruption [169 162 170]. However, two neutral AFPs (Gp-19 and Temporin-1PRb) lack charged amino acids and they exhibit activity via membrane disruption.

1.2.2 Posttranslational modifications of natural AFPs.

Several post-translation modifications have been observed in naturally occurring AFPs and each modification had an influence on the AFPs activity. The knowledge of these modifications is important to reach a full understanding about peptide activity and later, to be used to improve the activity or solve problems related to antifungal activity. This section will discuss the following modifications: glycosylation, amidation of C-terminus, isomerization which includes diasteromers and enantiomers, halogenation, phosphorylation, hydroxylation and cyclization. The data in this section are general and subjected to change at any time, as new AMPs could be published or added to the database. Furthermore, some of the AMPs have not yet been tested for antifungal activity.

Glycosylation. The addition of carbohydrates is one of the most common post-translational modifications and it is typically observed at asparagine or serine/threonine residues. N-linked and O-linked are both common types of glycosylation [171], and in rare cases S-linked peptides have been discovered [172]. In AMPs, glycosylation was observed in proline-rich peptides such as in Drosocin [173] and Pyrrhocoricin [174]. Moreover, the O-glycosylation of these peptides was determined to be essential for full antimicrobial activity; this is not always the case since S-linked glycosylation had no effect on the antimicrobial activity[175].

No natural AFPs have been found to be glycosylated in the antimicrobial peptide database [103]. In synthetic AFPs, several cases have been published. For example, the antifungal activities of caspofungin , a semi-synthetic lipopeptide, was improved after the addition of a monosaccharide [176].

Amidation of carboxy terminus. The main role of amidation is to improve peptide stability in the presence of aminopeptidases [177] and in some cases to increase the anti-fungal activity [178]. Several examples of AFPs with an amidated C-terminal were identified in nature such as Ctriporin from scorpions [179] and Ranacyclin from *Rana esculenta* skin [180]. Moreover, the amidation has also been a common approach in synthetic AFPs [100].

Isomerization (Diasteromers). This is the ability of peptide to exist in two conformations, cis and *trans*. In general, the isomerization increased the antimicrobial activity and improved the stability of multiple peptides [181]. Diasteromers have been identified in AMPs like Caenopore-5 [182] as well as in AFPs like Cyclo(L-Phe- 4-OH-L-Pro)[183] .

Isomerization (enantiomers): There are a few examples of naturally occurring AMPs with D-conformation, such as bombinin H4 [184], lactocin S [185] and gramicidin A [186]. However, none of them had antifungal activity. Several AFPs have been synthesized using D-amino acids in an attempt to improve the peptide stability against proteases [112, 113].

Halogenation. In nature, the most common halogenation is bromination and chlorination. Bromination mainly occurred in AMPs, and more precisely, in the tryptophan indole ring while chlorination was generally observed in AFPs. Bromination has been described in different AMPs such as *Hedistin* and in *hagfish* cathelicidins [100, 187]. The role of indole bromination in cathelicidins was suggested to reduce the peptide susceptibility to proteolysis via steric modifications [188]. Misgurin from the mudfish *Misgurnus anguillicaudatus* is an example of an antifungal peptide that has been modified by chlorination; however, the role of the chlorination was unclear and it did not alter the antifungal activity [100].

Phosphorylation. Nature has produced multiple examples of phosphorylated AMPs and AFPs. Phosphorylation has been found to be essential for AMPs activity. For instance, enkelytin, an antibacterial peptide derived from *proenkephalin A,* has two phosphoserines that are required for the full activity[189]. Prochromacin and chromacin are also examples in which the modifications were mandatory for peptide activity (glycosylation and phosphorylation)[190]. In AFPs, the situation was different and phosphorylation may be not required for the antifungal activity. Histatin 1 is an example of a phosphorylated AFP where the modification didn't have any effect on the peptide activity; however, it did increase the stability of the peptide in saliva [191].

Hydroxylation. In AMPs, hydroxylation was primarily observed at lysine, arginine, tryptophan and phenylalanine residues. The effect of hydroxylation on the peptides is unclear and it has been shown to have different effects. For example, the hydroxylation of the MGD-2 peptide was essential for antimicrobial activity; however, other studies have suggested that the peptide activity was not changed by the hydroxylation [192]. The Cecropins AMP family is an example of a hydroxylated lysine peptide and only cecropin B has shown anti-fungal activity [193]. Halocyamines, tetrapeptides from *Halocynthia roretzi*, are an example of dihydroxyphenylalanine modified AFP [194]. Styelin D and callinectin are examples of anti-microbial peptides with dihydroxyarginine and hydroxyl-tryptophan residues, respectively [192, 194], but both peptides did not display fungicidal activity.

Methylation: The most common sites of methylation are tyrosine and lysine residues. Clavanins are natural AMPs with a methylated tyrosine [195]; however, none of them has exhibited antifungal properties. The synthesized AFP cecropin A-melittin had trimethyl-lysines and a study has shown that the methylation decreased the hemolytic activity and promoted the selectivity against certain microorganisms[196].

Cyclization. Although not considered a posttranslational modification, cyclization has given unique properties to peptides, such as increasing antifungal activity, reducing the toxicity and improving the stability in proteases [197]. Tunicyclins B [198, 199] and cyclopsychotride A [200] are examples of cyclic naturally occurring AFPs. Moreover, there are several examples of synthetic antifungal peptides such as RTD-1, where the cyclization has improved the antifungal activity [201]

1.2.3 Disadvantages of antifungal peptides and proposed solutions.

The general disadvantages of AMPs, and AFPs specifically, could be summarized as: poor oral and tissue absorption, rapid in vivo degradation, poor stability (shelf-life) and potential immunogenicity. Moreover, most peptides are rapidly excreted, poorly bioavailable and salt sensitive. Human toxicity and hemolysis have also been observed with some AMPs. These issues will be addressed in detail below.

Stability. The stability is not an issue for naturally occurring AMPs within their environment for multiple reasons. First, most of the AMPs are derived by proteolysis of larger proteins or peptides; therefore, equilibrium exists between peptide generation and degradation. For example, buforin II is generated from histone 2A [202], lactoferricin from lactoferrin [203] and histatin 5 from histatin 1. Second, small peptide fragments that are generated via proteolysis of the active peptide, in some cases, maintain some antifungal activity. For example, 12 fragments of histatin 1 have been identified and most of them retain antifungal activity. Finally, there might be something within the physiological environment that supports peptide stability and increase the half-life. For instance, the ability of histatin 1 to bind to hydroxyapatite within the enamel pellicle decreases the proteolytic degradation [191].

For synthetic peptides, stability and bioavailability problems may be solved via peptide formulations or modifications. Several approaches have been used to improve the stability, such as liposome-encapsulation [204] , use of peptoids [205] , D-conformation-based peptide[206] , and β-peptides[207]. The use of different peptide formulations has been the major approach to improve peptide bioavalability, delivery, and stability. The melittin-lipid disk is an example of a formulated peptide in which polyethylene glycol-stabilized lipid was fused to the melittin peptide [208]. Carbon nanotubes and magnetics nanoparticles are useful tools for drug delivery; therefore, this may represent a promising avenue of research for peptide delivery [209] .

Toxicity. In therapeutics, drug toxicity is directly proportional to the concentration; however, the toxic concentration varies between the drugs. Natural AFPs, within their tissues, have shown a high target specificity and low toxicity. Most multicellular organisms express a cocktail of peptides within their `defensive' tissues, in which the cocktail contains several classes of AMPs and AFPs [93]. Furthermore, these peptides probably have a synergistic effect and work at low concentrations far from their toxic levels [210]. However, the synergistic effect is absent in most in vitro assays, since each AFP is tested individually. Furthermore, different isoforms of the same peptide could be present at the same time. For example, different forms of *Rhesus* θ defensins (RTD-1, RTD-2, and RTD-3) have been identified in leukocytes of *Rhesus macaques*, where the cellular abundance of the three peptides (RTD1, RTD-2 and RTD-3) differs at a ratio of 29:1:2, respectively. In spite of having multiple forms, all of the RTD isoforms have the same antifungal activity; however, they do display distinct antibacterial activity as well as differences in net charges RTD-1 (+5), RTD-2 (+6), and RTD-3 (+4) [201].

Specificity. One major challenge in designing new antifungal peptides is achieving high specificity toward fungal cells. The perfect AFP would ideally have an affinity for multiple

targets within the microbe and the targets must be accessible and relatively immutable. Unfortunately, this is not the case for most AFPs, either natural or synthetic. Host cell toxicity might be solved after a full understanding of the mechanism by which the peptides recognize their target [92, 117]. The literature has provided several examples in which the cytotoxicity of synthetic peptides was reduced successfully via modifications in the peptide composition [211-213]. However, each case is unique and the solutions have varied. An example of reduced toxicity was observed with the melittin peptide, where cytotoxicity was reduced by fusing melittin with cecropin A or the magainin peptide rather than by amino acids substitution [214, 215].

Salt sensitivity. Salt sensitivity may present the greatest challenge for the majority of AMPs as well as AFPs, for clinical use since they are typically salt-sensitive at physiological concentration of ions. Table 4 shows synthetic and natural AFPs that seem to maintain activity in the presence of salts. Since salt insensitivity is only found in a small number of peptides, the general biochemical properties of these peptides will be discusses briefly as potential solutions for other peptides that exhibit salt-sensitivity.

One parameter is the *net charge* that varies dramatically between anionic and cationic peptides; however, the majority of salt insensitive peptides are cationic with net charge greater or equal to +4 at pH 7. There is no preferred secondary structure, as it varies between α-helical and β-sheet. The amphipathicity has been observed in this group with an exception of RTD-1 which did not display any amphipathicity, and surface models have suggested a clustering of positive charges [138]. The second parameter of salt-insensitive peptide is the *secondary structure.* The fungicidal activity of these structured peptides is accomplished via pore formation or membrane permeabilization. This group can be subdivided into two groups, helices and sheets, where each group has something in common other than secondary structure. By looking to the amino acid

composition, most β-structure peptides have more arginine than lysine and also share the ability to form disulfide bonds. Studying the peptide structure and amino acid compositions of β-peptides sheds light on the role of disulfide bonds and cyclization in salt tolerance. It has been found the cyclization and disulfide bonds are essential for the salt insensitivity in RTD-1[216] , TP-1[217], and arenicin-1[218] peptides. However, the essential factor in potegrin 1 was structure rigidity and the presence of disulfide bonds did not have any effect [136]. The major general conclusion is from looking at salt-insensitive β-peptides is that disulfide bonds and/or structure rigidity may be crucial to salt insensitivity.

On the contrary, α-helical peptides did not have any cysteine nor disulfide bonds. Peptides in α-helical subclass did not show any common essential feature. The only observed similarity is that the majority of α-helices have lysine more than arginine in their sequence; however, it has been reported that the substitution of arginine to lysine in Ci-MAM-A24 increased salt sensitivity [219] . In addition, arginine was not always preferred and in some cases the increase in arginine percentages led to increase salt sensitivity [202]. DCD-1 is a unique example of α-helical peptide, that has been shown to be only 20% active at low salt conditions and the activity is retained by increase the salt content; however, this is not the case for the majority of AFPs. Furthermore, DCD-1 is also affected by pH since basic pH was the optimal [220].

1.3 Histatins

Human histatins are a family of small histidine-rich peptides secreted in saliva by parotid, submandibular, and sublingual glands[221]. Histatins have been also identified in some higher primates species including the great ape, *Hylobatidae* and *Cercopithecidae* [222]. Histatins are important members of innate immunity and are essential for dental and oral defense [223].

Moreover, histatins has been shown to have other functions including wound healing and formation of the acquired enamel pellicle [224]. Research has shown the entire histatin peptide family is encoded by two genes (*HIS1* and *HIS2*) which are localized on chromosome 4q13. Both *HIS1* and *HIS2* are exclusively expressed in the salivary glands where the full length precursors , histatin 1 and histatin 3, are synthesized, respectively [225]. The other members of histatin family are generated by an irresolute proteolytic pathway[226, 227] or via alternative splicing of the genes [228]. The size of human histatins ranges from 7 to 38 amino acids in length[103, 228]. The common feature in all histatin members is the fact they are enriched in histidine, lysine, and arginine residues. The predominant histatins are histatin 1, 3 and 5 consisting of 38, 32 and 24 residues, respectively. These three peptides comprise around 80 % of histatin family. Their average concentrations in the parotid saliva ranges from 7 to 28 µg/ml for histatin 1, 6 to 43 µg/ml for histatin 3, and 10 to 43 µg /ml for histatin 5. Furthermore, the mean concentrations for histatin 1, histatin 3, and histatin 5 in submandibular and sublingual saliva are 28 to 122 µg/ml, 5 to 75 µg/ml, and 26 to 90 µg/ml, respectively.

1.3.1 Histatin 5

Histatin 5 is composed of 24 amino acids and it is generated from a proteolytic processing of histatin 3. Moreover, histatin 5 is the most potent member of the histatin family against fungal species. Besides its role as an antimicrobial peptide (both fungicidal and bactericidal) [229-231], histatin 5 enhances the glycolytic activity of oral micro-organism [226], inhibits lipopolysaccharide mediated activities[232], prevents co-aggregation and hemagglutination in certain bacteria [228], inhibits bacterial enzymes such as collagenase and trypsin protease[233], inhibits *Candida albicans* colonization in oral cavity [234], reduces nickel allergy via direct binding with Ni(II) ions[235],

controls oral infection via down regulating the IL-8 responses [236] and promotes the secretion of histamine from mast cells[237].

1.3.1.1Unique features of histatin 5.

Histatin 5 has some unique features not found in other AMPs. First, histatin 5 is enriched in the amino acid histidine, which has a pKa of 6.0 for the side chain. Thus, this can result in the alteration of the peptide net charge as well as antimicrobial activities. Histatin 5 net charge is dramatically changed by altering the pH from 4 to7 to 9 , at pH 4.5 the net charge is +12.9, at pH 7 it is +6.6 and at pH 9 it is +3.6 [238]. Second, histatin 5 has an ability to form complexes with several ions metals that may promote histatin 5 killing activity via binding to divalent cations which are essential to microorganisms life cycle[239]. Finally, Histatin 5 is adsorbed to hydroxyapatite on the teeth[240]. This feature prolongs histatin 5 life via protection from proteolysis by proteases in the oral cavity. The binding to teeth lowers the overall antimicrobial activity, but it is sufficient to prevent *Candida albicans* colonization of the oral cavity[234].

1.3.1.2 Histatin 5 spectrum of antimicrobial activity.

Histatin 5 shows a broad spectrum of activity against oral and non-oral pathogens , including fungi (*Candida albicans, Cryptococcus neoformans, Candida stellatoidea, Candida tropicalis, Candida glabrada, Trichosporon pullulans, Candida guillermondii, Candida lambica, Candida pseudotropicalis, Candida krusei*, and *Candida parapsilosis)* , bacteria (*Streptococcus mutans*, *Porphyromonas gingivalis*, *Actinomyces viscosus*, *Actinomyces naeslundii*, methicillin resistant *Staphylococcus aureus* and *Actinomyces odontolyticus*)[228, 231, 241] as well as protozoa *(Leishmania donovani)* [242].

1.3.1.3 Secondary structure of histatin 5.

Histatin 5 has been studied extensively; therefore, the biophysical properties and NMR structure have been examined. Regarding the secondary structure, histatin 5 has no defined structure in water, but it adopts helical conformations in hydrophobic environments such as trifluoroethanol and synthetic lipids [139, 243, 244]. The NMR structure of histatin 5 in aqueous and non-aqueous solution has been solved[244]. The NMR studies have been also carried for some of histatin 5 derivatives including the C-16 peptide[243] and P-113 peptide[245]. The NMR studies supported the other biophysical studies suggesting that histatin 5 and its derivatives have a random coil structure in aqueous solution, but in a hydrophobic environment or biological membrane , they adopt a helical conformation and the positively charged residues are clustered together to form a hydrophilic patch. The electrostatic and hydrogen-bonding interaction of cationic and polar residues with the head groups of the plasma membranes of target cells are the reasons for induced helical structure [243]. The ability to form α-helical structures is essential for the activity in most of the cationic peptides[246, 247]; however, the role of helicity in histatin 5 activity remains a question. Situ et al. have demonstrated that reducing the ability to form a helical structure in histatin via replacing three amino acids with proline (a helix breaker) has little effect on the fungicidal activity [219].

1.3.1.4 Amphipathicity and hydrophobicity of histatin 5.

Amphipathicity is an essential requirement for AMPs activity, especially the pore-forming peptides [246, 248]; however, it has been suggested that histatin 5 exhibits a weak amphipathic structure as compared to other AMPs. Furthermore, increasing the amphipathic character of histatin 5 via histidine replacement did not improve the activity [249]. Another unique histatin 5

character is the hydrophobicity**.** In a comparative study between histatin 5 and other AMPs (PGLa, dhvar1, dhvar4, KL, CRAMP18, SPLN14-27, SPLN28-41, equinatoxin II and magainin 2) using a hydrophobicity plot that is generated by the mean of hydrophobicity, histatin 5 would appear as a "globular " protein region, while the other AMPs were ranked as "surface-seeking"[250] . The hypothetical difference between them is that part of histatin 5 has only little affinity to biological membranes while another portion contains sequences with high affinity to biological membrane [131, 139].

1.3.1.5 Charge of histatin 5.

The net charge of the cationic peptides plays an important role in target recognition as it initiates the interaction with the negatively charged plasma membrane and the AMPs. Therefore, increasing the peptide cationic charge might lead to increased activity. Unlike other AMPs, histatin 5 didn't follow this rule, as its fungicidal activity remained the same at different pH while the net charge shifted from +12.9 at pH 4 to + 6.6 at pH 9 [251].

1.3.1.6 The amino acids composition of histatin 5.

In some AMPs the full length peptide is not essential to achieve optimal antimicrobial activity and the activity can be restricted to a smaller fragment of the peptide [252-254]. Multiple studies have analyzed the amino acid composition of histatin 5 in attempts to identify the functional sequence relevant for fungicidal activity. To-date, multiple histatin 5 fragments have shown a comparable activity to the full peptide and one fragment has an activity exceeding histatin 5. The fungicidal activity of C-16 peptide, which represents the C-terminal 16 amino acid of histatin 5, and Dh-5 peptide, which represents the C-terminal 14 amino acid of histatin 5, was close in activity to the full length 24 amino acid histatin 5. Since that discovery, Dh-5 has been

referred to as the histatin 5 functional domain [139, 255, 256]. A study has claimed the identification of a six amino acid motif (KRKFHE) in all active histatins. In addition, the same study also identified H10-1 peptide, a 10 amino acid fragment with significant antifungal activity. However, the activity of H10-1 was only 30% of the full length histatin 5. Later, P-113, a twelve amino acid fragment, was identified with fungicidal activities higher than parent histatin 5 peptide. In a comparative analysis between histatin 5 and P-113, the fungicidal activity was improved in P-113 with the LD_{50} reduced nearly two-fold from 7.3 µg/ml to 3.9 µg/ml under the assay conditions used.

1.3.1.7 Structure-function relationship for histatin 5.

In spite of the fact that histidine is the predominant amino acid in histatin 5, replacing all of the histidines in the histatin 5 derivative termed P-113 with phenylalanine did not affect the fungicidal activity in vitro. Moreover, the histidine side chain will be uncharged at physiological pH. Nevertheless this finding did not exclude the role of histidine in vivo since the presence of the histidines could be essential for peptide stability and tissue binding [257].

Despite the above finding, the role of the positive charges that arise from lysine and arginine has not been eliminated. Any change in Lys-11 or Arg-12 or Lys-13 or Arg-22 in histatin 5 via replacing them with an uncharged amino acid leads to a reduction in the fungicidal activity or complete inhibition of activity[228]. Another study also confirmed this result by showing the replacement of Lys-11 and Arg-22 with glutamic acid and glycine, respectively, inhibits histatin 5 activity and eliminates peptide localization to the cytoplasm [238]. This phenomenon has been observed not only with the full length histatin 5, but also with the active derivatives (P-113 and Dh5). A single replacement of Lys-13 with threonine or glutamic acid in Dh5 was sufficient to

diminish the fungicidal activity [241]. Furthermore, replacement of Lys-2 and Lys-10 in another histatin 5 derivative (P-113) peptide with glutamine abolished activity. Additionally, replacing any of the arginines in P-113 with glutamic acid leads to reduced activity [257]. A single lysine substitution for a histidine in Dh5 peptide increases the candidacidal activity of the peptide by almost two-fold [249]. Taken together, these studies suggest that the lysine and arginine residues of histatin 5 are of greater relevance for fungicidal activity than the presence of the abundant amount of histidine residues.

1.3.1.8 Structural modifications of histatin 5.

Histatin 5, along with its derivatives, has undergone several structural modifications in an attempt to improve the antimicrobial activity and to increase the stability of the peptide. The modifications include: cyclization, acetylation of N-terminus and lysine residues, amidation of the C-terminus, the addition of a hydrophobic lipid tail, and the use of D-conformation amino acids as well as other amino acid derivatives.

Peptide cyclization has been used to improve the activity and serum stability of antimicrobial peptides[124] . For example, the cyclic form of histatin1 has a superior activity to the linear peptide [258]. The cyclization did not only increase the biological activity, the receptor affinity also improved through the stabilization of the peptide conformation[253]. This has not been the case with histatin 5 as head-to-tail cyclization did not affect its antimicrobial activity [259]. Nonetheless, DB2-121, a cyclic analog of histatin 5 has shown potent activity and less toxicity than the linear version [260].

One of the major problems with AMPs in general and histatin 5 specifically is the susceptibility to protease degradation. Modification of N-terminus and lysines via acetylation or methylation

is a method that has been used to improve the peptides stability [261]. The acetylation of histatin 5 N-terminus and methylation of lysine did not show any effect on histatin 5 activity, while the acetylation of the lysines abolished the fungicidal activity[262]. In general, it has been observed that amidated peptides exhibit higher antimicrobial activity over a peptide with a free C-terminus [263]. This improvement could be explained by the fact that the amidated peptides have a higher positive charge than those with a free C-terminus [263]. Furthermore, the amidation may enhance the antimicrobial activity by stabilizing the α-helical structure at the membrane interface and/or by improving the peptide stability[159, 264]. The amidation of histatin 5 analogs , Dh5 and P-113, increased the candidacidal activity almost twofold [249].

The conjugation of a fatty acid to antimicrobial peptides has been used as a method to enhance the activity and selectivity of the peptide[265]. This effect result from increasing the hydrophobic interaction between the plasma membrane and the peptide[266]. Research has shown that the addition of an eight carbon hydrophobic tail to histatin 5 derivative H10-2 increased the antifungal activity approximately by 50% in comparison with the native peptide [267].

Using peptide enantiomers has always been an ideal method to bypass peptide susceptibility to proteases and a way to improve peptide half-life. D-amino acids have been used in histatin 5 and its derivative P-113 and the results were the same for both of them; the enantiomers had improved stability in proteases and exhibited the same fungicidal activities [206, 257] .

As mentioned previously, all cationic antimicrobial peptides exhibit a similar problem, sensitivity to salts[270]. This problem leads to inactivation of the antimicrobial activity of peptides, even at physiological salt concentrations [271]. Histatin 5 and its fragments are also salt sensitive and their activity was totally abolished in a presence of 150 mM NaCl [257]. However, a strategy

to increase salt resistance of the histatin 5 derivative peptide P-113 has been published. The strategy is based on using uncommon amino acids via replacement of tryptophan or histidine residues with the bulky amino acids β-naphthylalanine and β-(4, 4′-biphenyl)-alanine [272, 273], respectively.

1.3.1.9 Histatin 5 mechanism of action.

The exact mode of action of histatin 5 remains unclear. The proposed antifungal activity of histatin 5 occurs through a multi-step mechanism that involves binding, translocation and toxicity to fungal cells. Unlike other AMPs, the binding of histatin 5 to the cell wall was proposed to be the first step [150, 238, 274, 275] with laminarin (β-1,3-glucan) reported to modulate histatin 5 binding to *Candida albicans* [275]. Besides carbohydrate, histatin 5 has been shown to bind to the cell wall protein (Ssa2), which may facilitate the intracellular translocation[150]. The Ssa proteins are conserved members of the heat shock protein 70 family in yeast. *Candida albicans* only has two members: Ssa1p and Ssa2p. It has been found that Ssa2, but not Ssa1, was required for histatin 5 translocation into the cell[276]. The role of Ssa2 in the fungicidal activity of histatin 5 has been the subject of debate since an *ssa2Δ* homozygous mutant was susceptible to histatin 5, demonstrating an activity that was only 25 % less than with an *SSA2* wild type strain. Moreover, the role of a specific protein receptor would seem unlikely given that a histatin 5 derivative containing only D-amino acids were as active as the L-conformation.

In addition other cell surface proteins, including polyamine transporters, have been suggested to have a role in histatin 5 translocation into cells. Six different polyamine transporters have been identified in *Candida albicans* and named the *Dur* gene family. However, the deletion of the *Dur* genes reduced the fungicidal activity of histatin 5, but did not completely abolish it. Nevertheless, the involvement of an energy-dependent process in histatin 5 killing mechanism

seems to be unanimously agreed upon, but at what stage in the process this occurs remains unresolved.

Several models have been proposed to explain histatin 5 activity and intracellular targets. The first model hypothesized that histatin 5 attaches and depolarizes energized mitochondria[238]. The cause of death in this model was assigned to the generation of reactive oxygen species[277] rather than loss of respiration, because *Candida spp.* are fully functional under anaerobic conditions [275]. However, the role of ROS is disputed, as other studies showed ROS has no role in histatin 5 induced yeast death [278, 279]. In another model, histatin 5 binds to cell membranes and increases the permeability, resulting in the efflux of cellular content, such as ATP[129], ions (magnesium and potassium)[280], and nucleotides[129]. Moreover, it has been shown that loss of intracellular contents was facilitated by Trk1, a membrane potassium transporter after exposure to histatin 5[281]. In this model, the fungicidal activity is achieved via disruption of cellular ionic balance instead of membrane integrity[273]. In another proposed mechanism, histatin 5 causes a disordered volume regulation and cell cycle arrest [273], which might be correlated to the loss of ATP[129]. In summary, the mechanism of histatin 5 antifungal activity remains unresolved. Any of the proposed models or a combination of the models may prove to be true.

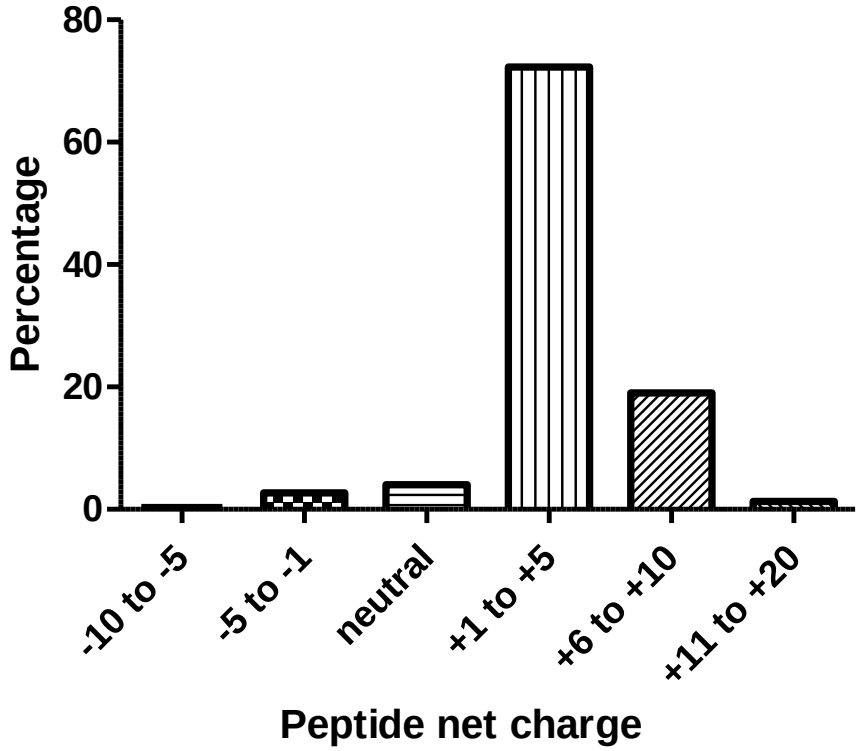

Figure 1: Distribution of the net charge within anti-fungal peptides. The data were generated using the antimicrobial peptide database [103].

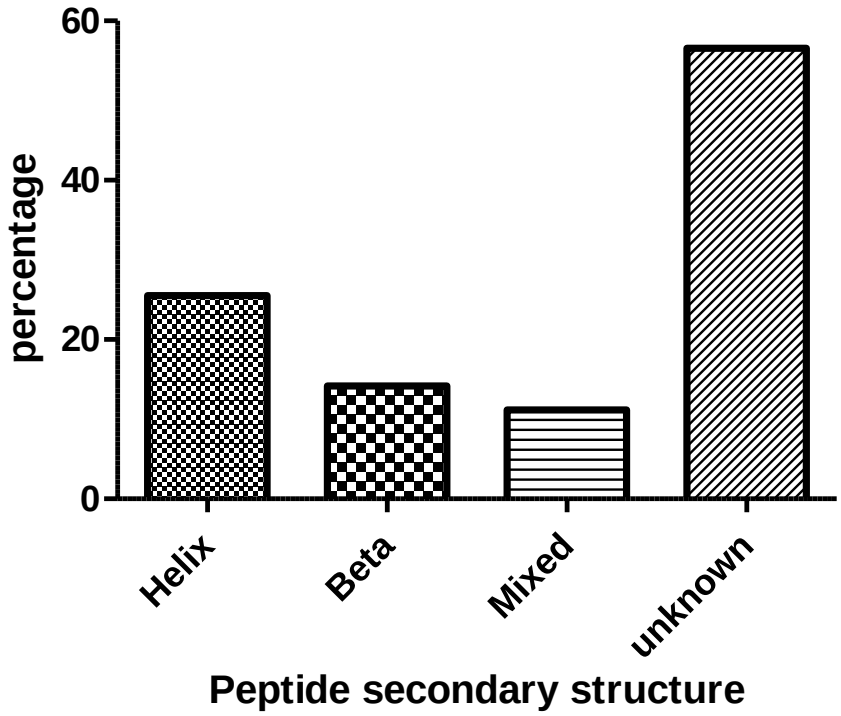

Figure 2: Distribution of the secondary structure within anti-fungal peptides. The data were generated using antimicrobial peptide database [103].

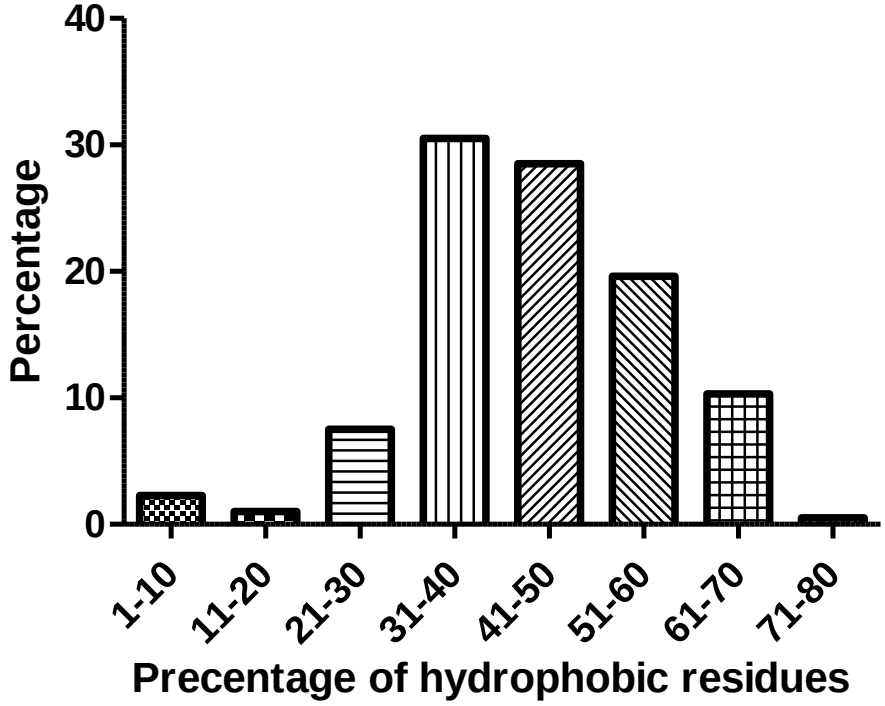

Figure 3: Percentage of the hydrophobic residues within anti-fungal peptides. The data were generated using the antimicrobial peptide database [103].

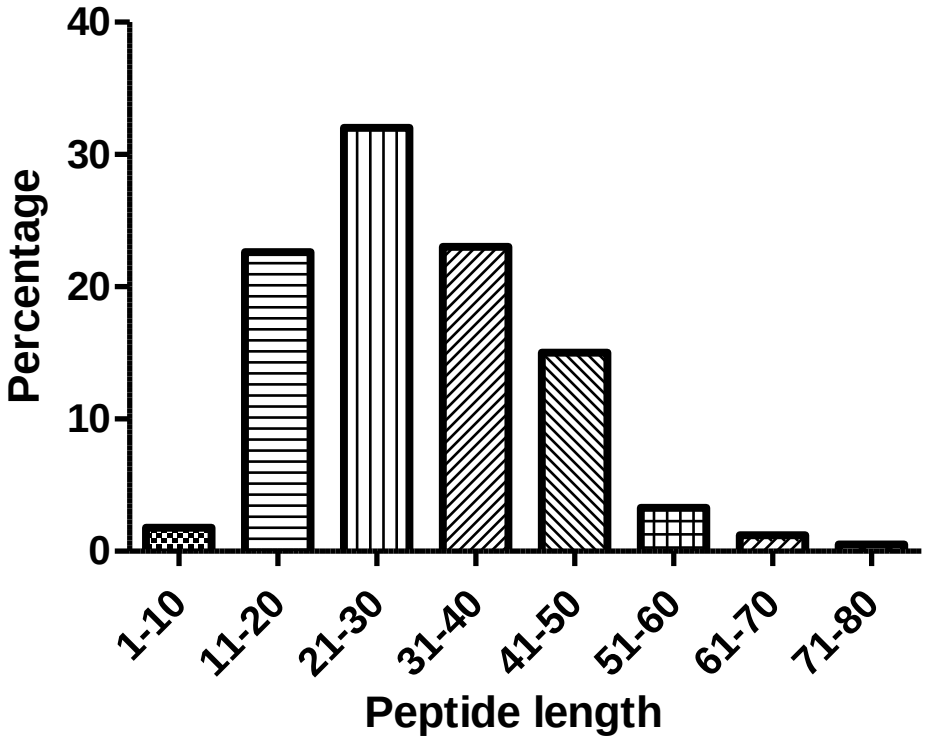

Figure 4: Percentage of peptide length within anti-fungal peptides. The data were generated using antimicrobial peptide database [103].

Table 1: Examples of cationic anti-fungal peptides sorted in ascending order by the net charge.

Name	Origin	Sequence	Activity	Secondary structure	Charge	Ref.
Heliomicin	*Heliothis virescens (worm)*	DKLIGSCVWGAVNY TSDCNGECKRRGYK GGHCGSFANVNCW CET	*C. albicans C. neoformans*	Helix and Beta-1I2U	+1	282
Metchnikowin	*Drosophila melanogaster*	HRHQGPIFDTRPSPF NPNQPRPGPIY	*F. graminearum*	unknown	+2	283
Maximin 1	*Bombina maxima (frog)*	GIGTKILGGVKTALK GALKELASTYAN	*C. albicans*	unknown	+3	284
Ranatuerin 1	*Rana catesbeiana (frog)*	SMLSVLKNLGKVGL GFVACKINKQC	*C. albicans*	unknown	+4	285
Melittin	*Apis mellifera (bee)*	GIGAVLKVLTTGLP ALISWIKRKRQQ	*C. albicans*	Helix-1MLT	+5	286
Tachystatin A2	*Achypleus tridentatus* (crab)	YSRCQLQGFNCVVR SYGLPTIPCCRGLTC RSYFPGSTYGRCQR Y	*C. albicans*	Beta-1CIX	+6	287
MBP-1	*Maize, Zea mays L (plant)*	RSGRGECRRQCLRR HEGQPWETQECMR RCRRRG	*F. graminearum F. moniliforme*	Helix-CD	+7	288
Lactoferricin B	*Bos Taurus (cow)*	FKCRRWQWRMKKL GAPSITCVRRAF	*C. albicans T. mentagrophytes T. rubrum (not all fungi)*	Beta-1LFC	+8	289
1AFP	*Aspergillus giganteus*	ATYNGKCYKKDNIC KYKAQSGKTAICKC YVKKCPRDGAKCEF DSYKGKCYC	*F. sambucinum N. crassa A. niger* (*not all fungi)*	Beta-1AFP	+9	290
BMAP-27	*Bos taurus (cow)*	GRFKRFRKKFKKLF KKLSPVIPLLHLG	*C. albicans C. neoformans*	Helix-2KET	+10	291
sBD-1	*Ovis arues (shep)*	NRLSCHRNKGVCVP SRCPRHMRQIGTCR GPPVKCCRKK	*C. albicans*	unknown	+11	292
Buforin I	*bufo gargarizans (frog)*	AGRGKQGGKVRAK AKTRSSRAGLQFPV GRVHRLLRKGNY	*C. albicans S. cerevisiae C. neoformance*	unknown	+12	293
CXCL14	*Homo sapiens*	SKCKCSRKGPKIRYS DVKKLEMKPKYPHC EEKMVIITTKSVSRY RGQEHCLHPKLQST KRFIKWYNAWNEK RRVYEE	*C. albicans*	unknown	+13	294
CodCath	*Gadus morhua (fish)*	SRSGRGSGKGGRGG SRGSSGSRGSKGPSG SRGSSGSRGSKGSRG GRSGRGSTIAGNGN RNNGGTRTA	*C. albicans*	unknown	+15	295

Table 2: Anionic anti-fungal peptides sorted in ascending order by the net charge.

Name	Origin	Sequence	Activity	Secondary structure	Charge	Ref.
Tn-AFP	*Trapa atans (Plant fruit)*	LMCTHPLDCSN	*C.tropicalis*	Unknown	-1	296
An-AFP	*Aspergillus niger (Fungi)*	SKYGGECSVEHNTCT YLKGGKDHIVSCPSAA NLRCKTERHHCEYDE HHKTVDCQTPV	*C. albicans S. cerevisiae T. beigelii F. solani F. oxysporum A. fumigatus A. flavus*	Unknown	-1	297
Kalata B1	*Viola betonicifolia (plant)*	GLPVCGETCFGGTCNT PGCTCTWPICTRD	*C. kefyr*	Helix and Beta- 1PT4	-1	298, 299
PvD1	*Phaseolus vulgaris (plant seed)*	KTCENLADTYKGPCFT TGSCD	*C. albicans, C. parapsilosis, C. tropicalis C. guilliermondii, K. marxiannus S. cerevisiae*	Expected Helix and Beta	-1	166, 300
human Dermcidin	*Homo sapiens (sweat)*	SSLLEKGLDGAKKAV GGLGKLGKDAVEDLE SVGKGAVHDVKDVLD SV	*C. albicans*	Helix-2KSG	-2	161
EP-20	*Xenorhabdus budapestensis (Bacteria)*	EGPVGLADPDGPASAP LGAP	*P. capsici V. dahliae*	Unknown	-3	162
Beta-amyloid peptide including beta -42	*Homo sapiens*	DAEFRHDSGYEVHHQ KLVFFAEDVGSNKGAI IGLMVGGVV	*C. albicans*	Helix -1IYT	-3	160
Ls-Stylicin1	*Litopenaeus stylirostris*	SSFSPPRGPPGWGPPC VQQPCPKCPYDDYKC PTCDKFPECEECPHISI GCECGYFSCECPKPVC EPCESPIAELIKKGGYK G	*F. oxysporum*	Unknown	-3	163
Gm anionic peptide-2	*Galleria mellonella (Moth)*	EADEPLWLYKGDNIER APTTADHPILPSIIDDV KLDPNRRYA	*P. pastoris P. stipites C. albicans C. fructus Z. marxianus*	Unknown	-4	302
Microplusin	*Rhipicephalus (Boophilus) Microplus (cattle tick)*	HHQELCTKGDDALVT ELECIRLRISPETNAAF DNAVQQLNCLNRACA YRKMCATNNLEQAMS VYFTNEQIKEIHDAAT ACDPEAHHEHDH	*S. cerevisiae C. neoformans*	Helix- 2KNJ	-8	164

Table 3: Example of neutral anti-fungal peptides.

Name	Origin	Sequence	Activity	Secondary structure	Ref.
Aurein 1.1	*Litoria raniformis (frog)*	GLFDIIKKIAESI GLFDIIKKIAESF	*C. albicans* *C. Tropicalis* *C. Krusi* *C. Parapsilosis* *C. glabrata*	Helix-2F3A 1VM5	303
Maculatin 1.1	*Litoria genimaculate (frog)*	GLFVGVLAKVAAH VVPAIAEHF	*C. albicans*	Helix	304
Skin peptide tyrosine-tyrosine	*Phyllomedusa bicolor (frog)*	YPPKPESPGEDASPE EMNKYLTALRHYIN LVTRQRY	*C. albicans* *A. fumigatus* *C. neoformans*	unknown	305
-Galleria defensin -Galleria defensin like	*Galleria mellonella (moth)*	DTLIGSCVWGATNY TSDCNAECKRRGYK GGHCGSFLNVNCW CE DKLIGSCVWGATNY TSDCNAECKRRGYK GGHCGSFWNVNCW CEE	*C. albicans* *G. candidum* *C. neoformans* *F. oxysporum*	unknown	306 302
Gm cecropin D-like peptide	*Galleria mellonella (moth)*	ENFFKEIERAGQRIR DAIISAAPAVETLAQ AQKIIKGGD	*A. niger*	unknown	302
Histatin 2	*Homo sapiens*	RKFHEKHHSHREFPF YGDYGSNYLYDN	*C. albicans*	unknown	307
Temporin-1PRb	*Rana pirica (frog)*	ILPILGNLLNSLL	*C. albicans* >100 µM	unknown	308
Neuropeptide Y	*Homo sapiens*	YPSKPDNPGEDAPA EDMARYYSALRHYI NLITRQRY	*C. neoformans* *C. albicans* *C. krusei* *C. utilis*	Helix-1RON	169 309
GHH20 Histidine rich glycoprotein	*Homo sapiens*	GHHPHGHHPHGHHP HGHHHPH	*C. parapsilosis* *C. albicans*	Helix	310
Ha-DEF1	*Helianthus annuus (sunflower)*	ELCEKASQTWSGTC GKTKHCDDQCKSW EGAAHGACHVRDG KHMCFCYFNC	*S. cerevisiae*	unknown	311
Drosomycin-2	*Drosophila melanogaster*	DCLSGKYKGPCAV WDNEMCRRICKEEG HISGHCSPSLKCWCE GC	*N. crassa* *G. candidum* *S. cerevisiae*	Helix and Beta-1MYN	312
Sm-AMP-D1	*Stellaria media L (plant)*	KICERASGTWKGICI HSNDCNNQCVKWE NAGSGSCHYQFPNY MCFCYFDC	*Phytopathogenic fungi*	unknown	313
Brevinin-1-OR3	*Odorrana rotodora (frog)*	IDPFVAGVAAEMMQ HVYCAASKKC	*C. albicans*	unknown	168

OR6 OR8 OR9		IIPFVAGVAAEMME HVYCAASKKC ILPFVAGVAAEMME HVYCAASKKC ILPFVAGVAAMEME HVYCAASKKC			
Andersonin-X1	*Odorrana andersonii (frog)*	GLFSKFAGKGIVNFL IEGVE	*C. albicans*	unknown	168
GP-19	*Xenorhabdus budapestensis NMC-10 (bacteria)*	GPVGLLSSPGSLPPV GGAP	*F. omysporum* *P. capsici* *V. dahlia* *F.graminearum*	unknown	162

Table 4: Salt-resistant anti-fungal peptides.

Name	Sequence	Source	Secondary structure	Disulfide bond	Activity (MIC)	Net charge	Ref.
Thanatin Thanatin-1 S-Thanatin	GSKKPVPIIY CNRRTGKC QRM GSKKPVPIIY CNRRGKCQ RM GSKKPVPIIY CNRRSGKCQ RM	*Podisus maculiventris* (insect) *recombinant*	beta	1(C11-C18)	-*N. crassa* 0.6-1.2 µM -*B. cinerea* 1.2-2.5 µM -*N. haematococca* 1.2-2.5 µM -*T. viride* 1.2-2.5 µM -*A. brassicola* 2.5-5 µM -*F. culmorumn* 2.5-5 µM -*A. pisi* 5-10 µM -*F. oxysporumn* 10-20 µM -*C.albicans* 25-50 µM	+6	314
RTD-1 (theta-defensin) RTD-2 RTD-3	GFCRCLCRR GVCRCICTR GVCRCLCRR GVCRCLCRR GFCRCICRR GFCRCICTR	*Rhesus Macaque* (monkey)	beta	3 (C3-C16) (C5-C14) (C7-C12)	-*C.albicans* 1µg/ml -*C. neoformans* 4µg/ml	+5 +6 +4	216 201
Tachyplesin I Tachyplesin II	KWCFRVCY RGICYRRCR RWCFRVCY RGICYRKCR	*Tachypleus tridentatus (crab)*	beta	2(C3-C16) (C7-C12)	-*C. albicans 3.1* µg/ml -*C. neoformans* 1.56 µg/ml -*C. kefyr* 0.9 µM *C. tropicalis*	+6	137 217

					0.5 uM		
Arenicin-1	RWCVYAYV RVRGVLVR YRRCW	*Arenicola marina* *Sand worm*	beta	1(C3-(20)	-*C. albicans* 4.5 μg/ml	+6	218
Protegrin 1	RGGRLCYCR RRFCVCVGR	*Pig*	beta	2 (C6-C15) (C8-C13)	-*C. neoformans* 2 μM -*C. albicans* 4 μM,	+6	315
Ci-MAM-A24	WRSLGRTLL RLSHALKPL ARRSGW	*Ciona intestinalis*	helix	no	-*C. albicans* 6 μM -*C. albicans (SC 5314)* 3.1 μM	+6	219
N-[RLLR]2-*C*	RLLRRLLR	*synthetic*	helix	no	-*C. albicans* 0.5 μg/ml -*S. cerevisia* 0.5 μg/ml -*C. neoformans* 0.5 μg/ml	+4	270
P-18	KWKLFKKIP KFLHLAKKF	*synthetic*	helix	no	*C. albicans* 2-4 μM	+7	297
human Dermcidin (DCD-1) rDCD-1L	SSLLEKGLD GAKKAVGG LGKLGKDA VEDLESVGK GAVHDVKD VLDSV SSLLEKGLD GAKKAVGG LGKLGKDA VEDLESVGK GAVHDVKD VLDSVL	*Homo sapiens* *recombinant*	helix	no	-*C. albicans* 10 μg /ml -*C. albicans* 12 μg /ml	-2	220 316
Melittin	GIGAVLKVL TTGLPALIS WIKRKRQQ	*Apis mellifera* (insect)	helix	no	NA	+5	286
Pelteobagrin	GKLNLFLSR LEILKLFVG AL	*Yellow catfish*	unknown	no	-*C. albicans* 5.4 μM	+2	317

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Chapter 2

Analysis of wild-type, enantio, retro and retroenantio derivatives of the Histatin 5 16mer

2.1 Introduction

Candida species are the fourth most common cause of nosocomial blood infections in the United States, resulting in a mortality rate of up to 40 % [1]. The estimated health care costs for the treatment of *Candida* infections is approximately 1.8 billion dollars annually [2]. Given the morbidity and mortality associated with *Candida* infections, and the increased incidence of drug-resistant *Candida* species, there is a strong emphasis on the development of new anti-fungal drugs with novel mechanisms-of-action [3]. To this end, antimicrobial peptides (AMPs) are considered a promising alternative to the traditional chemical antifungal drugs that are currently available. Antimicrobial peptides (AMPs) are known to play an important role in the human innate immune response against pathogenic and opportunistic microorganisms [2]. In addition, some of these naturally occurring peptides have displayed anti-fungal activities with distinct modes of action [4, 5]. AMPs also offer the advantage of higher selectivity against target organisms with reduced host toxicity as compared to the currently available anti-fungal drugs. Thus, a logical starting point in the development of novel anti-fungal peptides would be to identify a naturally occurring peptide with fungicidal activity and seek to enhance that activity.

Histatins are a family of naturally occurring peptides found on the mucosal surfaces of the oral cavity, a common location of *Candida* infections [7]. In fact, the oral cavity is one of the predominant sites of *Candida* infection in the human body, with *Candida albicans* being the most commonly isolated [8]. In oral cavity, there are actually five distinct AMPs; however, only three exhibit significant anti-fungal activity: HNP1-4 , histatins, and β-Defensins [9]. The histatins are a family of histidine-rich cationic peptides produced by the human parotid, submandibular, and sublingual salivary glands [10, 11] . Histatin 5 is one member of the histatin peptide family that has been shown to possess the most potent fungicidal activity and it has been extensively

characterized[4, 13]. Moreover, histatin 5 is among the smallest of the AMPs, making it economically suitable for development as an antifungal compound.

Histatin 5 is a twenty-four amino acid peptide whose mechanism of action has been examined in detail; however, the mechanism by which it kills fungi remains controversial. Two distinct mechanisms-of-action have been proposed, a membrane lysis mechanism and a nonlytic mechanism. In the membrane lysis mechanism, histatin 5 binds directly to the lipids of the *Candida* plasma membrane, increases membrane permeability, and consequently causing cell death due to loss of membrane integrity [14, 15]. In contrast, the non-lytic mechanism suggests that histatin 5 targets the metabolic activity of *C. albicans,* where the killing would result from the inhibition of cellular respiration via depolarization of the energized mitochondrion and the inhibition of ATP production[16,17].

To target the mitochondria for inhibition, histatin 5 must be transported across the plasma membrane. The translocation of histatin 5 across the plasma membrane has been proposed to occur by multiple mechanisms. Artificial liposome experiments have suggested that the translocation of histatin 5 is independent of any active endocytic pathways [14]; whereas, several in vivo studies have suggested that a plasma membrane protein Ssa2 is essential for the histatin 5 translocation process[18, 19]. However, the involvement of a specific protein receptor in histatin 5 translocation and subsequent antifungal activity seems unlikely since it was found that a histatin 5 derivative containing only D-amino acids was as active at the native peptide containing L-amino acids in fungicidal activity[20, 21].

Several studies have shown that the full-length twenty-four amino acid peptide is not required for histatin 5 fungicidal activities since a truncated peptide, termed C-16 (a 16 amino acid fragment of histatin 5), retains the full fungicidal activity [20]. Since histidine is a major substituent in histatin, the net charge for histatin 5 should be affected by pH. Moreover, changing the pH from acidic to basic is sufficient to shift the net charge of the peptide from +14 to +5 based on computational analyses. Surprisingly, histatin 5 and C-16 retain the same activity against *Candida albicans* over a broad range of pH (from pH 5 to 9); indicating the optimal number of positive charges and their role in fungicidal activity of histatin 5 remains unknown.

For the studies described in this chapter, histatin 5 has been selected as a model peptide. Moreover, the 16 amino acid derivative of histatin 5 (C-16) was used since it is smaller, yet retains antifungal activity indistinguishable from the 24 amino acid peptide found naturally [20]. The work presented in this chapter focuses on understanding the structural requirements for the fungicidal activity of histatin 5 using C-16 as a model. A simple approach was used to initially examine the structural requirements of the peptide, namely to evaluate whether the linear order of amino acids from N- to C-terminus or the enantiomeric form of the peptide affects the anti-fungal activity. To address these questions, four C-16 histatin 5 derivatives were synthesized: the wild-type C-16 peptide; C-16 peptide in which the amino acid sequence is reversed (retro-C-16); the wild-type C-16 with D-amino acids (enantio); and the retro-C-16 peptide with D-amino acids (retroenantio). The availability of these four histatin 5 derivatives allowed us to address the relevance of the specific amino acid sequence as well as the stereochemistry of the peptide as it relates to anti-fungal activity.

2.2 Materials and Methods

Peptide synthesis. N-Fmoc protected amino acids and Wang resin was purchased from NovaBiochem (San Diego, CA) and Advanced Chemtech (Louisville, KY), respectively. All peptides were synthesized on a Model 433A solid-phase peptide synthesizer (Applied Biosystems; Foster City, CA) using the Wang resin and Fmoc-protected amino acids. To improve the synthesis yields, modified FastMoc chemistry was used with extended deprotection and coupling times. After synthesis, the peptides were deprotected and cleaved from the Wang resin using a high concentration TFA (trifluroacetic acid) cleavage cocktail consisting of 85% TFA, 5% dH_2O, 5% triisopropylsilan, and 5% phenol. The resin was allowed to mix in the cocktail solution at room temperature for 3 h then precipitated in 50 ml 1:1 v/v methyl-t-butyl ether/hexane per ml of cleavage cocktail. The peptides were subsequently dissolved in 1:1 v/v acetonitrile/ddH_2O and recovered by lypholization under high vacuum. Crude peptides were purified on a PRP-3 reverse phase column (7 by 305 mm; Bio-Rad, Hercules, USA) on a Hitachi L7100 HPLC instrument using a linear gradient of 0 – 30% acetonitrile and water. Peptides were lyophilized multiple times from acetonitrile:water (1:1) to ensure complete removal of TFA. The peptide was then further purified by HPLC and peptide purity was verified by mass spectroscopy. Concentrations of the four peptides were determined by dried weight.

Fungicidal activity assays. The fungicidal activities of the peptides were determined by the microdilution plate assay using *Candida albicans* SC5314 as described previously [13]. *C. albicans* SC5314 was grown overnight on Sabouraud Dextrose agar plates at 30°C. Following overnight growth, a single *C. albicans* colony was diluted in 1 ml of 10 mM sodium phosphate buffer at pH 7.4. A hemocytometer was used to quantify the number of cells/ml and the cell concentration was adjusted to 1.8×10^5 cells/ml. Cell suspensions (20 µl) were mixed with 20 µl

of peptide dissolved in 10 mM sodium phosphate buffer at pH 7.4 and incubated for 2 h at 37°C with shaking at speeds of 550 rpm. The reactions were stopped by the addition of 360 µl yeast nitrogen base (3.4 g yeast nitrogen base without ammonium sulfate or amino acids and 10 g ammonium sulfate per liter) and 40 µl of cell suspension was spread on Sabouraud dextrose agar plates and incubated for 24 h at 37°C. The number of colony-forming units (CFUs) was qualified and each assay was repeated in triplicate. Loss of viability was calculated as [1-(CFUs in the presence of the peptide/CFUs with no peptide)] × 100.

Minimum Inhibitory Concentration (MIC) assay. MIC assays were carried following the CLSI M27-A2 standard with the modification as described below. Briefly, two-fold serial dilutions of the peptides were prepared with RPMI-1640 (Sigma R-7755) in 100 µl per well of a 96-well flat-bottom microtiter plates (Costar, Cambridge, MA). To demonstrate the effect of salt on peptide activity, different RPMI-1640 dilutions (1X, 0.5X, 0.25X, and 0.125X) were used. To each well of the microtiter plates containing the peptide, a 100 µl *C. albicans* cell suspension containing 1×10^4 cells /mL in a resazurin/water solution (0.01% w/v) was added. The final concentration of the peptides in the assay ranged from 0.2 to 100 µM (0.4 to 206.7µg/ml, respectively). The microtiter plates were subsequently incubated at 35°C and examined at both 24 h and 48 h after exposure to peptide. Each assay plate contained a positive control of *Candida albicans* without added peptide and the negative control of RPMI-1640 medium containing only the resazurin/water solution. For each peptide three independent assays were performed. The MIC assay results were interpreted visually using the criteria of the lowest peptide concentration that remained blue (indicating no growth) or the first dilution that changed from blue to slightly purple (equivalent to prominent growth inhibition). The peptide concentrations were determined based on the extinction coefficient of the four peptides.

Effect of pH, sodium chloride, EGTA, sodium azide and temperature on peptide killing activity. The effect of pH on the killing activity of the four peptides was tested by performing the fungicidal activity assays as described above using 10 mM sodium phosphate buffer at pH values ranging from 5 to 9. The pH of the buffer was adjusted using 2 M HCl or NaOH. The peptide net charge was calculated at the same pH range using Protein Calculator v3.3 server (http://www.scripps.edu/~cdputnam/protcalc.html) [24]. To study the effect of temperature on the killing activity, the fungicidal activity assay was performed in 10 mM phosphate buffer pH 7 at two different temperatures, 4°C and 37°C. The effect of sodium chloride (5 mM, 10mM and 25 mM), EGTA (10 µM, 50 µM, 100 µM, 250 µM, and 1000 µM) and sodium azide (5 mM) was evaluated using the fungicidal assays in 10 mM phosphate buffer pH= 7 at 37°C.

Circular dichroism spectroscopy. Circular dichroism spectroscopy measurements for the four peptides were performed using a Jasco-710 spectropolarimeter as described previously with some modifications [25]. The reading was made using a quartz cell with a 0.1cm path length at 25° C. The measurements were performed on the peptides at a final concentration of 240 µM (100 µg) in the presence of 50% trifluoroethanol (v/v) (TFE). The spectra were recorded every 0.2 nm between the absorbance range of 190 to 250 nm with a 1.0 nm bandwidth and a scan speed of 20 nm/min. Six scans were performed and averaged. The background was subtracted from all spectra, and curve smoothing applied. The CD spectra are reported as the mean residue ellipticity ($[\Theta]$) in degrees. cm^2. $dmol^{-1}$. The CD data were further analyzed using the web-based K2D2 program (http://www.ogic.ca/projects/k2d2/). The helical wheel projections were made with a tool created by Don Armstrong and Raphael Zidovetzki [26, 27].

Peptide Stability in Artificial Gastric Juice. The stability of each peptide was tested using artificial gastric juice prepared as described previously with slight modifications [28]. The artificial

gastric juice was prepared by dissolving 2 g NaCl and 3.2 g pepsin in 7.0 ml 0.085 M HCl and the volume was increased to 1000 ml with distilled water (pH 1.2). The peptide (5 µg in volume of 10 µl) was added to 90 µL of artificial gastric juice and incubated at 37^{o}C for 60 min. The reaction was terminated by boiling for 10 min and the sample was centrifuged at 13,000 rpm for 10 min. in a microcentrifuge. The supernatant was collected, neutralized with NaOH, and analyzed by reverse phase HPLC. The HPLC run time was 15 min with an injection volume of 50 µl. Chromatograms were recorded by UV detection at 220 nm. The individual peptides in 10 mM phosphate buffer (pH=7.4), were used as positive controls. Concentrations of the peptides were determined based on the extinction coefficient.

Peptide Stability in Human Saliva. Whole saliva samples (5 ml) were collected from three healthy donors ranging in age from 25 to 35 years in accordance with a protocol approved by the University of Arkansas Institutional Review Board. The stability of the peptides in human saliva was determined as described previously with slight modifications [28]. After the saliva was collected, it was immediately centrifuged at 13,000 rpm for 10 min at 4^{o}C in a microcentrifuge and the supernatant was aliquoted 1 ml portions and stored at -80^{o} C. One milliter of saliva from each volunteer was pooled, mixed and filtered through a 0.45 µm membrane filter. The stability assays was performed using a constant ratio of peptide to saliva (1:9). Twenty µl of peptide in 10 mM sodium phosphate buffer (pH=7.4) was mixed with 180 µl of saliva to give a final peptide concentration of 0.5 mg/ml. The mixture was incubated for 60 min at 37^{o}C, and the reaction terminated boiling the samples for 10 min. The samples were subsequently filtered and analyzed by reversed phase HPLC. The HPLC total run time was 15 min and the injection volume was 100 µl. Chromatograms were recorded by UV detection at 220 nm. The peptide dissolved in

artificial saliva was used as a control. The peptide concentration was determined based on the extinction coefficient of the peptides.

2.3 Results

Synthesis of the histatin 5 peptide derivatives. To understand the structural features and stereochemistry of histatin 5 that is important for the antifungal activity we synthesized the wild-type histatin 5 C-16 peptide (termed W) [20] along with three derivatives shown in Table 1. The first derivative was identical to the C-16 peptide except it contained only D-amino acids (termed WD). The second derivative is a "retro" isomer in which the order of amino acids is reversed from N- to C-terminus (termed R). The third derivative was identical to the retro isomer except D-amino acids were used for the synthesis. The W and R peptides had the identical amino acid composition with the difference being the N- to C- terminal linear order. The N- to C- terminal sequence of R is represented by 1, 2, 3...*n* amino acid, while R peptide is represented by the sequence n, n-1,..3, 2, 1. The WD and RD peptides are stereoisomers of W and R, respectively. All peptides were synthesized and the molecular mass of each peptide was evaluated by mass spectrometry and was shown to match the predicted mass based on the amino acid composition (Figure 1). The quality of each peptide was verified by HPLC to be >97% pure (Figure 2).

Fungicidal activity of the histatin 5 derivatives. To compare the relative antifungal activity of the four peptides, two different assays were performed with *C. albicans* SC5314. First, the dose-dependent killing activity of the four peptides was examined. In this assay, different concentrations (0.1, 1, 5, 10, 25, 50 and 100 μM) of the four peptides were evaluated and the dose-dependent killing activity after 2 h incubation was found to be indistinguishable (Figure 3). . Furthermore, all four peptide exhibited a similar fungicidal activity with an LD_{50} of less than 3μM (Table 2 and Figure 4).

Since the dose-dependent killing assay is not time dependent, it is possible that all of the peptides demonstrated similar killing activity, yet the rate at which they killed the fungal cells varied over

the 2 h incubation time. To address this possibility, a time-dependent fungicidal activity assay was performed in which *C. albicans* was incubated with 10 μM of each peptide and the fungicidal activity was determined at time intervals of 15, 30, 60 and 120 min (Figure 5). The kinetics of the anti-fungal activity of all four peptides was found to be similar at all data points ($p < 0.05$). Since the standard fungicidal assay is incubated for 2 h, it was relevant to determine whether the four peptides continued to demonstrate activity beyond the 2 h incubation; alternatively, the activity of the peptides may plateau at some point prior to 2 h. To address this question, another fungicidal activity assay was performed over a period to 8 h (Figure 6). These data demonstrate that the activity of all four peptides plateau at approximately 2 h with 20% of the cells remaining viable when exposed to 10 μM of each peptide.

On the basis of the fungicidal activity data, the R, RD, W, and WD peptides demonstrated similar killing activity, suggesting the linear order of amino acids from the N- to the C-terminus is not relevant for the fungicidal activity. Moreover, these data also demonstrate that the enantiomeric form of the peptide is also not significant for fungal killing activity. These observations strongly argue against a cell surface protein receptor playing a role in the killing activity as will be discussed later.

The effect histatin 5 inhibitors on fungicidal activity. To further explore the similarities or differences between the four histatin 5 peptide derivatives, a series of experiments were performed using conditions known to inhibit the fungicidal activity of histatin 5, namely reduced temperature [29], increased concentrations of sodium chloride and the respiratory inhibitor sodium azide [30]. The goal was to determine whether the four peptides display any differences in killing activity as compared to the native histatin 5.

Previous studies have demonstrated that the killing of *C. albicans* cells by histatin 5 is halted at 4^{o}C by presumably preventing translocation of the peptide into the cytoplasm[29, 31] or by altering the fluidity of the plasma membrane. To evaluate the effect of temperature on the activity of the W, R, WD and RD peptides, each peptide was incubated with *Candida albicans* at two different temperatures, 4^{o}C and 37^{o}C. The activity of all four peptides was found to be dramatically inhibited at 4^{o}C as compared to 37^{o}C (Figure 7).

As common with many peptide-based antimicrobials, it has been shown previously that increasing concentrations of sodium chloride (NaCl) can inhibit the killing activity of histatin 5. To examine the inhibitory effect of NaCl on the W, R, WD, and RD peptides, two different assays were performed. First, the peptides were tested at different concentrations of NaCl up to 25 mM, which mimics the physiological concentration of NaCl in human saliva [32]. Second, a minimum inhibitory concentration assay (MIC) was performed for each peptide to evaluate the fungicidal activity at physiological concentration of various salts [33]. As shown in Figure 8, NaCl has the same inhibitory effect on fungicidal activity for the four peptides using 5 µM of each peptide. The decrease in the activity was clearly observed at 10 mM NaCl or higher. For the MIC assay, the standard RPMI-1640 medium used at 1X, 0.5X, 0.25X and 0.125X normal concentrations. The logic was to set up a visual assay typically found in a clinical setting that would reflect the effect of physiologic salts on the activity of candidate fungicidal peptides. As expected, the activity of the four 16mer peptides was completely inhibited in 1X, 0.5X, and 0.25 X RPMI-1640 medium. The only observed activity was at the lowest RPMI-1640 concentration (0.125X) (Table 3). Interestingly, the D-conformation peptides (WD and RD) displayed slightly better activity in 0.125X RPMI. The MIC value for WD and RD peptides was 103-207 µg/ml, while the W and R peptide activity was outside the range of the assay (>207 µg/ml).

Sodium azide, an inhibitor of cytochrome oxidase activity, has been shown previously to inhibit histatin 5 fungicidal activity [30] which is likely reflective of its killing mechanism. Hence, the sensitivity of the four histatin 5 peptide derivatives W, R, WD, and RD to the treatment of cells with sodium azide would provide preliminary evidence suggesting that all four peptides are killing by similar mechanisms. Thus, the fungicidal activity of the four peptides was evaluated in the presence of 5 mM sodium azide. To compensate for ionic strength effects caused by the presence of sodium and azide ions, the control reactions without sodium azide were performed in buffer supplemented with 5 mM of sodium chloride. These data demonstrated that the inhibitory activity of sodium azide was similar for all four peptides, providing support for the model that all four peptides function via similar killing mechanisms (Figure 9).

pH sensitivity of the histatin 5 derivatives. As mentioned in the Introduction, histatin 5 is a cationic peptide containing a large number of histidine residues (29% histidine). If the histidines are structurally important for the killing activity, and the fact that pKa of imidazole sidechain is approximately 6.0, it would not be surprising that the fungicidal activity of histatin 5 is pH-sensitive. Consistent with this hypothesis, the predicted peptide net charge was calculated across a range of pH values (Figure 10A). It is predicted that the net charge changes significantly as the pH shifts from acidic to basic, from +8 net charge at pH 5 to +3 net charge at pH 9. To evaluate the relevance of pH in the activity of the histatin 5 derivatives, the fungicidal activity of the peptides were determined at various pH values ranging from 5 to 9 (Figure 10B). It was found that no significant difference in the fungicidal activity occurred across the range of pH values, suggesting the histidine residues within histatin 5 may not be a primary factor in the killing activity of histatin 5.

The role of zinc in the fungicidal activity of the histatin 5 derivatives. It has been previously demonstrated that Histatin 5 contains a zinc-binding domain of a general sequence HEXXH [34, 35] that is part of the C-16 peptide used in the studies reported in this chapter. However, it is unclear whether the retro peptides retain the ability to bind zinc. Moreover, the overall contribution of zinc-binding to the fungicidal activity of histatin 5 remains controversial. To evaluate whether zinc-binding contributes to the killing activity of the W, WD, R, and RD peptides, EGTA was added as a metal chelator to remove any metal ions, including Zn^{+2} in the fungicidal activity assay. EGTA was chosen in preference to EDTA because EGTA shows no fungicidal activity alone in concentrations up to 10 mM. As shown in Figure 8, the addition of EGTA to the fungicidal activity assay had only a slight inhibitory effect on the killing activity of the four peptides, suggesting that zinc is not a major contributing cofactor to the killing mechanism of the histatin 5 peptide derivatives.

Structural analysis of the histatin 5 derivatives. To evaluate whether there were any major differences in secondary structure of the four histatin peptide derivatives, circular dichroism (CD) spectroscopy was performed (Figure 12). The data were acquired for each peptide in the presence of 50% trifluoroethanol. As illustrated in Figure 12, all four peptides folded into alpha helical structures and the deconvolution of the spectrum using the K2D2 software program [38] yielded a similar percentage of helical structure in all peptides (41.1 % in W and WD, while the value was 40.8% in R and RD). As expected, the CD spectra of WD and RD were mirror images of W and R, respectively, with the same ellipticity value but with the opposite sign. The distribution of charges on a hypothesized alpha helical structure was evaluated using helical wheel projections (Figure 13) [39]. The charge distribution remained the same in the R peptide

versus the W peptide keeping the basic amino acids biased to one side of the helix, and uncharged and nonpolar on the other side.

The stability of the histatin 5 derivatives in human saliva. Histatin 5 is secreted from the salivary glands into the oral cavity where it is active in controlling microbial cell growth. Thus, the stability in human saliva of the wild-type C-16 versus the retro-C16 peptide was compared to determine whether they displayed any differences. The stability assay was performed by incubating the W and R peptides with human saliva for 60 min and concentration of the peptide after the incubation period was monitored using HPLC (Figure 14) as described in the Materials and Methods. These data demonstrated that both peptides were completely degraded after 60 min incubation in human saliva.

The stability of histatin 5 derivatives in simulated gastric juice. Since histatin 5 is present in saliva, it is likely to pass through to the stomach, and to the small bowel. Since *Candida spp*. are a common commensal of the gastrointestinal tract, the stability of the histatin 5 peptide may be a relevant issue for the control of *Candida spp*. growth in the gastrointestinal tract. To examine the stability of the histatin 5 related peptides, artificial gastric juice was prepared as described in the Materials and Methods. The simulated gastric juice was subsequently used to evaluate the stability of the peptides after 60 min at 37^{o}C. Surprisingly, these data demonstrated that the wild-type 16mer peptide (W) was extremely stable in gastric juice, while the retro (R) peptide was degraded completely during the 60 min incubation (Figure 15). The results were confirmed by mass spectrometry. The W peptide with a mass of 2067 g/mole was the only molecule detected, which represents the full-length 16 amino acid peptide (Figure 17). In contrast, two fragments were detected in R peptide samples with the full-length peptide barely detectable (Figure18). The analysis of mass spectrometry data revealed that the R peptide was cleaved only

in one location, immediately after the single phenylalanine in the peptide (Table 4). The D-isomers of both the W and R peptides were stable in artificial gastric juice as expected (Figure16). The results of peptide stability in artificial gastric juice are summarized in Figure 19.

2.4 Discussion

Currently available antifungal agents have been effective against fungal infection for an extended number of years; however, the increased use of these agents has been associated with a development of new resistant strains, including *Candida* species. The increase in resistant fungal pathogens has emphasized the need for new antifungal agents. AMPs have been used widely as a base for the development of new clinically approved antibacterial and antifungal drugs [40,41,42]. Studying the structure and mode of action of AMPs could lead to a development of new drugs via chemically mimicking AMPs in structural features such as the net charge, amphipathicity, and selectivity for the pathogen. For example, Ceragenix, a squalamine peptide based antibiotic, is effective against a broad spectrum of bacterial infections [43]and mPE (PMX70004), a phenylethynylene derivative of the maganin peptide, exhibits a broad-spectrum of activity against oral cavity pathogens[44].

Despite the lack of understanding the mechanism of action of histatin 5, all of the proposed lytic or nonlytic mechanisms for histatins would imply that they have a target that is distinct from the current antifungal agents. After a careful analysis of the histatin 5 amino acid sequence and studying the published data, we developed the hypothesis that the distribution of positively charged amino acid residues and secondary structure are the critical factors in histatin 5 fungicidal activity rather than the total net charge or the specific N- to C-terminal amino acid sequence. The question was how to effectively begin to address the proposed hypothesis. Several prior studies with different peptides have shown that using the retro peptide model has been a successfully strategy in dissecting the structural requirements of antimicrobial peptides. In most cases retro and diastereo analogs retained the same activity as the original peptides [49-53]. Thus, the generation of retro peptides and stereoisomers was adopted as a strategy to understand

the structure features of histatin 5 important for fungicidal activity. We chose to use the C-16 histatin 5 peptide derivative since several publications have shown that the C-16 peptide of histatin 5, an N-terminal truncated derivative containing 16 amino acid residues, retains the same fungicidal activity as the full-length 24 amino acid peptide. Thus, the C-16 peptide, referred to as the W peptide in this study, was used as a model due to the shorter sequence that eliminates amino acid residues that did not influence antifungal activity. The optimal way to test our hypothesis that the linear N- to C-terminal order of amino acids was not relevant was by generating a retro peptide, where the amino acid composition and the total net charge remained the same as the W peptide. The retro peptide, referred to as the R peptide, was synthesized using same amino acid sequence as the W peptide but the amino acid sequence was reversed (Table 1). To evaluate stereospecificity, two additional peptides with D-amino acids were synthesized, and termed the WD and RD peptides (Table 1).

The first step was to examine the fungicidal activity of the four histatin 5 derivatives. The fungicidal activity assays showed that the W, WD, R and RD peptides were equally active over a range of concentrations against *Candida albicans*. The differences in activity were found to be insignificant ($P<0.05$) (Figure 1). The LD_{50} value for histatin 5 and C-16 peptide has been published previously, and the values range from 2 to 7.3 µM against most *Candia albicans* strains [10, 45-48]. The LD_{50} values for W, WD, R, and RD peptides were calculated and shown to be similar to each other and to the previously published data (Table 2). Since there was the possibility that the four peptides may kill *C. albicans* at different rates, the kinetics of killing was examined in a time course assay. These studies indicated that all four peptides reached a maximum killing activity after 2 h of incubation (Figure 5, 6). The kinetic studies indicated no significant difference between the four histatin 5 peptide derivatives.

While the four histatin 5 peptides derivative demonstrated similar fungicidal activities, it was plausible that they were functioning via distinct mechanisms. Thus, we examined the activity of the four histatin 5 peptide derivatives under conditions known to effect histatin 5 killing activity. Low temperature has been shown to inhibit histatin 5 fungicidal activity via one of two suggested mechanisms: affecting cell metabolism by reducing cell respiration and ATP production [31], and increasing membrane rigidity. This in turn leads to a decrease in the binding of histatin 5 to the yeast cell membrane and reduces or eliminates the translocation to cytoplasm [30]. Our data showed that low temperature displayed the same effect on all four peptides, inhibiting 70% of their fungicidal activity against *Candida albicans*. This result demonstrates that regardless of the exact effect of temperature on histatins 5 activity, the retro peptides likely work through the same pathways as W peptide. The inhibitory effect of low temperature was not unique to histatin 5, as it has been observed in several antibacterial and antifungal peptides[54-56].

Histatin 5 activity can also be blocked by the inhibition of cellular respiration using sodium azide[17]. Sodium azide is a potent inhibitor of mitochondrial respiration as it inactive cytochrome c oxidase via intercalating between the home a_3 iron and Cu_3 at the oxygen reduction site [57]. Also, sodium azide binds to the F1 catalytic domain within mitochondrial F-ATPase's and inhibits the hydrolyase activity [58,59]. Multiple previous studies have shown that 5 mM sodium azide inhibits both the conventional and the alternative respiratory pathways in *Candida albicans*[17, 60].

This observation led to the hypothesis that histatin 5 may target energized mitochondria. Thus, the influence of azide on *Candida albicans* susceptibility to the four histatin 5 peptide derivatives

was examined. As illustrated in Figure 9, the activity of the peptides was equally inhibited by azide. Taken together, the inhibition by low temperature and azide suggests that the fungicidal activity of the four peptides is occurs via a similar or identical mechanism.

In general, the killing activities of cationic AMPs are sensitive to ionic strength, with some limited exceptions [32, 61, 62, 63]. Because of the nature of the ionic interaction between cationic AMPs and the negatively charged membrane of the target organism, it can be weakened by monovalent and divalent cations to reduce their activity [64]. Histatin 5 activity has been evaluated under different physiological conditions and it was found to be affected by the presence of salts including sodium chloride [32]. In this study, the four peptides were evaluated in a presence of NaCl simulating two different physiological environments: oral cavity with salt concentration ranges from 5 to 25 mM sodium chloride [65] and RPMI1640 tissue culture medium. The RPMI-1640 medium contains multiple salts along with 150 mM sodium chloride, which simulates the human cellular environment[29]. At 25 mM NaCl, the four histatin 5 peptide derivatives were all inhibited by 35%, while it was totally impaired in RPMI-1640 medium. Published data showed that the activity of histatin 5 against *Candida albicans* (SC5314) was abolished totally in 150 mM NaCl [66] and was undetectable in RPMI medium [67]. It's important to mention that besides salt content, RPMI 1640 medium also induces the hyphal growth of *C. albicans* [68, 69]. The role of hyphae will be discussed later. Our results show the effect of NaCl on R and RD peptides matches W and WD peptide. This finding further supports the hypothesis that these peptides work via similar mechanisms that require an ionic interaction for activity.

In addition to salt sensitivity, cationic AMPs activity is also pH dependent in many cases[61]. The pH has an effect on AMPs activities by interfering with the target cell or the peptide. *C. albicans* has the ability to grow either as unicellular budding spherical yeast or as filamentous

pseudohyphal and hyphal forms [70]. The ability to switch between these two forms plays an important role in *Candida* pathogenicity [71]. This switching process can be stimulated by changes in pH [72], namely moving toward pH 7 or greater. Previously published data suggests that the activity of antifungal agents against different *Candida* forms varies with the hyphae being more resistant [71]. This factor must be taken into consideration when developing an effective therapeutic agent against *Candida spp*. In the case of histatin 5, prior studies have suggested the peptide has the same activity against all of the morphological forms of *Candida*[46].

The net charge of cationic peptides plays an important role in their activity and an increase in net charge usually leads to an increase in the antimicrobial activity [15, 73] . The net charge on peptides is dictated by the presence and abundance of basic amino acids (arginine, lysine and histidine) and acidic amino acids (aspartic acid and glutamic acid). The pH interferes directly with the peptide net charge as each amino acid has a unique pKa and isoelectric value. Thus, the vast majority of cationic AMPs are more active at acidic medium than basic medium [32]. Unlike other AMPs, pH has little effect on histatin 5 fungicidal activity [74]. As the data shown in Figure 10A the net charge decreased dramatically as the pH shifted from acidic to basic and that change can be related to the abundance of histidine residues. The pKa of histidine side chain (imidazole ring) is 6 [75] which means it will lose the positive charge and become neutral at physiological pH (7.4) and above. Changing the pH from 5 to 9 is sufficient to shift the net charge of histatin 5 from +14 to +5; however, the published data suggest that this change in histatin 5 charge did not correlate with any significant effect on the fungicidal activity. The same observation was made with bot the W and R peptides in our study; they retained fungicidal activity over the same range of pH from 5 to 9 (Figure10B). After excluding the positive charges contributed by histidine residues, only few positive charges remained in histatin 5 or the derivatives examined in this

study. Thus, if there is a role for positive charge in histatin 5 activity, it would be represented by lysine and arginine residues. Several published results have supported this conclusion. For example, the replacement of lysine-13 with glutamic acid and arginine-22 with glycine was enough to reduce histatin 5 killing activity [76]. A single substitution of histidine for a lysine increases the fungicidal activity of histatin 5 by 50 % [5]. Because pH did have an effect on our four peptides, not all positive charges may be required for R and W peptide. These results are not unique to histatin 5, as similar observations have been reported with other peptides [32].

Histatin 5 peptide sequence contains two different metal binding motifs, ATCUN and a zinc binding domain [77]. Binding to Cu^{2+} and Ni^{2+} ions, the ATCUN motif is located at the N-terminal and it is represented by a sequence of DSH. On the other hand, the HEXXH sequence represents the zinc binding motif. The C-16 peptide sequence used in our study still contains the zinc binding motif, but not the ATCUN motif. The zinc-binding motif could improve histatin 5 activity by binding with metal ions[78]. Since reversing the C-16 sequence in the retro peptide may influence zinc-binding, it was important to evaluate the relevance of metal ion binding to the activity of the four peptides. Thus, the influence of zinc-binding on the activity of the four histatin 5 peptide derivatives was examined by the inclusion of EGTA in the fungicidal activity assay in an effort to chelate any divalent ions in the medium. EGTA was chosen over EDTA because the latter displays some antifungal activity of its own, while EGTA lacked any antifungal activity up to 10mM [37]. The data demonstrated that the fungicidal activity of all four peptides was not affected significantly over a wide range of EGTA concentrations (10 μM to 1mM) (Figure 11). This finding strongly suggests that metal binding is not a major factor in the fungicidal activity of histatin 5.

Histatins in general and the C-16 (W) peptide specifically have been shown to form an alpha helical structure in a presence of trifluoroethanol (TFE) and synthetic lipids [5, 79]. The formation of a helical structure leads to the amphipathic structure by which the hydrophobic residues are biased to one side of helix and the hydrophilic residues to the opposite side. Furthermore, the ability to form an amphipathic alpha helix was found to be essential for the fungicidal activity of histatins[5]. To evaluate the effect of reversing the sequence on peptide structure and to determine whether the R peptides retained the ability to form an amphipathic alpha helix, CD spectra were performed in 50% TFE to mimic a hydrophobic environment [80]. The percentage of alpha helix in each peptide was calculated using K2D2 software. CD spectra have been used in several publications to determine the secondary structure of retro peptides and the results have varied. In some cases the percentage of α-helix was similar in the retro peptide [81] while it was different in others [51, 52, 82]. The CD data obtained with the four histatin 5 derivatives demonstrated that reversing the sequence of the W peptide, generating R peptide, did not interfere with the ability of the peptide to form an alpha helical structure as the percentage of helix was very similar (Figure 12). The amphipathicity of R peptide was evaluated using a helical wheel model and the results for the R peptide suggested that this peptide could form an amphipathic structure (Figure 13). It should be noted that the exact region of the peptides that form the helix at 50% TFE is undefined. The CD data confirm the similarity between the tested peptides and proof that reversing the sequence of W peptide didn't interfere with the potential amphipathicity or the propensity to form an alpha helix.

Human saliva contains a pool of protease enzymes, more than thirteen, with various activities such as trypsin-like, chymotrypsin-like and histidine peptidase [83, 84]. These enzymes are mostly secreted from white blood cells and microflora, but some are produced by the salivary glands [85,

[86]. The main cleavage sites of salivary proteases are lysine and arginine residues for trypsin-like enzymes, aromatic amino acids for chymotrypsin-like, and histidine is the primary target for histidine protease [83, 84]. The stability of histatin 5 in saliva has been evaluated in multiple publication and the kinetics of histatin proteolysis in saliva have also been measured [78, 83]. The rate of histatin 5 degradation in saliva was approximately17.8 µg/ml/h and the $T_{1/2}$ was approximately 8 h [33]. However these studies didn't use whole saliva, instead they used a diluted version (1:10) [33]. Histatin 5 is not highly stable in saliva due to the abundance of histidine, lysine and arginine residues. In this study, the stability of the four histatin 5 derivatives was evaluated using undiluted saliva. Not surprisingly, D-amino acid peptides (WD and RD) showed extreme stability, while the L-peptides (W and R) were totally degraded after a sixty minute period of time. The stability of D conformation AMPs in saliva and protease enzymes has been confirmed in multiple occasions [28, 86-90]. The stability of other AMPs has been evaluated in saliva and in several cases they were degraded completely in less than 60 min [86, 87]. For example, the $T_{1/2}$ for the KSL peptide, in saliva was less than 5 minutes and the peptide was totally degraded within 10 minutes [28]. In summary, no superior stability was observed for the retro peptide in human saliva; however, the D-amino acid peptides are highly stable.

Since the histatin 5 that is secreted from the oral cavity will travel to the stomach, the stability in simulated gastric juice was measured. Pepsin, a major digestive enzyme found in gastric juice, cleaves peptides and proteins before and after any hydrophobic residue, such as phenylalanine, tryptophan, and tyrosine. Therefore, there were three possible cleavage sites within the W and R peptides (two tyrosine and one phenylalanine), which could generate six possible proteolytic fragments (Table 4). Unexpectedly, HPLC data showed that the W peptide is stable in the simulated gastric juice for over 60 minutes, while the R peptide was totally degraded within the

same period of time (Figure15). The mass spectrometry data revealed that pepsin selectively cleaved the R peptide at phenylalanine and it did not cleave at tyrosine residues as anticipated. This is not the first reported case in which pepsin selectively cleaved one predicted residue over another [28, 91]. In addition, the stability of the W peptide is not the first AMP to be stable in the stomach as several peptides that target *H. pylori* [92, 93] have proved to be stable in the stomach. Because we are the first to report a stability of histatin 5 analogs in gastric juice, we cannot generalize these data or compare it to the full-length histatin 5. As predicted, the D-conformation peptides were stable in simulated gastric juice [28].

In an attempt to understand the stability of the W peptide, we used the software program Expasy Peptide Cutter to predict the stability of the W peptide over the R peptide in pepsin. The possible explanation could be the accumulation of positively charged amino acid residues before the susceptible residue because in W peptide there are three basic amino acid residues before the phenylalanine ($K^+R^+K^+F$) while in R peptide the basic residues occur after the phenylalanine ($FK^+R^+K^+$). Thus, the stretch of positively charged amino acids may interfere with pepsin activity with the W peptide. It is important to mention that this observation was considered in the design of other antifungal peptides under development in our laboratory.

2.5 Conclusions

In summary, reversing the sequence of the W peptide to generate the R peptide did not interfere with the fungicidal activity or the peptide secondary structure. The results of the killing assays in a presence of common histatin 5 inhibitors (NaCl, sodium azide, EGTA and low temperature) endorse the similarity between the W and R peptides, and support the hypothesis that both work through the same pathway. An ionic interaction step is required for all peptides to achieve their activity. Chirality and stereospecificity have no role in the peptide activity as the D-conformation peptides retained the full activity. The secondary structure and distribution of certain positive charges are essential factors for the fungicidal activity rather than the specific N- to C-terminal amino acid sequence.

Table 1: Histatin 5 peptide derivatives synthesized.

Peptide	Sequence[1]
Histatin 5 (24 amino acid natural peptide	DSHAKRHHGYKRKFHEKHHSHRGY
Normal (C-16) Histatin 5 (**W**)	GYKRKFHEKHHSHRGY
Normal (C-16) Histatin 5 with D-amino acids (**WD**)	Gykrkfhekhhshr**G**y
Retro (C-16) Histatin 5 (**R**)	YGRHSHHKEHFKRKYG
Retro (C-16) Histatin 5 with D-amino acids (**RD**)	y**G**rhshhkehfkrkyG

[1] *Lower case sequence is used to designated D-amino acids*

Table 2: The LD_{50} for the W, WD, R, and RD peptides against *Candida albicans* (SC5314).

Peptide	LD_{50} (μM)[1]
Normal (C-16) Histatin 5 (**W**)	2.705 ± 0.445
Normal (C-16) Histatin 5 with D-amino acids (**WD**)	2.400 ± 0.289
Retro (C-16) Histatin 5 (**R**)	2.928 ± 0.472
Retro (C-16) Histatin 5 with D-amino acids (**RD**)	2.477 ± 0.177

[1]*Data represents three independent experiments and the error represents the standard deviations. LD_{50} is defined as the peptide concentration at which 50% of the viable cells were killed under the assay conditions and was determined using linear regression equations as shown in Figure 4.*

Table 3: Minimum Inhibitory Concentration assay for the W, WD, R and RD peptides RPMI-1640 medium[1].

Peptide	1X	0.5X	0.25X	0.125X
W	>206.7	>206.7	>206.7	206.7
R	>206.7	>206.7	>206.7	>206.7
WD	>206.7	>206.7	>206.7	103-206.7
RD	>206.7	>206.7	>206.7	103-206.7

[1] *Data are expressed in µg/ml.*

Table 4: Mass Spectrometric identification of the degraded R and W peptides in simulated gastric juice as determined by MALDI-TOF.

Peptide	Sequence[1]	Mass *(m/z)*
R	**Y**-G-R-H-S-H-H-K-E-H-[redacted]K-R-K-**Y**-G	2067
Degraded R-1	**Y**-G-R-H-S-H-H-K-E-H-**F**	1434
Degraded R-2	K-R-K-**Y**-G	651
W	G-**Y**-K-R-K-**F**-H-E-K-H-H-S-H-R-G-**Y**	2067

[1] Slash (/) represents the detected cleavage site .The bold underlined amino acids represent the possible cleavage sites for pepsin.

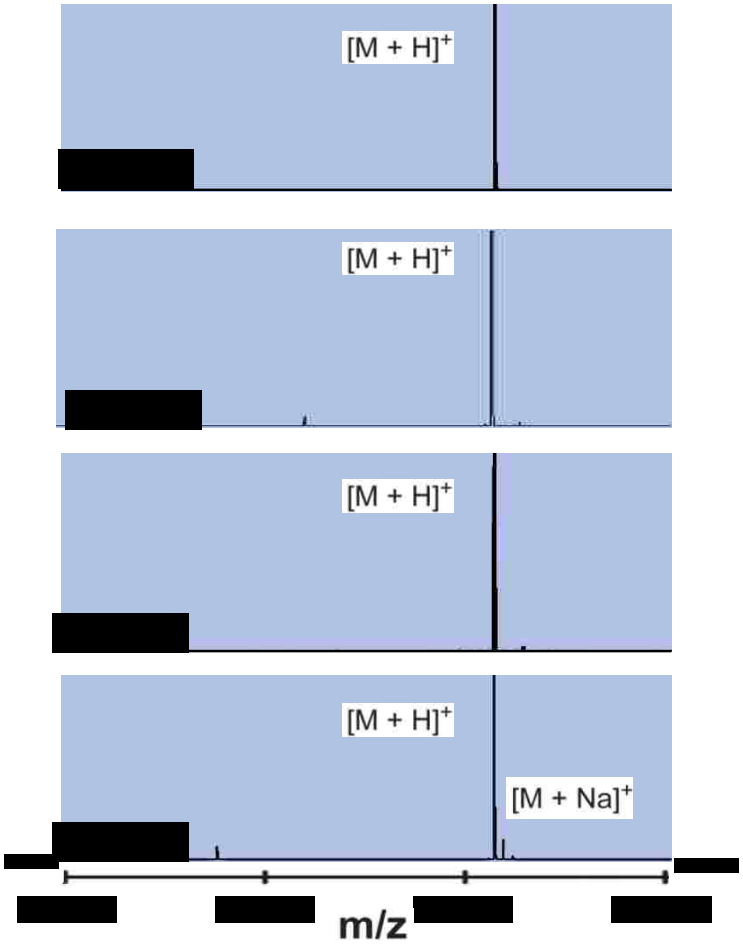

Figure 1: **Mass spectrometry of the four peptides (W, WD, R, RD) using matrix-assisted MALDI-TOF mass spectrometry.** The expected mass of the four peptides is 2067 g/mol. W is normal (C-16) histatin 5 peptide while R is the retro peptide. WD and RD are enantiomers of W and R peptide, respectively.

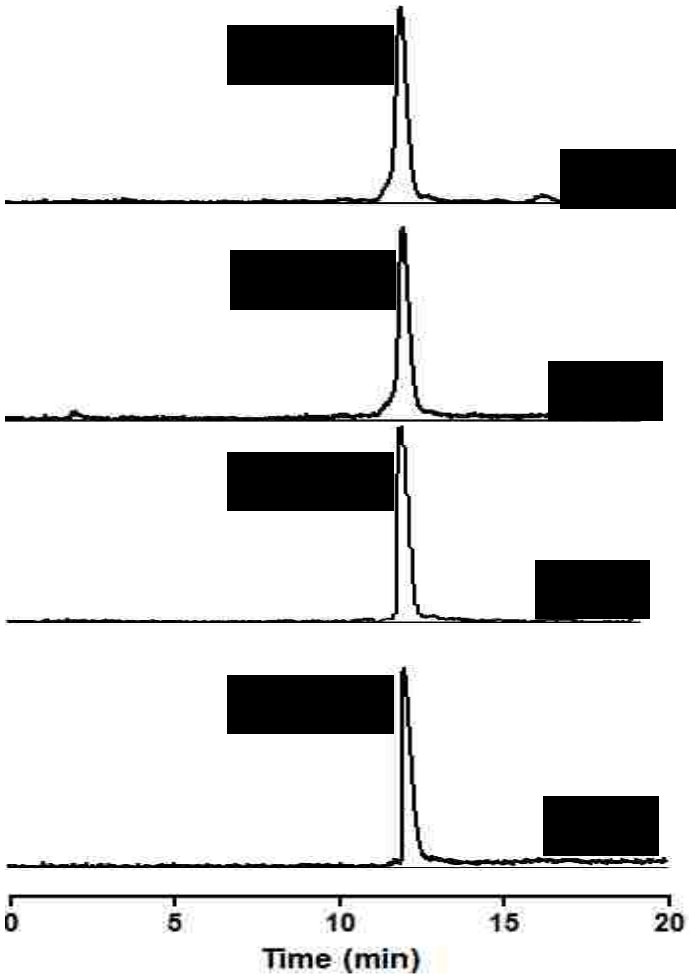

Figure 2: **HPLC elution profiles of the four peptides.** Elution time (minute: seconds) of the main peak is shown for each peptide. The peptides are W is normal (C-16) histatin 5 peptide while R is the retro peptide. WD and RD are enantiomers of W and R peptide, respectively.

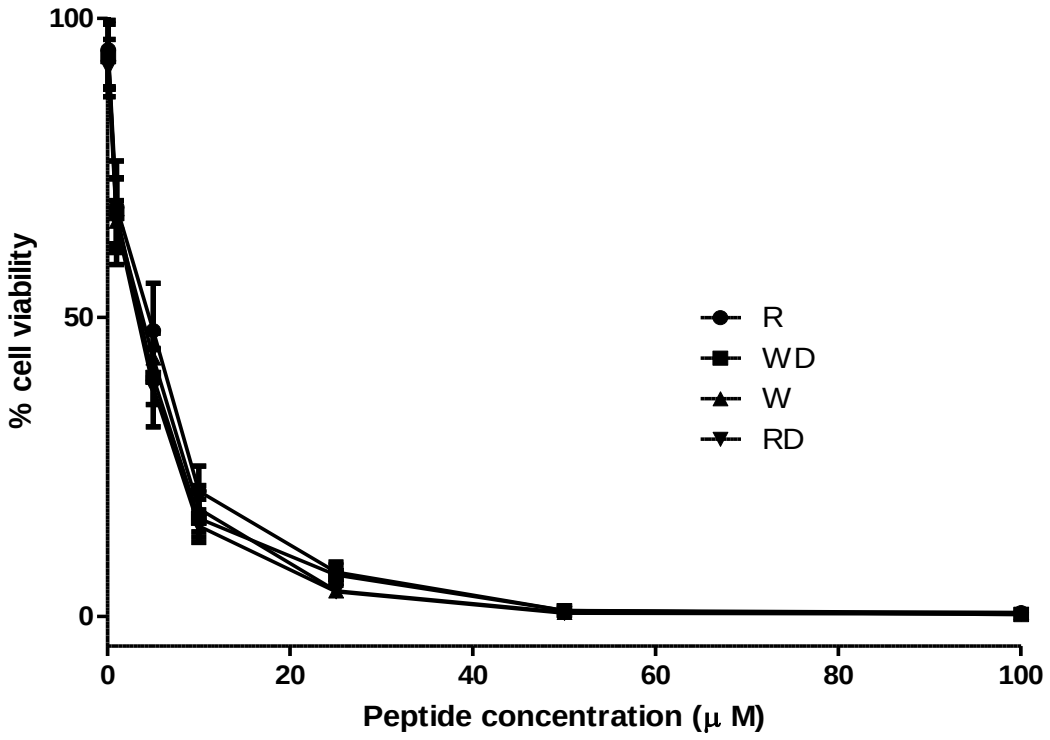

Figure 3: **Relative fungicidal activity of W, WD, R and RD peptides against *C. albicans* SC5314.** Different concentrations of each peptide were incubated with *C. albicans* (1.8×10^5 cells/ml) in 10 mM Sodium phosphate buffer for 2 h at 37^oC. The percent fungal cell viability was determined by counting the viable colonies on Sabouraud dextrose agar (SDA) as compared to the number of colonies on peptide-free control plate. Data represents the mean of three independent experiments and the error bar represents the standard deviation.

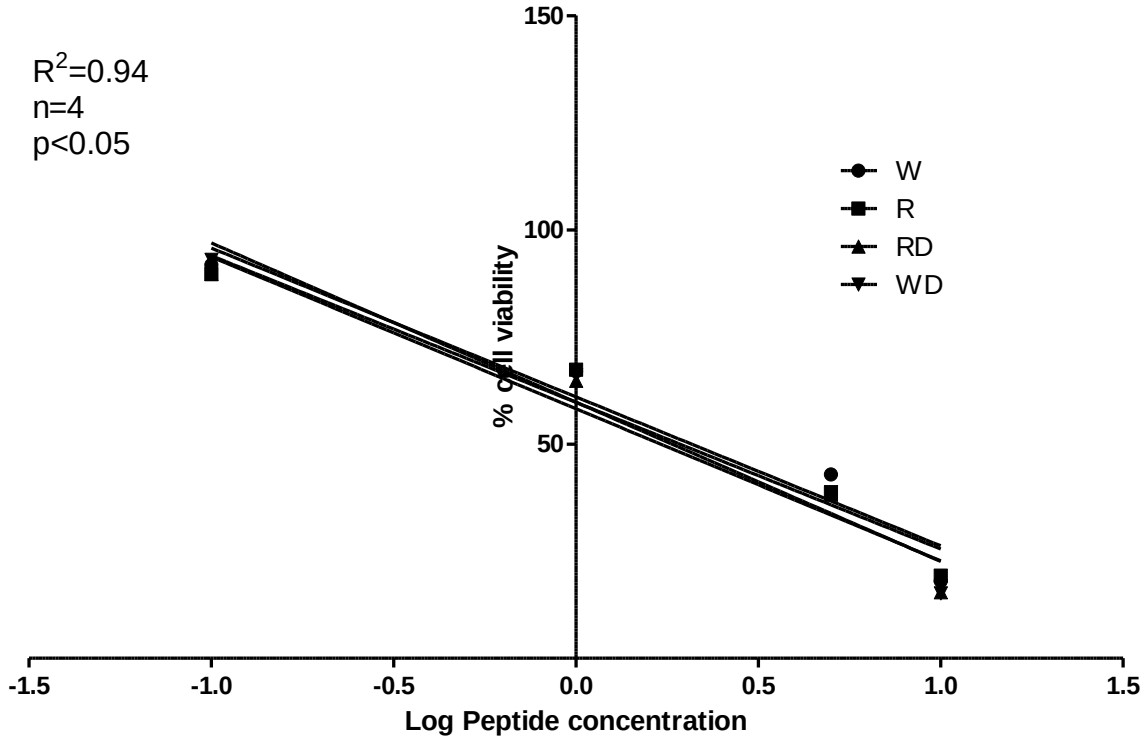

Figure 4: Determination of the LD_{50} for W, WD, R and RD. The data generated in Figure 3 was used to calculate the LD_{50} of each peptide using linear regression equations and the results are summarized in Table 2.

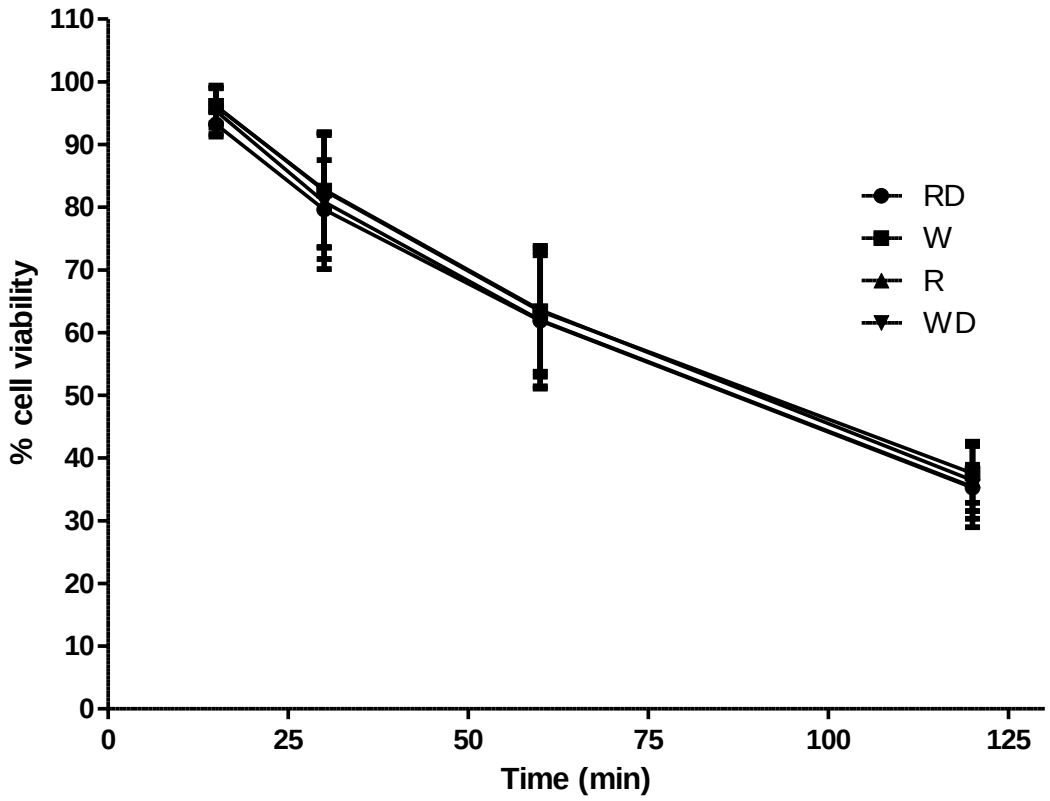

Figure 5: Kinetics of fungicidal activity against *C. albicans* for the W, WD, R, and RD peptides. For each assay, 10 µM peptide was incubated with *Candida albicans* (1.8×10^5 cells/ml) in 10 mM sodium phosphate buffer for different time periods (15 ,30 ,60 and 120 min). The percentage of viable cells at each time point was calculated relative to a control without peptide incubated for an identical time. Data represents the mean of three independent experiments and the error bar represents the standard deviation.

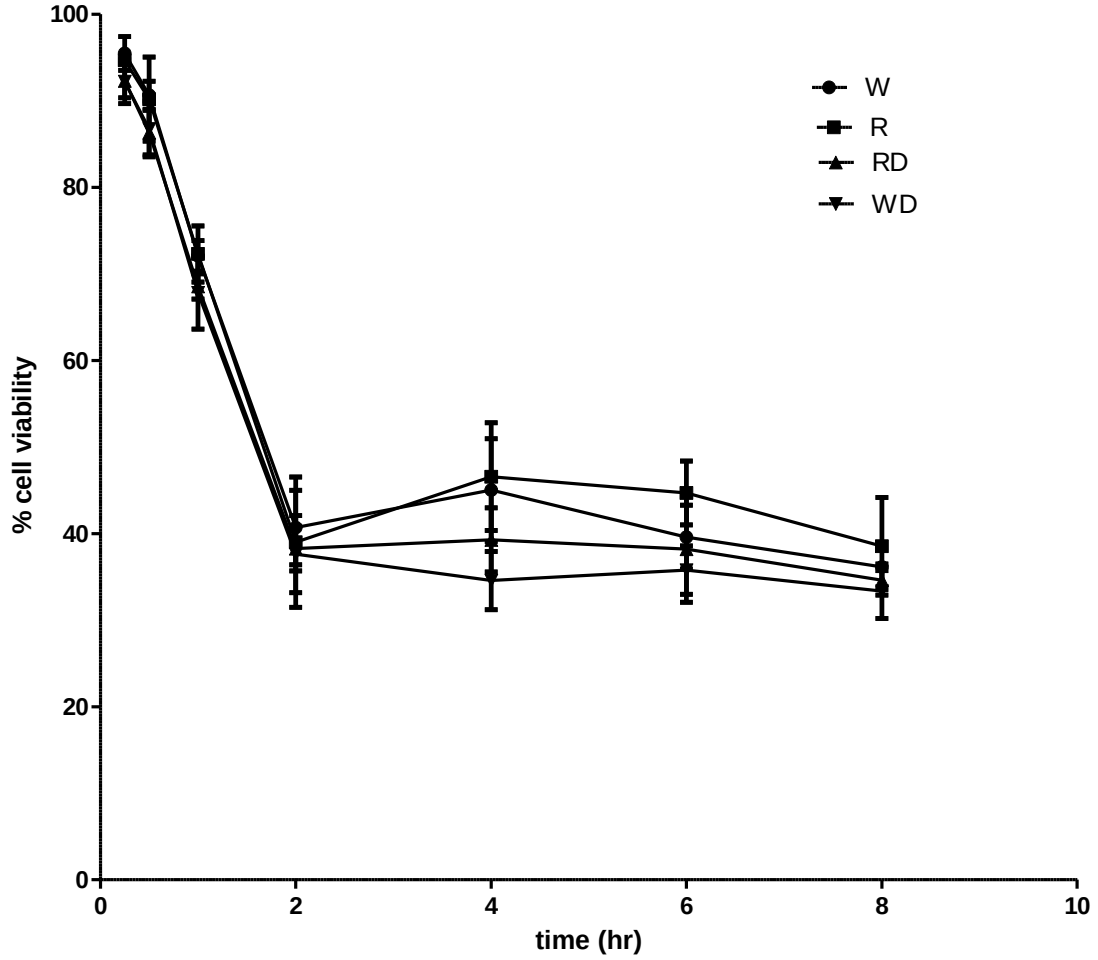

Figure 6: Extended kinetics of fungicidal activity against *C. albicans* for the W, WD, R, and RD peptides. For each assay, 10 μM peptide was incubated with *Candida albicans* (1.8×10^5 cells/ml) in 10 mM sodium phosphate buffer for different time periods (0.25, 0.5, 1, 2, 4, 6, and 8 h). The percentage of viable cells at each time point was calculated relative to a control without peptide incubated for an identical time. Data represents the mean of three independent experiments and the error bar represents the standard deviation.

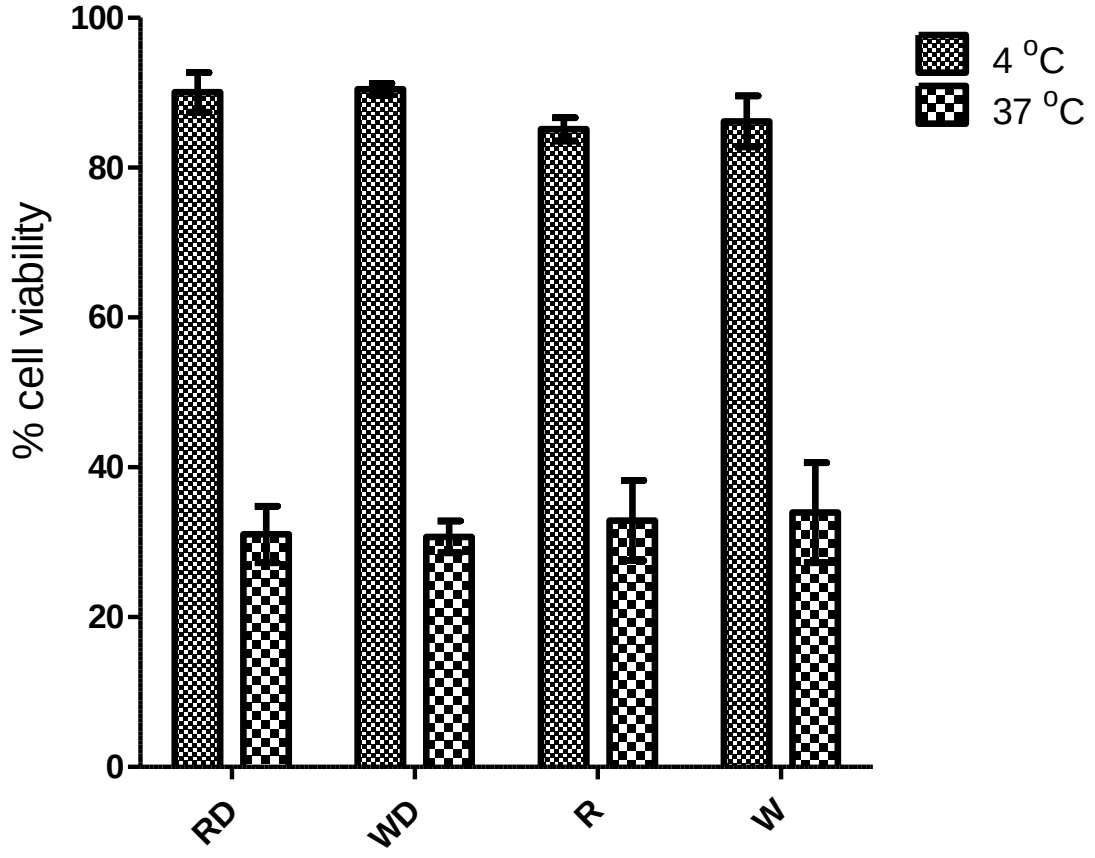

Figure 7: The effect of temperature on fungicidal activity. For each assay, 10 μM of W, R, WD, and RD peptides were incubated with *C. albicans* (1.8×10^5 cells/ml) in 10 mM sodium phosphate buffer for 2 h at either 4^oC or 37^oC as indicated. The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. Data represents the mean of three independent experiments and the error bar represents the standard deviation.

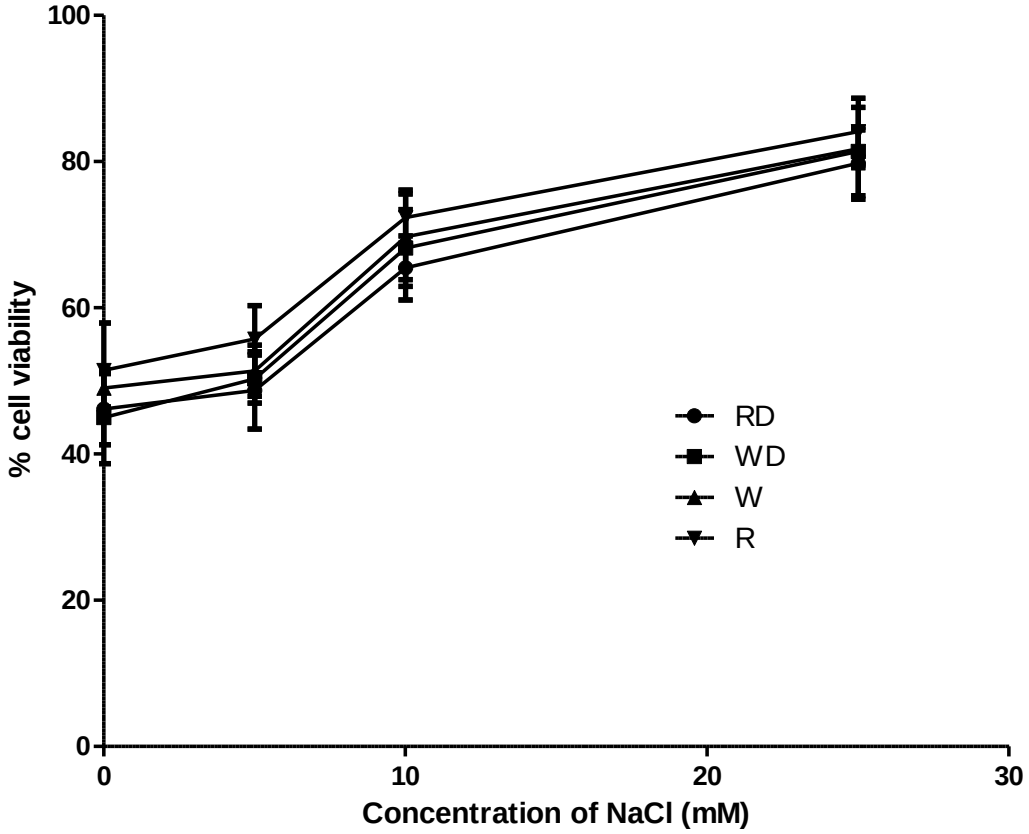

Figure 8: The effect of sodium chloride on fungicidal activity. For each assay, 5 µM of W, R, WD, and RD peptides were incubated with *C. albicans* (1.8×10^5 cells/ml) in 10 mM sodium phosphate buffer for 2 h in the presence of increasing concentrations of sodium chloride (0, 5 ,10, and 25 mM). The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. Data represents the mean of three independent experiments and the error bar represents the standard deviations.

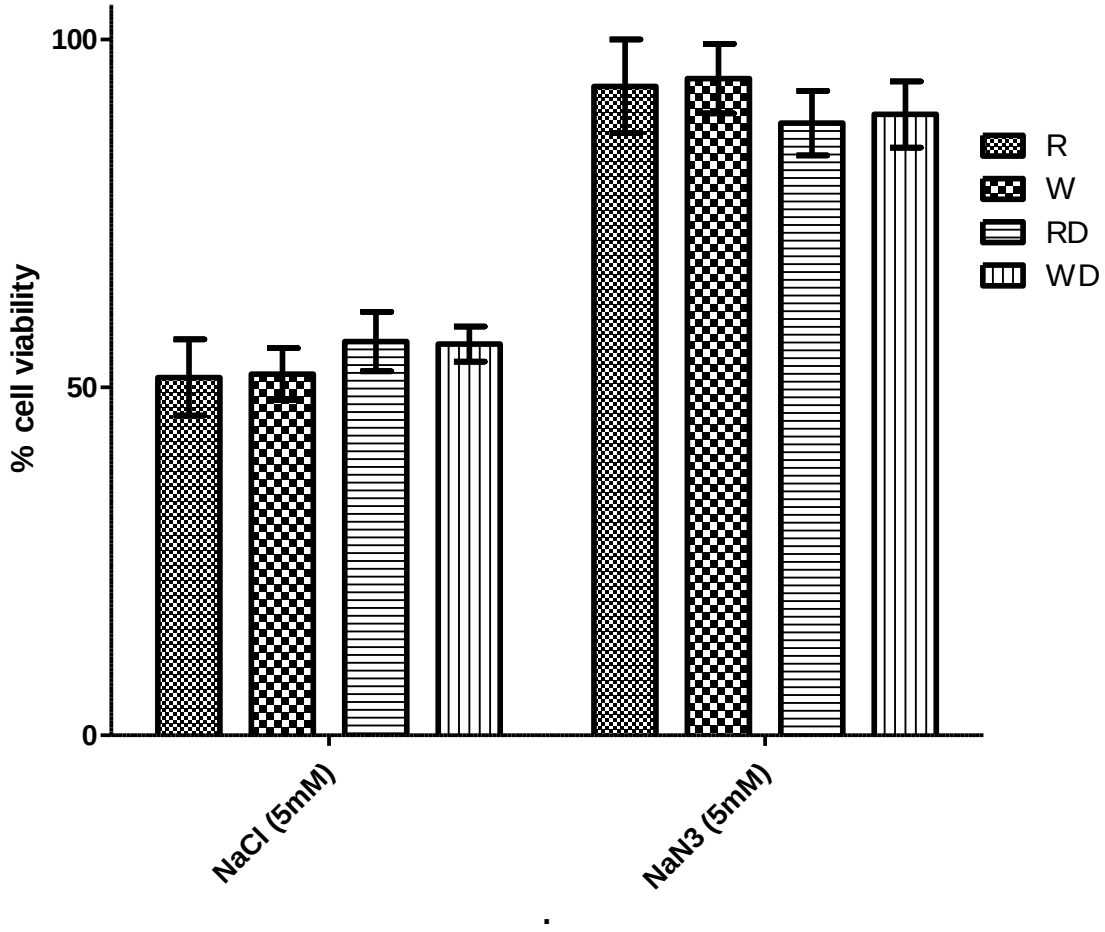

Figure 9: The effect of sodium azide on fungicidal activity of W, WD, R and RD peptide against *C. albicans*. 5µM of each peptide was incubated with *Candida albicans* (1.8×10^5 cells/ml) in 10 mM Sodium phosphate buffer for 2 hours in the presence of 5mM sodium azide. A control contains 5mM sodium chloride was used. The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. Data represents the mean of three independent experiments and the error bar represents the standard deviations.

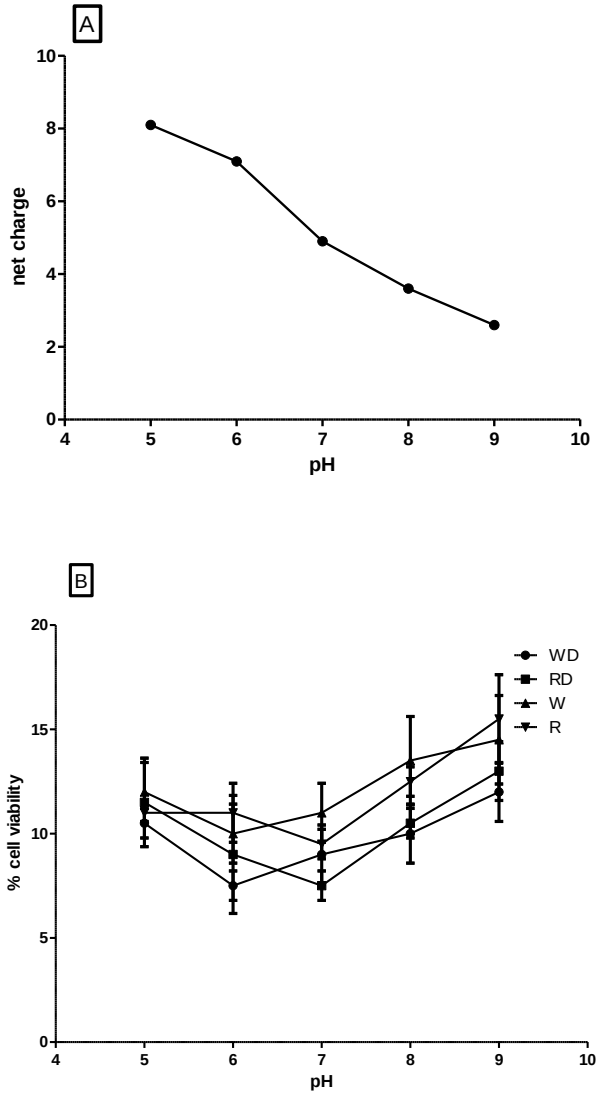

Figure 10: The pH dependence of fungicidal activity of the four histatin 5 peptide derivatives. (**A**) The calculated net charge of the peptides at different pH values. The net charges were calculated using Protein Calculator v3.3 - Scripps Research Institute. (**B**) For each assay, 20 µM of the indicated peptide was incubated with *Candida albicans* (1.8×10^5 cells/ml) for 2 h at 37^{o}C in 10 mM sodium phosphate buffer at different pH values (5, 6, 7, 8 and 9). The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. The data represents the mean of three independent experiments and the error bar represents the standard deviation.

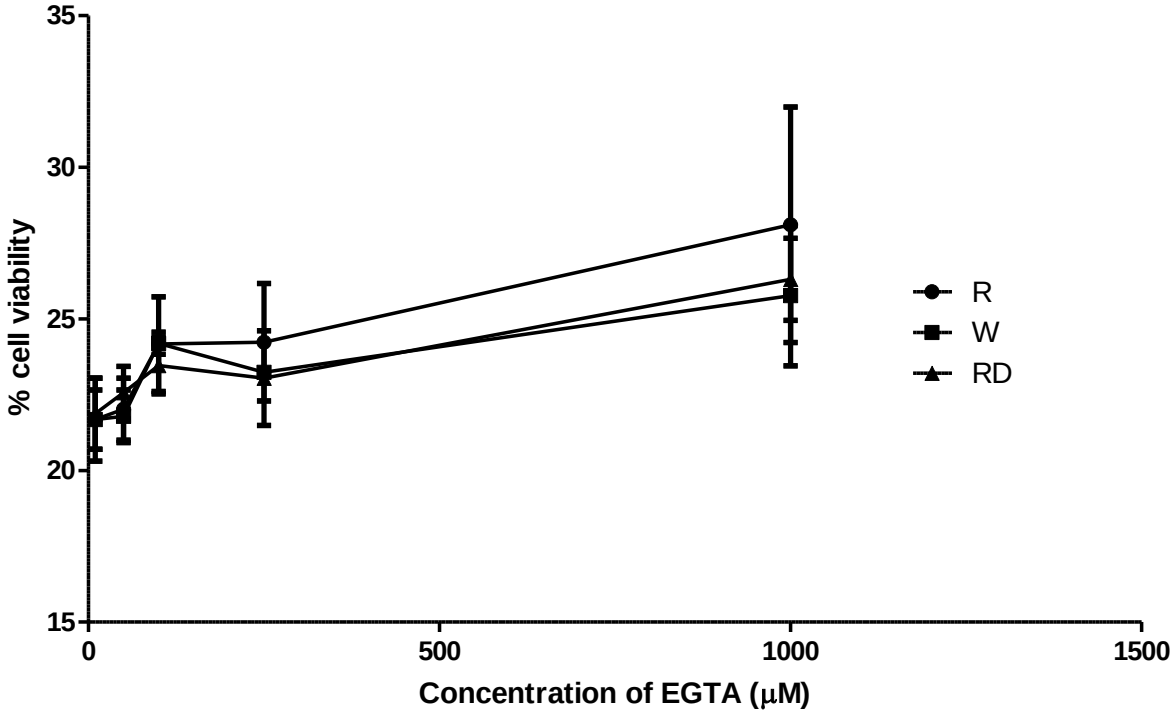

Figure 11: Role of zinc binding in fungicidal activity of the histatin 5 peptide derivatives.

For each assay, 10 μM of peptide was incubated with *Candida albicans* (1.8×10^5 cells/ml) in 10 mM sodium phosphate buffer in a presence of increasing concentrations of EGTA as indicated. The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. The data represents the mean of three independent experiments each and the error bars represents the standard deviation.

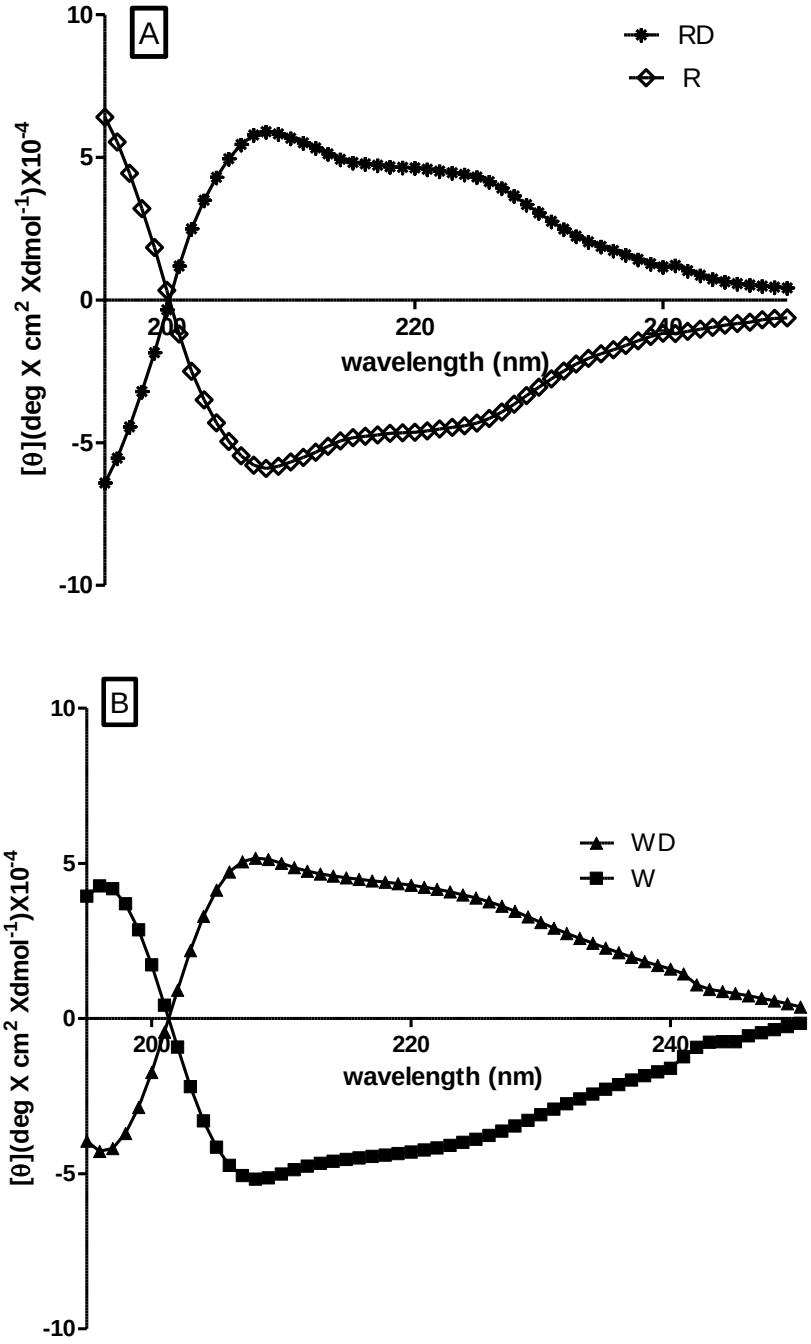

Figure 12: CD spectra in 10 mM phosphate (pH 7.4) at 25^{o}C in the presence of 50% TFE (A) R and RD peptides (B) W and WD peptides. The samples were prepared as described in Materials and Methods. CD spectra are displayed in mean residue ellipticity [θ].

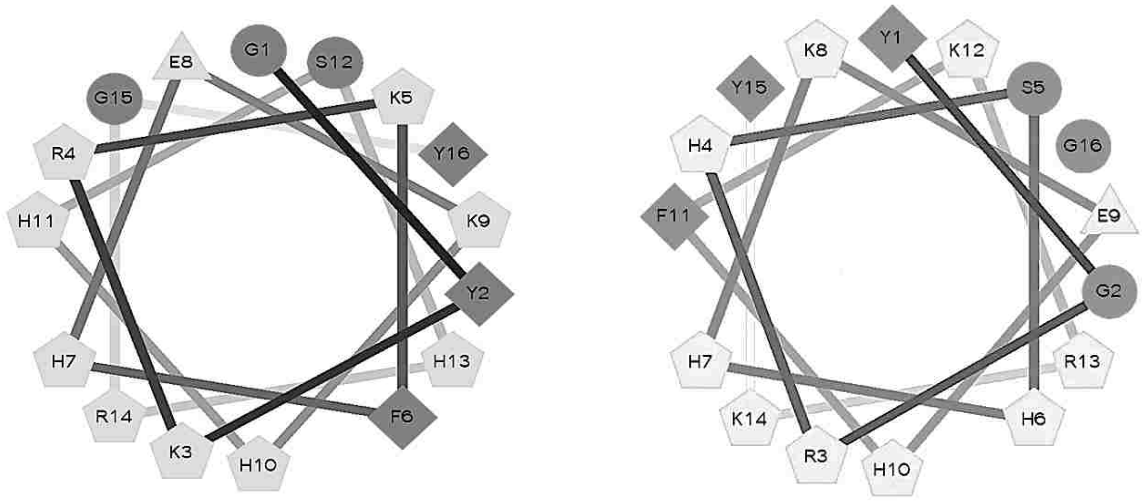

Figure 13: Predicted helical wheel projections of the R peptides (left) and the W peptides (right). The helical wheel projections were prepared with the tool created by Don Armstrong and Raphael Zidovetzki. Amino acids illustrated in the light background are charged while those amino acids depicted in the dark background represent non- polar and polar uncharged amino acids.

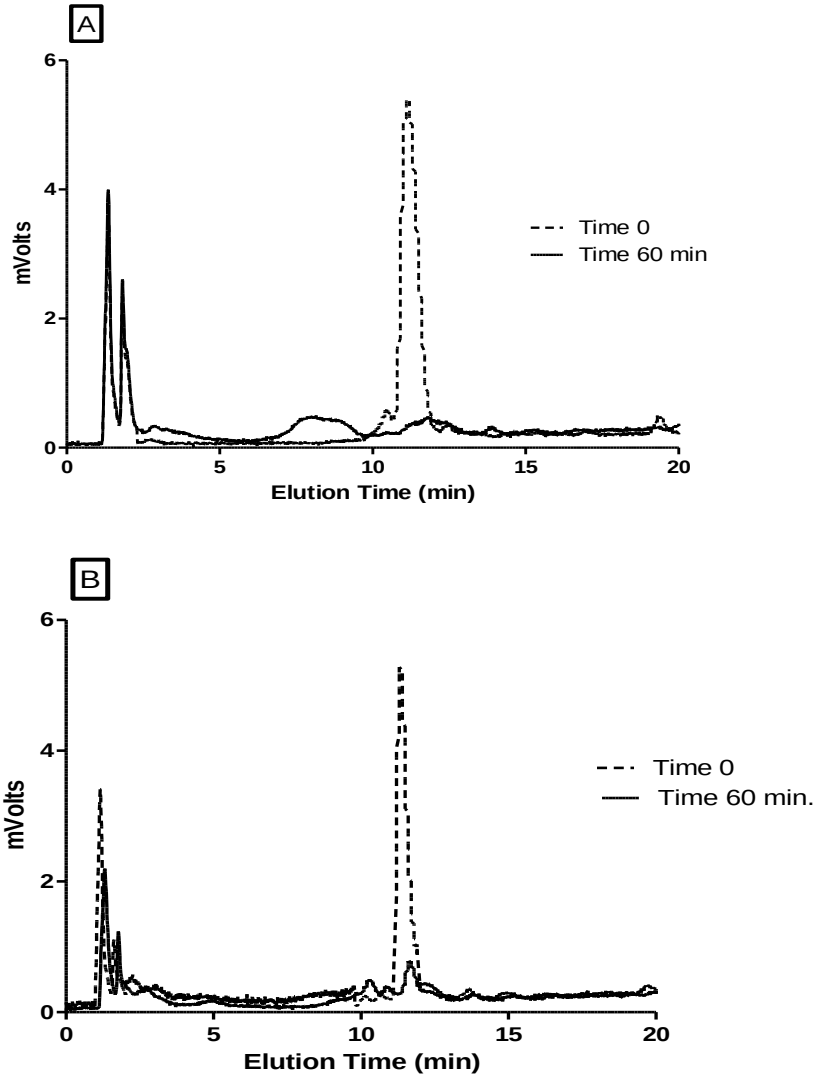

Figure 14: Stability of the wild-type (W) versus retro (R) peptide in human saliva. The R (panel A) and the W (panel B) peptides were incubated in human saliva for 60 min at 37^{o}C and the level of peptide degradation was determined by HPLC. The samples were prepared and processed as described in Materials and Methods.

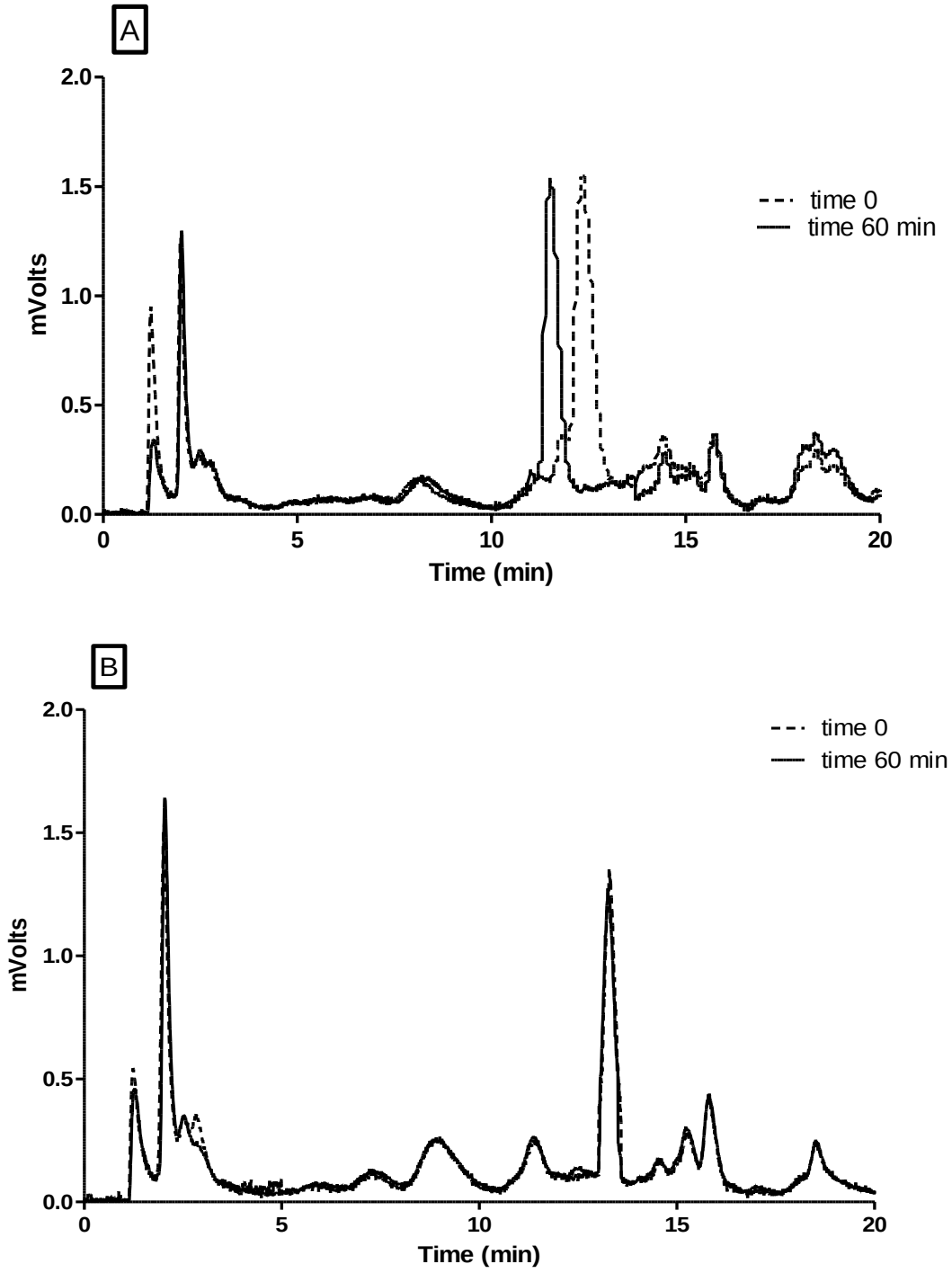

Figure 15: **Stability of the wild-type (W) versus retro (R) peptide in artificial gastric juice**. The R (panel A) and the W (panel B) peptides were incubated in artificial gastric juice for 60 min at 37^{o}C and the level of peptide degradation was determined by HPLC. The samples were prepared and processed as described in Materials and Methods.

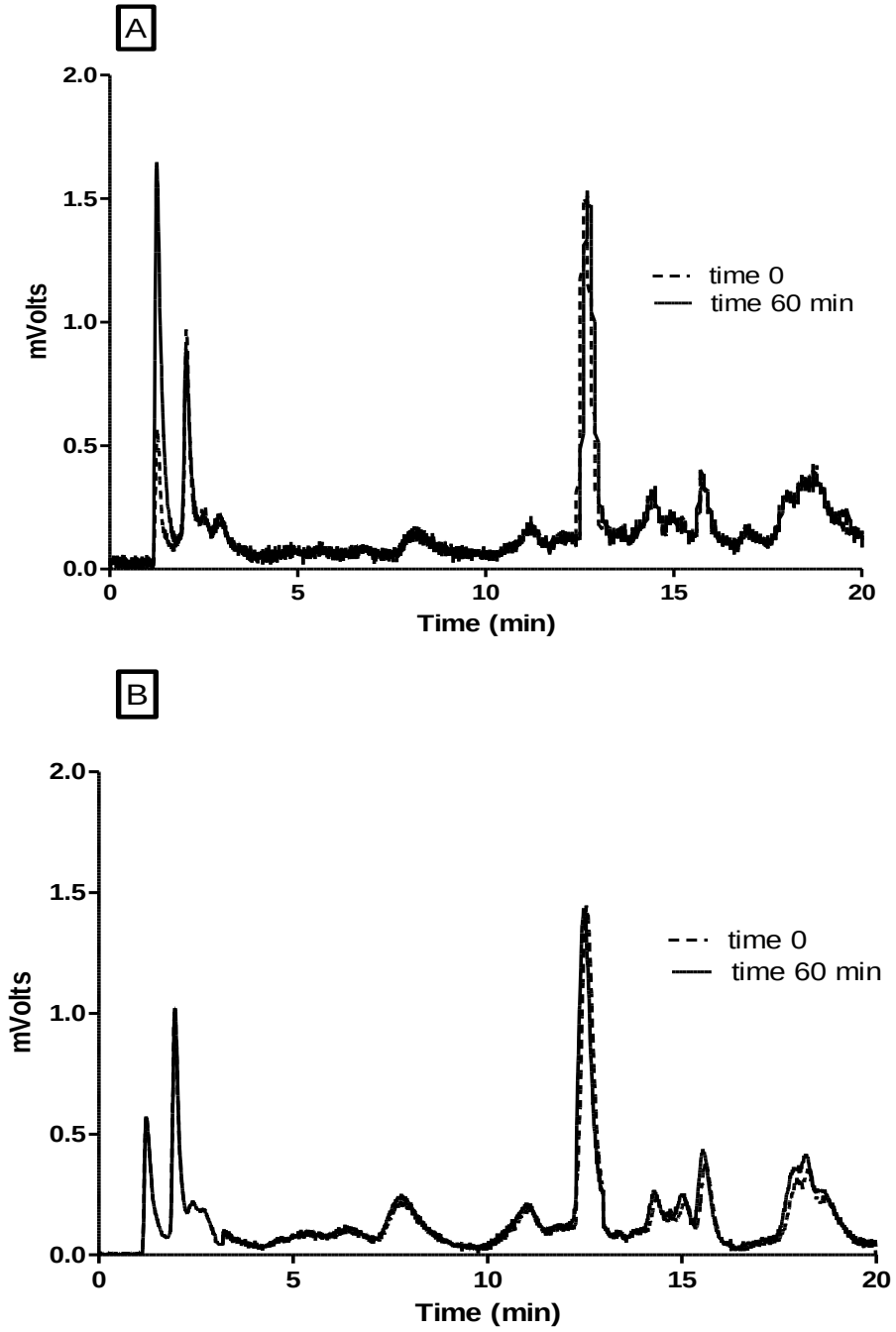

Figure 16: **Stability of D-isomers of the wild-type (WD) versus retro (RD) peptide in artificial gastric juice**. The RD (panel A) and the WD (panel B) peptides were incubated in artificial gastric juice for 60 min at 37^{o}C and the level of peptide degradation was determined by HPLC. The samples were prepared and processed as described in Materials and Methods.

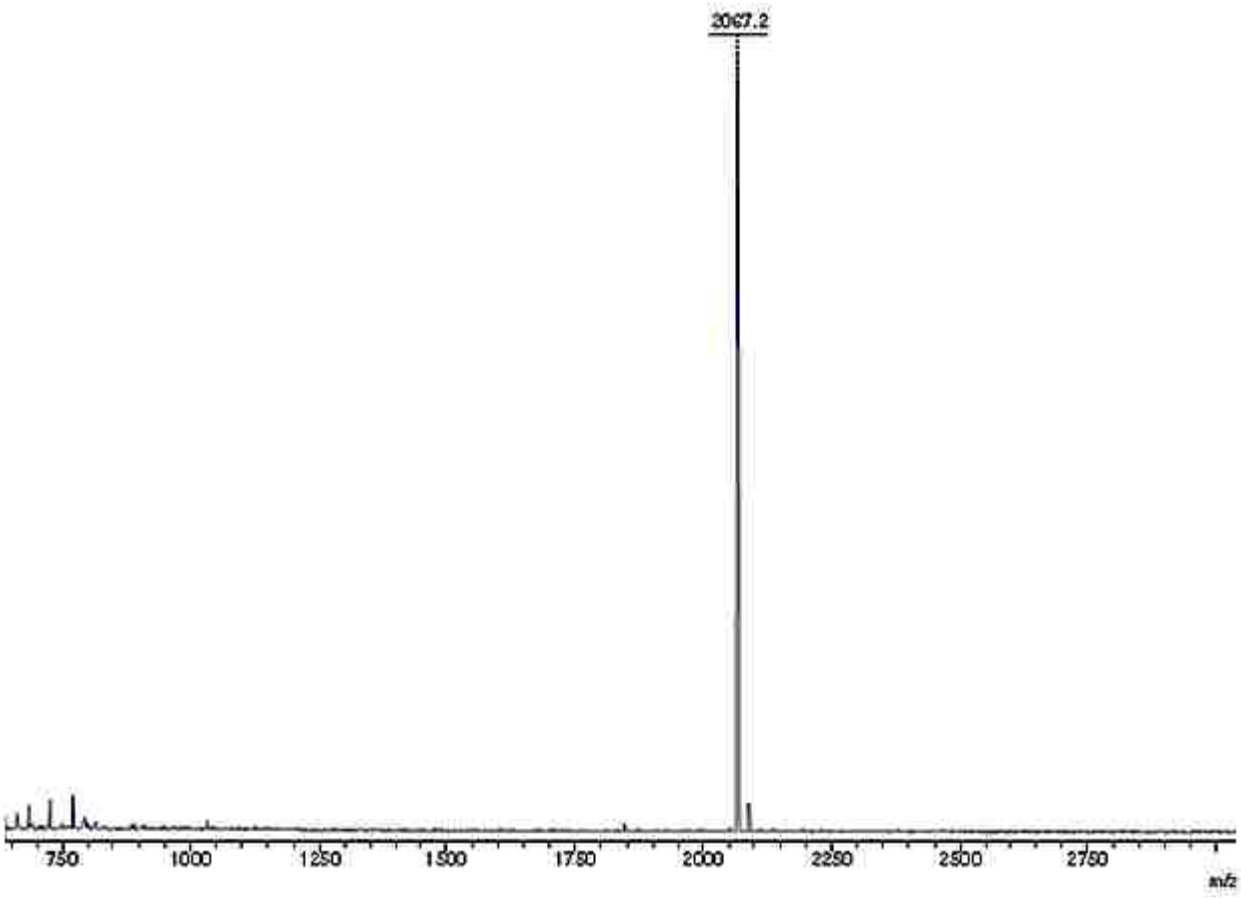

Figure 17: Mass spectrum analysis of the wild-type (W) 16mer peptide. The W peptide was incubated in artificial gastric juice for 60 min at 37^{o}C. The peak shown in Figure 15B was analyzed by mass spectrometry.

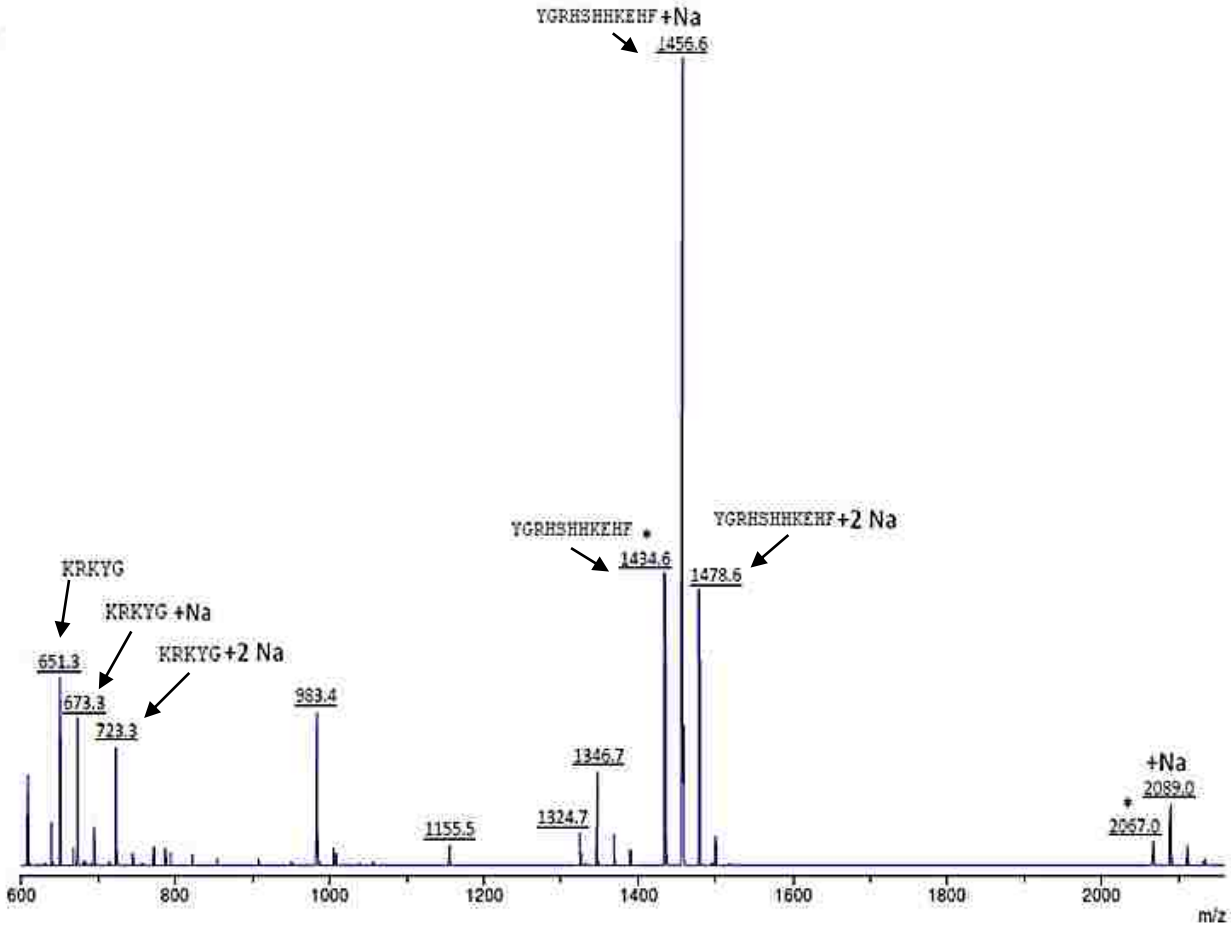

Figure 18: Mass spectrum analysis of the retro (R) 16mer peptide. The R peptide was incubated in artificial gastric juice for 60 min at 37^{o}C. The peak shown in Figure 15A was analyzed by mass spectrometry. The relevant peaks are indicated by the arrows with the appropriate amino acid sequence.

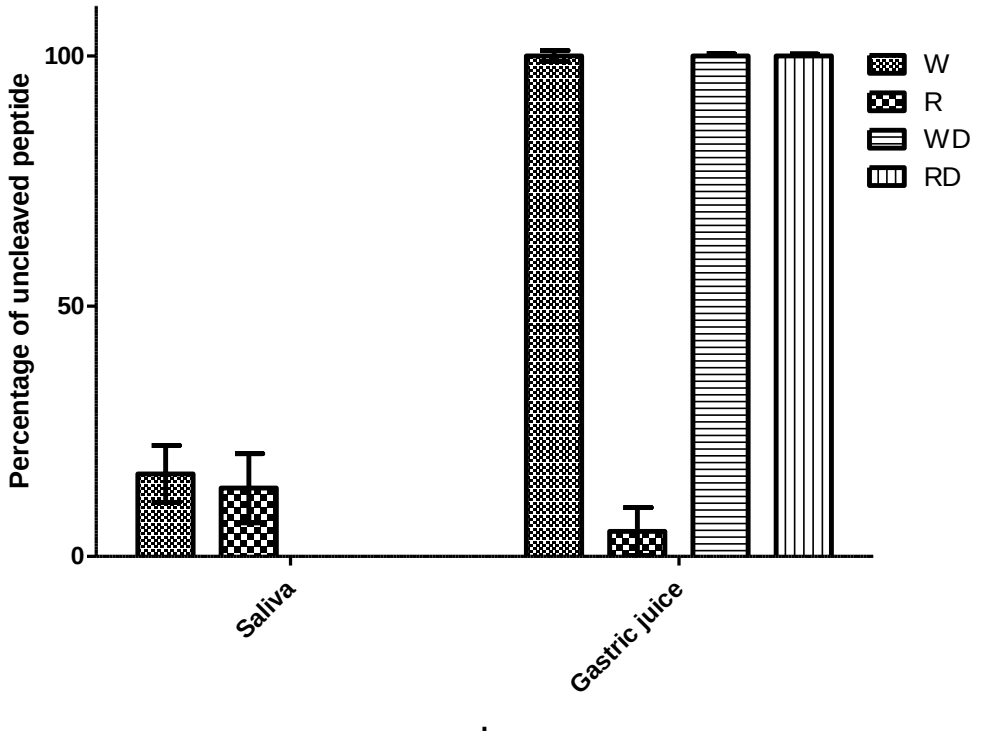

Figure 19: The relative stability of the four histatin 5 peptide derivatives. Following exposure of the peptides to human saliva or artificial gastric juice for 60 min at 37^{o}C, the relative stability of the peptides were evaluated by HPLC and the percent of peptide present a 0 min versus 60 min at exposure to saliva or gastric juice is shown. The error bars represent the standard deviation from three independent experiments.

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CHAPTER 3

Identification of a small domain within histatin 5 essential for fungicidal activity

3.1 Introduction

Candida species are one of the most common opportunistic pathogens in humans, and the fourth leading cause of nosocomial blood infection that lead to a high level of mortality. *Candida albicans* is the major cause of invasive mucosal fungal infections observed in humans. The increased incidence of drug-resistant *Candida* and the high frequency of fungal infections in immune compromised patients emphasize the need for a new class of antifungal drugs with novel mechanisms of action.

Naturally occurring antimicrobial peptides (AMPs) play an important role in the human innate immune response against pathogenic and opportunistic microorganisms. Moreover, some of these peptides exhibit antifungal activities with distinct modes of action [1, 2]. AMPs could represent promising candidates for the treatment of fungal infections, and they may serve as an alternative to chemical therapeutics. These peptides are also advantageous because they exhibit a selective toxicity against the target microorganism and target organisms are less likely to acquire resistance to the peptides due to their common mechanism of action at the plasma membrane. Nevertheless, AMPs cannot enter the therapeutic drug market unless some general application problems are solved, including the susceptibility to enzymatic degradation, pharmacokinetic problems, salt sensitivity, and manufacturing costs.

Human histatins are a family of histidine-rich peptides that are secreted into the saliva by the parotid, submandibular and sublingual glands. They are important members of the innate immune response that is essential for dental and oral microbial defense. Thus, the histatins possess significant antimicrobial activities, and they are considered to be the first line of defense against *Candida* infections of the oral cavity. Histatin 5 has the most potent fungicidal activity

among the histatin family as well as compared to other oral antimicrobial peptides. Histatin 5 structure has been well-characterized, so it is a useful model for designing new antifungal drugs.

Unfortunately, prior studies have failed to identify a region of histatin 5 less than 12 amino acids that maintains antifungal activity. It has been shown previously that the fungicidal activity of histatin 5 does not require the full length peptide as several fragments exhibit similar activity. C-16 (16 amino acid) is an example of an active histatin 5 fragment that retains full activity (Chapter 2). The previous chapter demonstrated that the fungicidal activity of C-16 peptide depends on the distribution of positive charges over an alpha helical structure, as the retro C-16 retained a similar activity. In addition, the essential positive charges seem to be those derived from lysine and arginine residues, but not the histidine residues, as suggested by the lack of sensitivity to pH (Chapter 2).

The studies described in this chapter seek to further delineate the structural requirement for the fungicidal activity of histatin 5. After our previous finding that retro C-16 is as active as the normal peptide (C-16), we identified a small symmetrical sequence within the C-16 peptide that would not appear to be affected by the orientation of the amino acid sequence (retro versus normal), and the sequence of this fragment is -YKRKF- , later referred to as the KM motif. The role of the KM motif in histatin5 antifungal activity was further investigated by comparing with the sequence of known histatin 5 derivatives published over the past decade (see Table 1 and 3 with references therein). The result revealed that the KM motif was found in nearly every peptide fragment that displayed antifungal activity, and any change in the five amino acid KM sequence led to the abolishment or decrease in antifungal activity.

The studies described in this chapter seek to evaluate the importance of the KM motif in antifungal activity. This was accomplished by initially synthesizing and evaluated two peptides, KM-5 and KM-6. Both peptides were pentameric and contained the same amino acid composition as KM; however, KM-5 had a retro sequence (FKRKY) while KM-6 a normal sequence (YKRKF). We then evaluated and compared the activity of these pentamers against the C-16 peptide. To date, the KM motif appears to be the shortest active histatin 5 fragment identified and it will serve as a model for designing a therapeutic peptide.

3.2 Materials and methods

Strains : *Candida albicans* (SC5314)[3], and *Saccharomyces cerevisiae* BY4741(*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 pat1::natMX4*) were used. *S. cerevisiae* strain was a generous gift from Dr. Ines Pinto (University of Arkansas-Fayetteville).

Peptide synthesis. N-Fmoc protected amino acids and Rink resin was purchased from NovaBiochem (San Diego, CA) and Advanced Chemtech (Louisville, KY). All KM peptides were synthesized with an acetylated N-terminus and amidated C-terminus. The peptides were synthesized on a model 433A solid-phase peptide synthesizer (Applied Biosystems; Foster City, CA) using Rink resin and Fmoc-protected amino acids (NovaBiochem). After synthesis, the peptides were deprotected and cleaved from the resin using a high TFA (trifluroacetic acid) cleavage cocktail consisting of 85% TFA, 5% dH2O, 5% triisopropylsilan, and 5% phenol. The resin was mixed in the cocktail solution at room temperature for 3 hours, after which the peptide was precipitated into 50 ml 1:1 v/v methyl-t-butyl ether/hexane per ml of cleavage cocktail. The peptides were subsequently dissolved in 1:1 v/v acetonitrile/ddH2O and recovered by lypholization under high vacuum. Crude peptides were purified on PRP-3 reverse phase column (7 by 305 mm; Bio-Rad, Hercules, USA) on a Hitachi L7100 HPLC instrument using a linear gradient of 0 – 30 % acetonitrile and water (both were contained 0.1% TFA). The purity of each peptide was evaluated by mass spectroscopy .The peptides concentrations were determined by the extinction coefficient.

Fungicidal activity assays. The fungicidal activities to *C. albicans* and *S. cerevisiae* were examined by micro dilution plate assay as described previously[4]. *C. albicans* and S. cerevisiae was grown overnight on agar plates at 30^{o}C. Sabouraud dextrose agar plates were used for *C.*

albicans, and YPD (2% yeast extract, 1% Bacto-peptone and 2% glucose)[5] and YPL (2% yeast extract, 1% Bacto-peptone and 2% lactate)[6] plates for *S. cerevisiae*. Following overnight growth, a single *C. albicans* colony was diluted in 1 ml of 10 mM sodium phosphate buffer at pH 7.4. A hemocytometer was used to quantify the number of cells/ml and the cell concentration was adjusted to 1.8×10^5 cells/ml. Cell suspensions (20 µl) were mixed with 20 µl of peptide dissolved in 10 mM sodium phosphate buffer at pH 7.4 and incubated for 2 h at 37°C with shaking at speeds of 550 rpm. The reactions were stopped by the addition of 360 µl yeast nitrogen base (3.4 g yeast nitrogen base without ammonium sulfate or amino acids and 10 g ammonium sulfate per liter) and 40 µl of cell suspension was spread on the appropriate growth medium plates and incubated for 24 h at 37°C. The number of colony-forming units (CFUs) was counted and each assay was repeated in triplicate. Loss of viability was calculated as [1-(colonies from suspension with peptide/colonies from suspension with no peptide)] × 100.

Effect of pH, low temperature, and sodium chloride on KM-5 activity. The effect of pH on the fungicidal activity of KM-5 peptide was tested at three distinct pH values: 5 (acidic), 7 (neutral) and 9 (basic). Briefly, the fungicidal activity assays were performed using 25µM of each peptide. The pH was adjusted using 2 M HCl or 2 M NaOH. The influence of pH on the peptide net charge was evaluated using Protein Calculator v3.3 serve (http://www.scripps.edu/~cdputnam/protcalc.html)[7]. To study the effect of temperature on the fungicidal activity, fungicidal activity assays were performed at neutral pH at two different temperatures, 4^oC and 37^oC. The effect of sodium chloride on peptide activity was evaluated using the fungicidal activity assays in phosphate buffer (10 mM, pH 7) at 37°C. Different concentrations of sodium chloride were added to the phosphate buffer: 5mM and 10mM. In all assays, C-16 (W) was used as a control for a direct comparison.

Circular dichroism spectroscopy. Circular dichroism spectroscopy measurements were performed on a Jasco-710 spectropolarimeter as described previously with some modifications[8]. The reading was made using a quartz cell of 0.1cm path length and at 25^{o} C. KM-5 was measured in water and in the presence of 50% TFE (v/v), and all samples had a 120 µM final concentration. The spectra were recorded every 0.2 nm between the absorbance range of 190 to 250 nm with a 1.0 nm bandwidth and a scan speed of 20 nm/min. Six scans were performed and averaged. The background was subtracted from all spectra, and curve smoothing applied. The CD spectra are reported as the mean residue ellipticity ([Θ]) in degrees.cm^{2}.dmol^{-1}. CD data were further analyzed using the web-based K2D3 program (http://www.ogic.ca/projects/k2d2/).

3.3 Results

Analysis of histatin 5 and derivative peptides. After the studies described in Chapter 2, namely that the retro C-16 showed activity similar to the normal peptide (C-16), we examined the sequence of both versions of the peptide and we identified a small symmetrical sequence within the peptides that would not appear to be dramatically affected by the orientation of the amino acid sequence (retro versus normal), and the sequence of this region was -YKRKF- , later referred to as the KM motif. Our hypothesis was that this small five amino acid peptide could be responsible for a significant portion of the antifungal activity. This was supported by the observation that the antifungal activity of histatin 5 was not dramatically influenced by pH, suggesting the abundance of histidine residues within histatin 5 were not relevant for fungal killing activity.

To further support the proposed hypothesis, the published sequence of various histatin 5 derivatives that were generated over the past several years were examined. The comparison was divided into two categories: peptides derived directly from histatin 5 (Table 1) and histatin 5 substituted analogs (Table 2). Since the published data for each peptide was reported from different laboratories in which the threshold of peptide concentration and LD_{50} was unique to each laboratory, the activity of each peptide was correlated to the maximum concentration used in that particular study. Any peptide exhibiting an activity at a concentration lower than the threshold concentration was considered active, while all other peptides that did not show any activity at the maximum concentration were classified inactive.

In the first step of the comparative analysis, histatin 5 peptide derivatives were compared to each other and to the retro C-16 peptide, R described in Chapter 2 (Table 1). The result of the first

comparison suggested that the sequence YKRKF (later named as KM) is found in all active fragments, and an incomplete KM sequence caused significant reduction in the peptide activity.

The next step in the comparison was to examine histatin 5 derived peptides that contained amino acid substitutions, and the activity correlated to the presence of KM motif. This comparison demonstrated that a single amino acid substitution within the KM motif was sufficient to reduce or totally abolish the antifungal activity of the peptide, further emphasizing the significance of KM motif in antifungal activity (Table 2). Nevertheless, there were some exceptions identified in which peptides were either not active, despite the presence of KM motif, or the existence of KM motif didn't promote 50% killing activity (Table 3).

Fungicidal activity of the KM motif. Given the comparative sequence data described above, we hypothesized that the fragment YKRKF or the KM motif may be solely responsible for the antifungal activity, and the major functional domain within histatin 5. To evaluate this hypothesis, two peptides were synthesized KM-5 (FKRKY) and KM-6 (YKRKF), and the antifungal activity of the peptides was evaluated. Both peptides were pentamers containing the KM motif, but KM-5 had the retro sequence. In addition, both peptides were modified by N-terminal acetylation and C-terminal amidation to improve the stability. The fungicidal activity of both peptides was evaluated with *C. albicans* using 25μM peptide and correlated to the activity of the C-16 histatin 5 peptide derivative. As Figure 1 illustrates, both KM-5 and KM-6 possessed equivalent antifungal activity as 25 μM of peptide killed approximately 80% of the cells; however, both peptides were less potent than the C-16 that killed 95% of the fungal cells at the same concentration.

Since KM-5 and KM-6 had identical results in the fungicidal activity assays, we proceeded in studying the activity of only one of them: KM-5. Dose-dependent and killing kinetic experiments were performed to determine the LD_{50} and the kinetics of antifungal activity by KM-5. The LD_{50} was found to be 5.2 μM as determined from a dose response curve (Figure 2). The results of the kinetic studies are shown in Figure 3. The fungicidal activity against *C.albicans* was time-dependent, reaching the maximum killing activity in two hours, similar to the C-16 histatin peptide (see Chapter 2).

Fungicidal Activity of KM-5 substitution analogs. Since the relevant sequence of histatin 5 was delimited to five amino acids, it was important to next determine the contribution of the various amino acids to the antifungal activity of the peptide. Thus, several amino acid substitutions within KM-5 were evaluated for fungicidal activity to optimize the sequence for the maximum antifungal activity (Table 4). The fungicidal activity of these analogs was tested with *C.albicans* using a standard 25 μM concentration of each peptide. In general, increasing the positive charge in cationic antimicrobial peptides (AMPs) enhances the activity, especially if the positive charges are derived from arginine or lysine residues. Besides the net charge, the secondary structure of AMPs plays an important role by inducing the amphipathic character essential for antifungal activity.

Role of positively charged amino acids as well as secondary structure on the antifungal activity was assessed using two different KM-5 analogs, KM-7 and KM-8, where the central arginine was replaced by either serine or proline, respectively. In KM-7, this alteration should only change the charge and preserve the symmetry, size and amphipathic character of the peptide. On the other hand, the proline-substituted peptide (KM-8) was synthesized by introducing a nonpolar alpha-helix breaker. In KM-8, proline will interfere with any peptide secondary

structure as well as the amphipathic character. For both peptides, KM-7 and KM-8, the fungicidal activity was abolished (Figure 4).

To evaluate the role of the tyrosine in the antifungal activity as well as to increase the symmetry within KM-5, KM-9 was generated, in which tyrosine was substituted with phenylalanine. As illustrated in Figure 4, KM-9 exhibited a slight decrease in the activity. The same results were observed using KM-10, an analog in which the amino acids were randomly shuffled within the peptide (Figure 4). To further compare the antifungal activity of KM-5, KM-9, and KM-10 a dose-dependent assay was performed to distinguish slight differences between these three peptides. As shown in Figure 5, the activity of the three peptides similar at multiple concentrations; however, the antifungal activity of KM-5 was consistently stronger.

Finally, the role of stereospecificity was evaluated by generating KM-5-D, in which the peptide was synthesized using D-enantiomers of the amino acids. The fungicidal activity assays demonstrated that using D-enantiomers did not affect the antifungal activity as both KM-5 and KM-5-D possessed the same activity (Figure 4).

Effect of pH on KM-5 fungicidal activity. Many AMPs display pH-dependent antimicrobial activity via interfering with the target cell or with the peptides itself. To evaluate the pH-dependency of KM-5, the fungicidal activity was examined at pH values of 5, 7, and 9. For comparison, the C-16 histatin peptide was analyzed in parallel. The results showed that the antifungal activity of C-16 did not change across the range of pH levels as shown in Chapter 2; however, KM-5 demonstrated slightly greater activity under neutral or basic pH (Figure 6). At acidic pH, the activity of KM-5 was inhibited by approximately 30% compared to neutral and basic conditions than at acidic pH. As shown in Figure 6B, changes in the pH from acidic to

basic had a large effect on C-16 net charge, from +12 to +4 when the pH changed from 5 to 9, respectively. However, under the same circumstances the net charge of KM-5 was slightly reduced from +3 to +2.

Effect of temperature and salt on KM-5 fungicidal activity. It has been reported that the activity of antimicrobial peptides in general and histatin 5 specifically are often weaker in a presence of NaCl as well as at low temperature. In the studies shown in Chapter 2, it was demonstrated that the activity of C-16 is affected by NaCl and low temperature. Since KM-5 is a derivative of histatin 5, its antifungal activity was evaluated under the same conditions. To study the role of temperature, the antifungal activity was measured for KM-5 and C-16 at 4^{o}C and 37^{o}C. As Figure 7A shows, the activity of both peptides was inhibited dramatically at 4^{o}C.

The salt effect was measured by incubating KM-5 and C-16 at 5 mM and 10 mM NaCl (Figure 7B). As was expected, the antifungal activity of KM-5 and C-16 were attenuated by the addition of NaCl in a dose dependent manner. While 5 mM NaCl was sufficient to inhibit the activity of KM-5 by 50%, the activity of C-16 was not affected. However, the inhibitory effect of NaCl on both peptides was obvious at a concentration of 10 mM NaCl.

Role of respiration in the fungicidal activity of KM-5. It is known that the activity of histatin 5 is affected by the metabolic activity of *Candida* and the activity was completely inhibited in a presence of inhibitors of respiratory metabolism such as sodium azide, as shown in Chapter 2. Furthermore, several studies have shown that the yeast *Saccharomyces cerevisiae*, which produces ATP primarily by fermentation, is resistant to the antifungal activity of histatin 5[10].

To evaluate the role of respiratory metabolism in the fungicidal activity of KM-5, *S. cerevisiae* and *C. albicans* were grown in rich medium containing glucose; whereby, *S. cerevisiae* would produce ATP primarily by fermentation while *C. albicans* would use respiration. Thus, the prediction would be that *S. cerevisiae* would be more resistant to KM-5 antifungal activity than *C. albicans*. As shown in Figure 8A, *S. cerevisiae* was resistant to killing by KM-5 at 25 μM concentration; whereas, *C. albicans* was sensitive at the same concentration.

To further explore the role of respiratory metabolism in the KM-5 killing mechanism, *S. cerevisiae* was grown on rich medium containing either glucose to induce fermentation or lactate to induce respiratory metabolism. As shown in Figure 8B, when *S. cerevisiae* cells are grown under conditions that induce respiratory metabolism (lactate as a carbon source), they become more sensitive to the fungicidal activity of KM-5, suggesting respiratory metabolism is important for the killing activity.

Analysis of the secondary structure of KM-5 by circular dichroism spectroscopy. To study the possible secondary structure of KM-5, CD spectroscopy experiments were conducted. The data were acquired for KM-5 in aqueous solution as well as in the presence of 50% TFE. As illustrated in Figure 9, KM-5 did not obtain a defined secondary structure, but maintain a random coil structure in both water and 50% TFE. However, the possibility of beta-turn structure was observed. The deconvolution of the spectrum using K2D3 program [13] yielded a similar percentage of helical and beta structure in both environments (in water: 1 % helix and 26% beta while in TFE: 1% helix and 24% beta).

3.4 Discussion

The current therapeutic agents have been effective in treating fungal infection for a long period of time; however, the increased use of these agents has been associated with the development of new resistant strains. The increase of resistant fungal pathogens underscores the search for new antifungal agents with different mechanism of action. AMPs have been used widely as a base for the development of new clinically approved antibacterial and antifungal drugs [14,15, 16]. Histatin 5, a salivary antimicrobial peptide , may serve as a good model for new therapeutic peptides [2, 17-20]. While the histatin 5 mechanism of action has not been fully explained, all suggested modes of actions would imply histatin 5 has a distinct target from the currently available antifungal agents [2] on the commercial market.

One of the general problems limiting the use of therapeutic peptides as drugs is the cost effectiveness. In order to utilize a peptide as a commercial drug it must be cost effective to produce. Several attempts were carried out to optimize the histatin 5 peptide and attain the smallest active fragment. To date, P-113 is the most effective and smallest fragment of histatin 5, and it is composed of 12 amino acids. However, using histatin 5 peptide derivatives coupled with our prior studies (Chapter 2), we have identified a shorter antifungal peptide, referred to as KM-5; it is less than half the size of P-113.

Previously, we reported that the retro C-16 peptide, retained fungicidal activity equivalent to the as non-retro peptide. Following this finding, a direct comparison between the retro and non-retro peptide showed the existence of a short symmetrical sequence. This fragment contains five amino acids with a sequence of YKRKF, later named as the KM motif, where arginine is the dyad of symmetry between two lysine residues and two aromatic amino acids. We hypothesized

that this sequence might be the active motif of C-16 as well as histatin 5, yet this sequence would remain the same in both the retro and non-retro peptide.

Before we proceed with testing this hypothesis, a sequence comparison of several histatin 5 peptide derivatives and analogs was carried out, focusing on the presence of the KM motif and how its presence correlated fungicidal activity. It was found that the KM motif was present in the majority of peptides that maintained antifungal activity. Given these observations, the putative fungicidal activity of this small peptide was tested using KM-5 and KM-6, and the activity of both peptides was compared to C-16. The fungicidal activity assays performed with *Candida albicans* demonstrated that both KM-5 and KM-6 have an antifungal activity and they are equivalent, and this finding endorses the hypothesis of symmetry. On a molar basis, both KM-5 and KM-6 was found to be less active than the C-16 peptide. However, the fungicidal activity of KM-5 against *Candida albicans* in mg/mL is equal to if not better than C-16.

In order to optimize KM-5 activity, several KM-5 analogs were synthesized and their fungicidal activity against *C. albicans* was measured. The results showed that KM-5 is the optimal sequence and any interference with the positive charge, secondary structure or the amino acid sequence reduces the antifungal activity. The single arginine residue in KM-5 is equivalent to Arg-22 in histatin 5. There are other studies that have shown the importance of this residue, as a substitution leads to a decrease in the activity. It is not surprising that the removal of a single positive charge from a five amino acid peptide would have a dramatic effect on activity due to the change in the net charge of the peptide. In general, positive charges in AMPs have an essential role in membrane binding because of the ionic interaction between the cationic peptide and the negatively charged membrane. Introducing a helix breaker in KM-5 abolished the activity completely; however, this change might also be contributed to the change in the peptide

net charge. In addition, generating an analog of KM-5 that was symmetrical, via replacing tyrosine with phenylalanine, had a slightly inhibitory effect on fungicidal activity. This could result from the lack of hydrogen-bonding with phenylalanine. Using D-enantiomer of KM-5 did not affect the antifungal activity, eliminating the role of stereospecficity in KM-5 activity. Similar results were reported in several antimicrobial peptides including full length histatin 5 and C-16 peptide (see Chapter 2).

The secondary structure of KM-5 was evaluated by circular dichroism spectroscopy. It was found that KM-5 in aqueous solution was largely a random coil structure with a possibility to form a beta-turn. Unexpectedly, the presence of 50% TFE did not induce a change in the structure as it remained a random coil. Similar cases of small AMPs (even tetramer) adopting a beta-turn have been reported [26, 28-32]. Besides, TFE has been reported to induce less effect on short peptides that adapted a beta-turn structure. For example, increasing the percentage of TFE up to 90% showed a small effect on Prp peptides. On the contrary, increasing TFE percentage resulted in an increase in the percentage of beta-turn [33].

The study of secondary structure suggests that KM-5 has significantly less α-helical character than histatin 5 and C-16 histatin. This reduction in helical content of KM-5 could be due to the fact that KM-5 is too small to adapt a thermodynamically stable helical structure as the potential number of hydrogen bonds along the backbone for KM-5 is reduced to five. The same pattern of secondary structure was also observed in P-113 peptide, a dodecameric derivative of histatin 5.

As previously shown, NaCl and low temperature have an inhibitory effect on C-16 histatin as well as on full length histatin 5[34]. The antifungal activity of KM-5 was also found to be salt and temperature dependent. With some exceptions, the killing activities of cationic AMPs are

sensitive to the ionic strength of the surrounding medium [34, 35, 36, 37]. As the nature of interaction between cationic AMPs and the negatively charged plasma membrane is ionic, it can be weakened by monovalent and divalent cations, consequently the fungicidal activity will be reduced [38]. As Figure 7 illustrates, the activity of KM-5 follows the same trend at C-16 as both were inhibited by NaCl; however, the NaCl inhibitory effect was more pronounced in KM-5. This might be related to the fact that C-16 has more basic amino acids than in KM-5, hence the interaction between C-16 and the membrane is stronger, and a higher concentration of the salt is required to interrupt this interaction.

Besides salt and temperature dependence, the antimicrobial activity of most cationic AMPs is pH dependent [35]. Changing the pH typically has an effect on AMPs activity through two different mechanisms, either interfering with the target microorganism or directly with the peptide. *Candida albicans* has the ability to grow either as unicellular budding yeast or filamentous, as in pseudohyphal and hyphal forms[41]. This ability, which plays an important role in *Candida* pathogenicity [42] can be stimulated by pH [43]. Moreover, the effect of AMPs on each *Candida* morphological form is not equivalent[42]. The pH can also directly influence the antimicrobial peptide as several studies have shown the secondary structure and the net charge of AMP are to change at different pH [44-46].

Unlike other AMPs, the antifungal activity of histatin 5 and the C-16 peptide against *C. albicans* was similar over a wide range of pH (from 5 to 9) (see Chapter 2). On the contrary, the activity of KM-5 was observed to change slightly by a pH shifting from acidic to basic as the activity decreased in acidic medium. The net charge of KM-5, within the tested range, would not change since all positive charges in KM-5 come from lysine and arginine residues. It is unlikely that the pH made a significant change on the secondary structure because KM-5 adopted a partial

secondary structure and has largely remained a random coil. We tested the possibility that KM-5 might be losing the C-terminal amide under the influence of acidic or basic medium, but the mass spectrometry results did not detect any changes (data not shown). Similar observations have been reported for several other AMPs[34, 47, 48].

The mode of action of histatin 5 is unclear as numerous studies have suggested the involvement of multiple pathways. However, it has been demonstrated that cellular respiration and mitochondrial ATP synthesis are necessary for the antifungal activity of histatin 5. The role of respiration was clearly observed in *Saccharomyces* as it is resistant to killing by histatin 5. There is an important metabolic difference between *Saccharomyces* and *Candida*. *S. cerevisiae* is classified as a Crabtree-positive yeast; when it is grown on a fermentable medium (glucose) under aerobic conditions, the sugar is largely fermented to ethanol. On the other hand, *C. albicans* is a Crabtree-negative yeast; when grown under the same conditions the cells respire, even when the carbon source is fermentable. This difference could explain the observed susceptibility of *Candida albicans* to KM-5 and C-16. Fungicidal activity of both peptides requires the presence of active mitochondria[49]. To evaluate the effect of respiration on KM-5 activity, the activity was measured on *S. cerevisiae* in non-fermentable medium (YPL) and compared to the fermentable medium YPD. The results showed that KM-5 has more effective antifungal activity in YPL over YPD. These data strongly suggest that oxidative phosphorylation is required for KM-5-induced cell death.

Although small peptides (5 or 6 amino acids) are traditionally not considered long enough to form a distinctive secondary structure or pass through the membrane of microorganisms, several small antifungal[22-24], antibacterial[25, 26]and antiviral [27]peptides have been reported including a

currently available antifungal drug class, the echinocandins. The KM5 peptide represents an excellent lead peptide for the future development of an effective antifungal therapeutic peptide.

3.5 Conclusion

KM-5 is the shortest active fragment in histatin 5 and our results show that it might work through the same mechanism as histatin 5. The activity of KM-5 was inhibited in the presence of histatin 5 inhibitors: low temperature and sodium chloride. Moreover, KM-5 activity requires respiratory metabolism to be active in killing fungi. Although KM-5 is less active than the C-16 peptide, it offers a model target peptide to be further developed as an antifungal therapeutic. The smaller size makes it attractive for development because of the lower cost of production.

Table 1: The sequence and activity of histatin 5 peptide derivatives

Name	Sequence	Activity[1]	Reference
Histatin 5	DSHAKRHHG**YKRKF**HEKHHSHRGY	Yes	*50*
Fragment 1	DSHAKRHHG**YKRK**	No	*51*
N-16	DSHAKRHHG**YKRKF**HE	Yes	*19, 50*
P-118	AKRHHG**YKRKF**	Yes	*2*
P-119	AKRHHG**YKRK**	No	*2*
P-113	AKRHHG**YKRKF**H	Yes	*2*
P-103	KRHHG**YKRKF**HEKHHSHR	Yes	*2*
P-117	KRHHG**YKRKF**H	Yes	*2*
Fragment 2	HG**YKRK**	No	*51*
C-16	G**YKRKF**HEKHHSHRGY	Yes	*50*
Dh5	**KRKF**HEKHHSHRGY	Yes	*50, 52*
H10-1	**KRKF**HEKHHS	Yes	*21*
Histatin 9	**RKF**HEKHHSHRGYR	No	*2*
C-12	**KF**HEKHHSHRGY	No	*2, 50*
Fragment 3	**F**HEKHHSHR	No	*51*
C-10	HEKHHSHRGY	No	*50*
Retro C-16 (R)	YGRHSHHKEH**FKRKY**G	Yes	Our study

Table 2: The histatin 5 substitution derivatives.

Name	Sequence	Activity	Reference
m68	DSHAKRHHG**YKRE**F**H**EKHHSH**G**GY	No	*53*
reHsn-5	**G**SHAKRHHG**YKRKF**HEKHHSHRGY	Yes	*19*
reHsn-5- K13T	**G**SHAKRHHG**YKRTF**HEKHHSHRGY	Yes	*19*
reHsn-5- K13T/R22G	**G**SHAKRHHG**YKRE**F**H**EKHHSH**G**GY	Yes	*19*
reHsn-5- K13E	**G**SHAKRHHG**YKRE**FHEKHHSHRGY	Yes	*19*
reHsn-5-F14A/H15A	DSHAKRHHG**YKRKAA**EKHHSHRGY	No	*19*
3P	DSHAKRHHG**YKRKF**H**P**KH**P**S**P**RGY	Yes	*54*
P-113-Q2.10	A**Q**RHHG**YKRQF**H	No	*2*
P-113-Q2.3.9.10	A**QQ**HHG**YKQQF**H	No	*2*
P-113-Q3.9	AK**Q**HHG**YKQKF**H	Yes	*2*

Table 3: Inactive histatin 5 substitution derivatives containing the KM motif.

Name	Sequence	Activity	Reference
P-114	HHG**YKRKF**HEKH	No	*2*
P-115	**YKRKF**HEKHHSH	No	*2*
P-123	DSHAKRHHG**YKRKF**	No	*55*
M10	HHG**YKRKF**HE	No	*19, 21*
Fragment 4	HG**YKRKF**HEK	No	*51*
1P	DSHAKRHHG**YKRKF**HEKHHS**P**RGY	Yes	*54*
2P	DSHAKRHHG**YKRKF**HEKH**P**S**P**RGY	Yes	*54*

Table 4: KM peptides containing amino acid substitutions.

Name	Sequence	Activity[1]
KM-5	Ac-FKRKY-NH2	Yes
KM-5-D	Ac-fkrky-NH2	Yes
KM-6	Ac-YKRKF-NH2	Yes
KM-7	Ac-FK**S**KY-NH2	No
KM-8	Ac-FK**P**KY-NH2	No
KM-9	Ac-FKRK**F**-NH2	Yes
KM-10	Ac-KFRYK-NH2	Yes

1 Fungicidal activity summarized based on data in Figure 5.

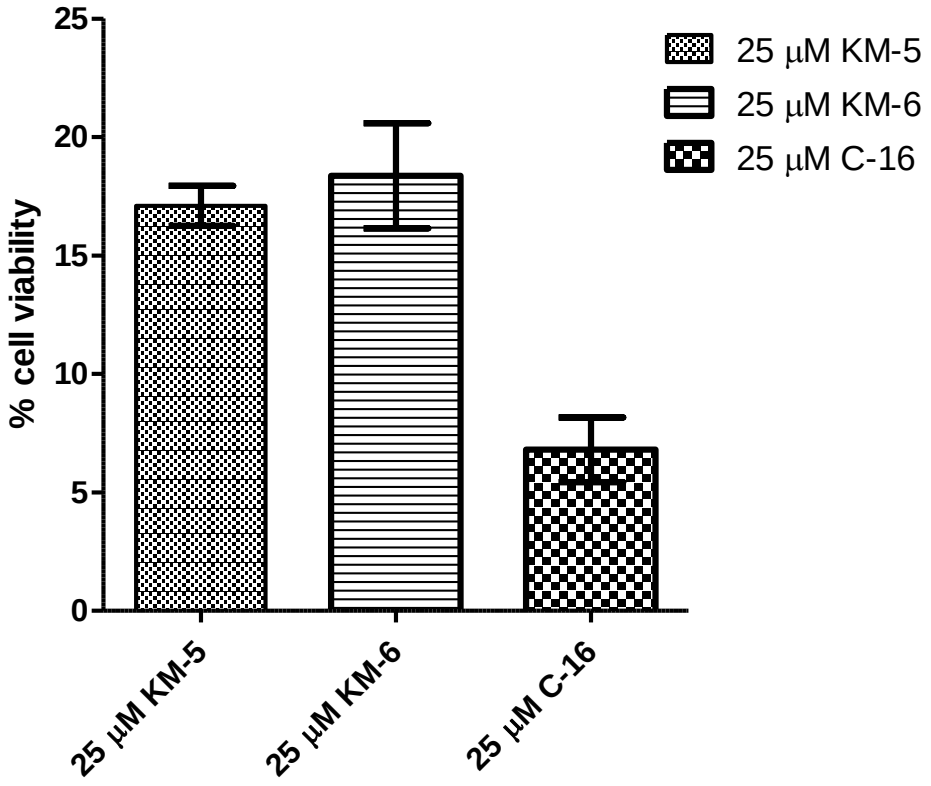

Figure 1: Comparison of the fungicidal activity of KM-5, KM-6 and histatin C-16.

Fungicidal activity assays were performed using 25 μM of each peptide incubated with *C. albicans* (1.8×10^5 cells/ml) in 10 mM sodium phosphate buffer for 2 hours at 37°C. The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. The data represents the mean of three independent experiments with the error bars representing the standard deviation.

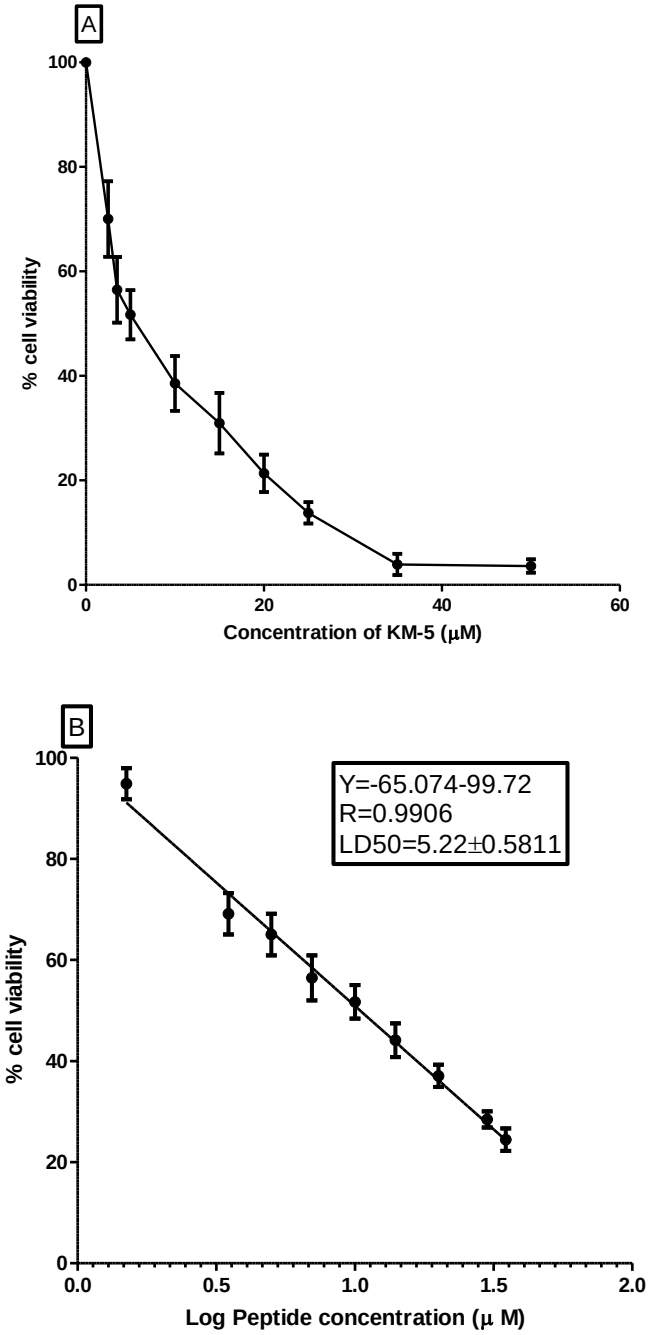

Figure 2: Determination of the LD_{50} for KM-5. A) A dose-dependent assay was performed in which *C. albicans* cells were exposed to varying concentrations of KM5 (2.5, 3.5, 5, 10, 15, 20, 25, 35 and 50 μM) for 2 hours at 37^{o}C. B) The LD_{50} of the peptide was calculated using a linear regression equation. The data represents the mean of three independent experiments with the error bars representing the standard deviation.

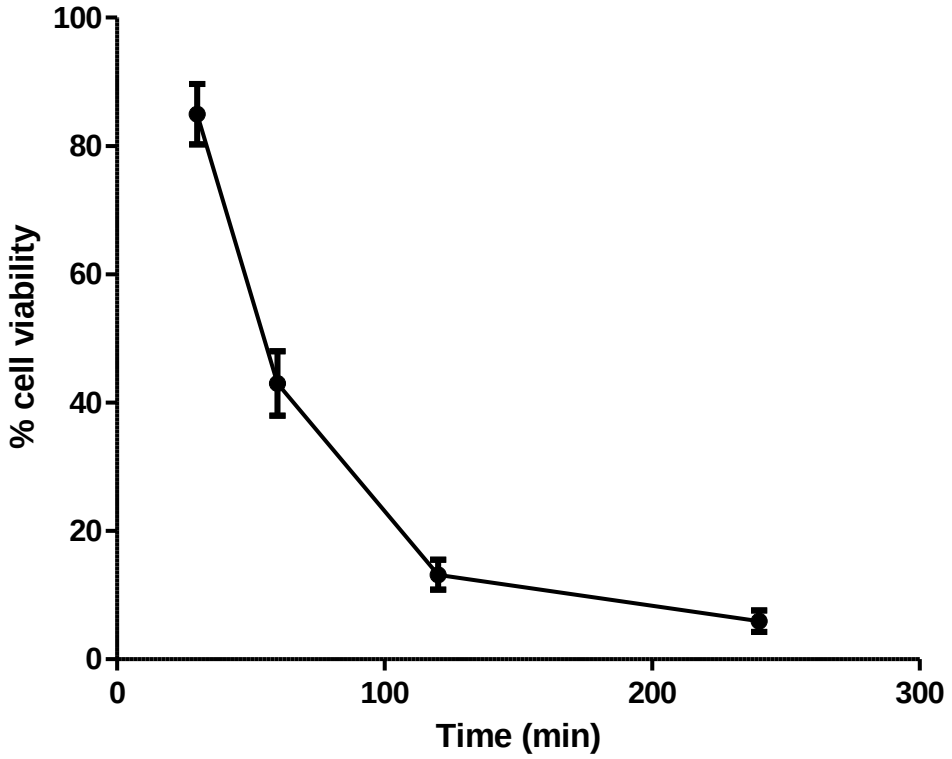

Figure 3: The kinetics of KM-5 fungicidal activity. The kinetics of KM-5 fungicidal activity was determined using 10 μM KM-5. KM-5 was incubated with *C. albicans* (1.8×10^5 cells/ml) in 10 mM sodium phosphate buffer for different time periods (30, 60 ,120 and 240 min). The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. The data represents the mean of three independent experiments with the error bar representing the standard deviation.

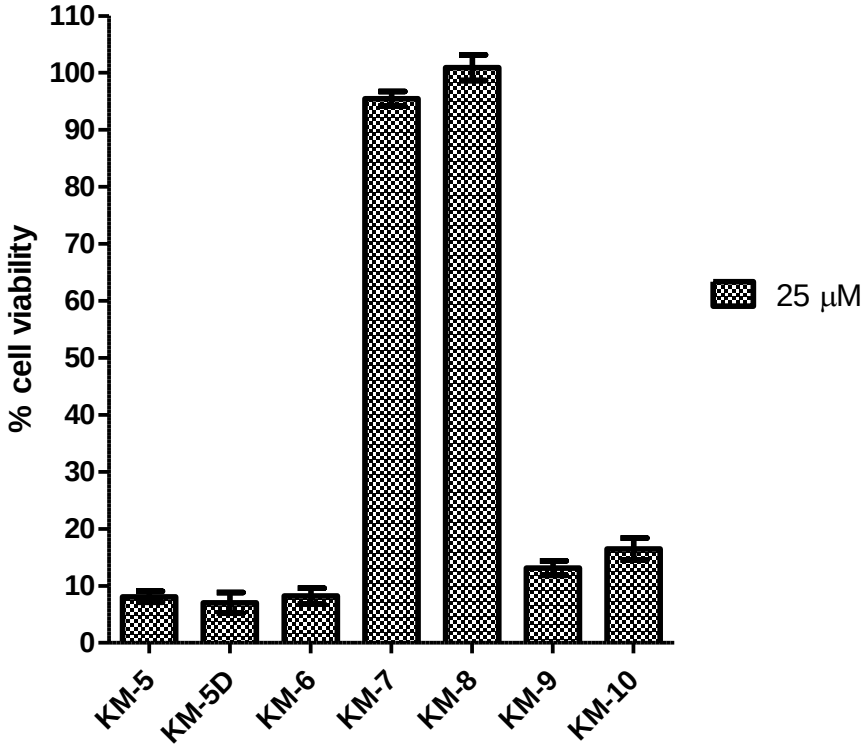

Figure 4: Fungicidal activity of KM-5 substitution analogs. The fungicidal activity of the KM-5 peptide analogs described in Table 4. For each peptide (25μM) was incubated with *C. albicans* (1.8×10^5 cells/ml) in 10 mM sodium phosphate buffer for 2 hours at 37°C. The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. The data represents the mean of three independent experiments with the error bar indicating the standard deviation.

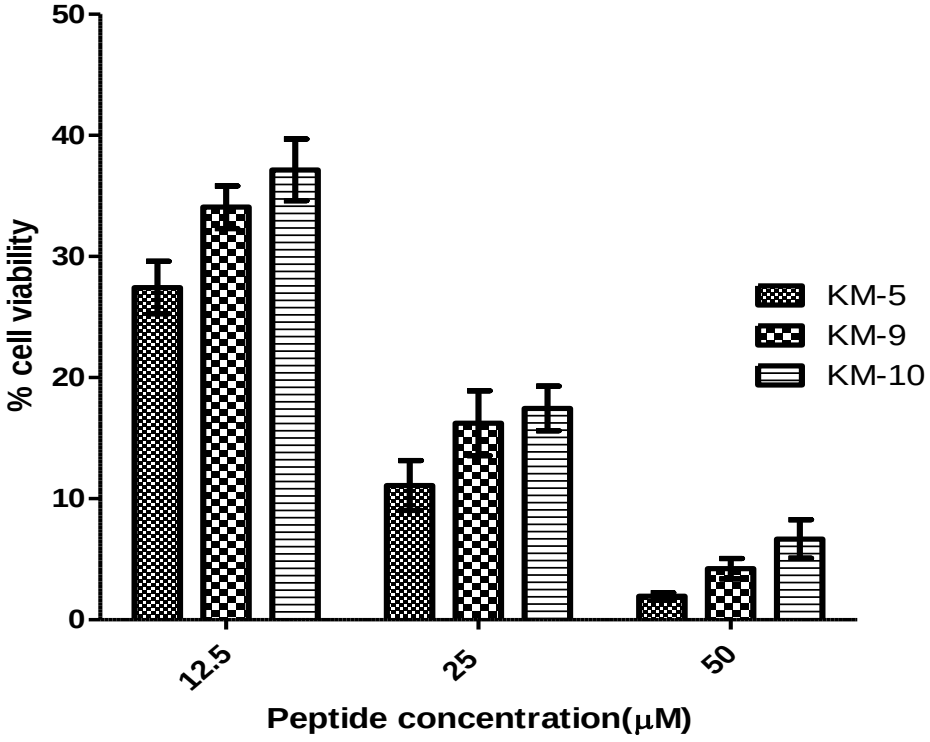

Figure 5: Dose-dependent comparison of the fungicidal activity of KM-5, KM-9 and KM-10. The indicated concentrations of each peptide were incubated with *C. albicans* (1.8×10^5 cells/ml) in 10 mM sodium phosphate buffer for 2 hours at 37^oC. The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. The data represents the mean of three independent experiments with the error bars representing the standard deviation.

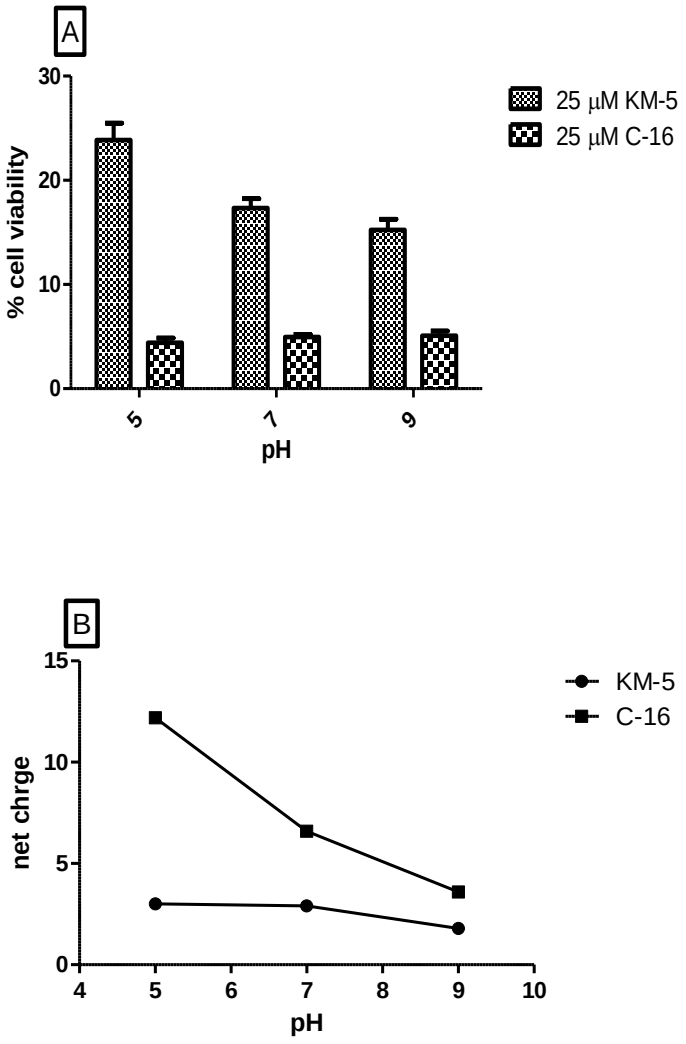

Figure 6: Effect of pH on fungicidal activity of KM-5. (Panel A) 25 μM of the indicated peptides were incubated with *Candida albicans* (1.8×10^5 cells/ml) for 2 hours in 10 mM sodium phosphate buffer adjusted to the indicated pH (5 , 7 and 9). The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. The data represents three independent experiments and the error bar representing the standard deviation. (Panel B) The calculated net charge of each peptide at different pH values as determined by the Protein Calculator v3.3 software.

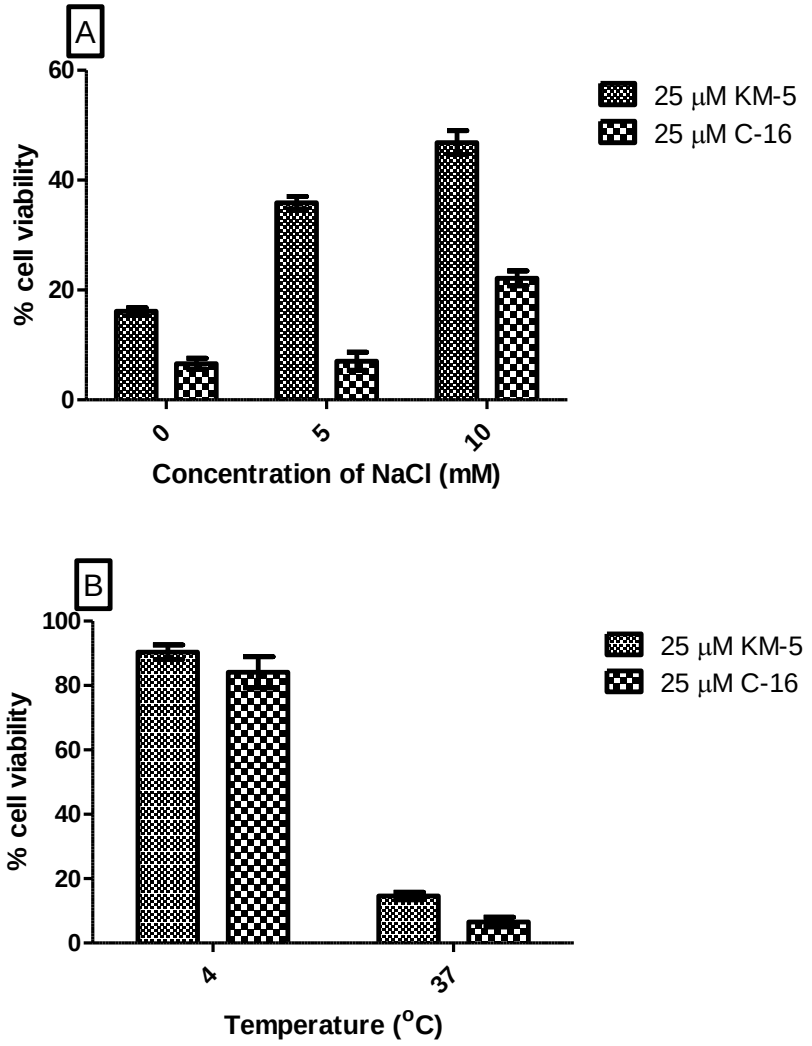

Figure 7: The effect of sodium chloride and low temperature on the fungicidal activity of KM-5. For each assay, 25 μM of peptide was incubated with *C. albicans* (1.8×10^5 cells/ml) in 10 mM sodium phosphate buffer for 2 hours at 37°C. (Panel A) Fungicidal assay performed in the presence of the indicated concentrations of sodium chloride. (B) Fungicidal assay performed at either 4°C or 37°C. The percentage of viable cells was calculated as (viable colonies in the presence of peptide /viable colonies without peptide) × 100. The data represents the mean of three independent experiments and the error bars indicate the standard deviation.

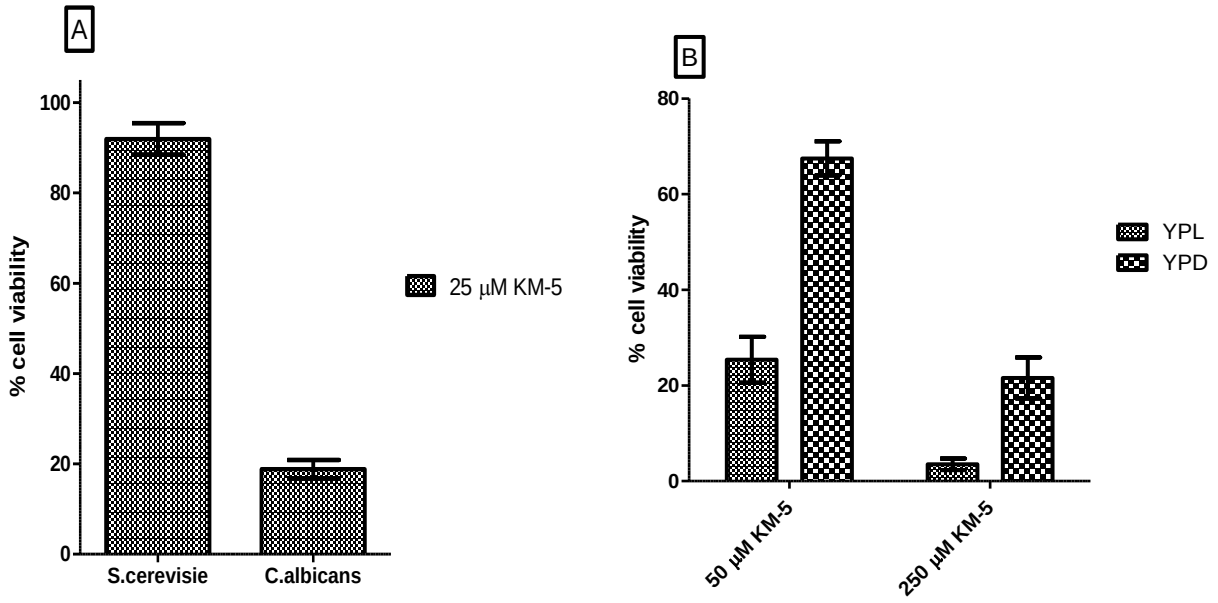

Figure 8: The role of respiratory metabolism in the fungicidal activity of KM-5. A) Comparison of the fungicidal activity of 25 μM KM-5 on *S. cerevisiae* versus *C. albicans*. B) Comparison of the fungicidal activity of KM-5 on *S. cerevisiae* grown in the presence of glucose (YPD) or lactate (YPL) as the carbon source. The activity was measured in 10 mM sodium phosphate buffer for 2 hours at 37^{o}C. The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. The data represents the mean of three independent experiments with the error bars indicating the standard deviation.

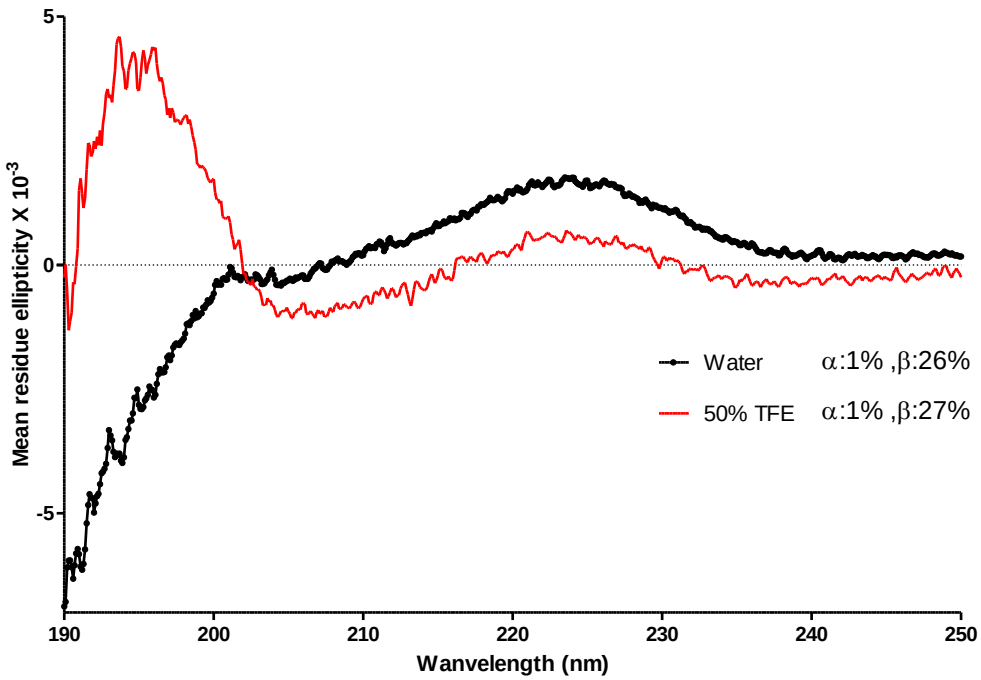

Figure 9: CD spectra of KM-5. The CD spectra were determined in aqueous solution using 120 µM KM-5, and in the presence of 50% TFE at 25°C as indicated. The samples were prepared as described in Materials and Methods. The CD spectra are displayed in mean residue ellipticity [θ]. The percentage of secondary structure is indicated in the graph legend in the inset.

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CHAPTER 4

The development and characterization of KM-12 as a potent fungicidal peptide.

4.1 Introduction

Although *Candida* species are primarily commensal microorganisms in the gastrointestinal, urinary, and vaginal tracts of healthy individuals [1,2], they are also opportunistic pathogens that can be associated with high mortality especially in immune compromised patients [3]. In fact, *Candida* species are the most common fungal pathogens that cause disease in humans [1]. Recently, infections by *Candida spp.* have increased as drug resistant strains have emerged. The drug resistance mainly developed from the overuse of antifungal drugs, as well as cross-resistance between *Candida spp.*[4]. Moreover, some *Candida spp.* exhibit inherent resistance to the available fungal drugs. For example, *Candida glabrata* is resistant to fluconazole at therapeutic concentrations and *Candida lusitaniae* shows resistance to amphotericin B[5]. Due to the toxicity issues associated some antifungal agents, such as amphotericin B: the triazoles are considered the drug of choice for treating *Candida* infections[6]. Unfortunately, the widespread use of the azoles has resulted in an increase in azole-resistance[7]. These circumstances have led to a renewed interest in the development novel antifungal drugs with different mechanisms of action.

Histatin 5 is a cationic antimicrobial peptide (AMP) produced in human saliva and it is the first line of defense against oral fungal infection. Histatin 5 possesses the most potent antifungal activity among the oral antimicrobial peptides and it has been a potential candidate for drug therapy or as a template for antifungal drug design[8]. Previously, we identified the KM motif (Chapter 3), as a possible functional motif in histatin 5 and its antifungal activity has been examined on *C. albicans*. In this chapter, we describe the further development of a potent antifungal peptide utilizing the KM motif. The peptide, termed KM-12 is a dimeric peptide composed of two KM motifs, with a cysteine residue introduced to facilitate dimerization of the

monomeric KM motif. It was hypothesized that the antifungal activity displayed by the KM motif may be improved additively by using two copies of the motif. To our surprise, the dimerization of two KM motifs caused a synergistic effect, with fungicidal activity that was much greater than the additive prediction. Our results show that KM-12 exhibited a potent activity against multiple *Candida* species and that the dimerization of the KM motif improved the fungicidal activity by almost 15-fold. The pharmacological and fungicidal activity of KM-12 was examined. These studies include: an evaluation of the dose dependency, the kinetics of activity, the effect of general histatin 5 inhibitors, the secondary structure of the peptide, and the stability of KM-12 in saliva, serum and artificial gastric juice.

4.2 Material and method

Strains : *Candida albicans* SC5314 [9] and *Saccharomyces cerevisiae* BY4741(*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 pat1::natMX4*) are strains available in the McNabb laboratory. *Candida glabrata* ATCC90030, *Candida parapsilosis* ATCC22019, *Candida tropicalis* ATCC750, *Candida krusei* ATCC6258, *Candida lusitaniae* (ATCC200951), *Candida kefyr* (ATCC4135) and *Candida dubliniensis* MYA-646 were purchased from the American Type Culture Collection (ATCC).

Peptide synthesis. N-Fmoc protected amino acids and Rink resin was purchased from NovaBiochem (San Diego, CA) and Advanced Chemtech (Louisville, KY). All peptides were synthesized with an acetylated N-terminus and amidated C-terminus. The peptides were synthesized on a model 433A solid-phase peptide synthesizer (Applied Biosystems; Foster City, CA) using Rink resin and Fmoc-protected amino acids (NovaBiochem).

Cleavage of the peptide from the Rink resin. After synthesis, the peptides were deprotected and cleaved from the resin using two high TFA (trifluoroacetic acid) cleavage cocktails. *Method A.* The cleavage cocktail consisted of 85% TFA, 5% dH2O, 5% triisopropylsilan, and 5% phenol. The resin was mixed in the cocktail solution at room temperature for 3 h at 480 rpm, after which the peptide was precipitated into 50 ml 1:1 v/v methyl-t-butyl ether/hexane (MTBE) per ml of cleavage cocktail. The peptides were dissolved in 1:1 v/v acetonitrile/ddH2O and recovered by lypholization under high vacuum. Crude peptides were then purified on a PRP-3 reverse phase column (7 by 305 mm; Bio-Rad, Hercules, USA) on a Hitachi L7100 HPLC instrument with a linear gradient of 0 – 30 % acetonitrile and water (both were contained 0.1% TFA). The purity of each peptide was evaluated by mass spectroscopy (MALDI and ESI). The

peptides concentrations were determined by the extinction coefficient. *Method B.* KM peptides were cleaved from the resin using 90% TFA, 5% Thioanisol, 3% Ethandithiol, and 2% anisole. The cleavage mixture was shaken for 4 h at 480 rpm at room temperature, and then precipitated in ether. Two different solutions were evaluated; 1:1 v/v methyl-t-butyl ether/hexane and diethyl ether, where 50 ml of ice-cold solution was used per ml of cleavage cocktail. After which the crude peptides were purified, processed and identified as described above.

Dimerization of the peptides. The 10 mg/ml concentration of peptides in reduced form, except for KM-18, was oxidized in an aqueous solution (10 mM sodium phosphate buffer) containing 10% DMSO at pH 8.5. For KM-18, the intramolecular disulfide bond was initiated at a peptide concentration of 1 mg/10ml. The mixture was incubated in a shaking incubator at 550 rpm overnight at 37^{o}C. The dimerization was monitored by Ellman reagent (Sigma-Aldrich), reverse phase-HPLC, and verified by ESI mass spectroscopy. The sample was subsequently lyophilized three times and precipitated in isopropanol (10% v/v) to ensure the removal of any trace amounts of TFA and DMSO. The reduction of KM-12 dimer to the monomeric form was achieved by the addition of 5 mM DTT and incubated at room temperature overnight [10, 11]. The oxidation state of the peptide was verified by reverse phase-HPLC.

Fungicidal activity assays. The fungicidal activity of the peptides against *C. albicans* and *S. cerevisiae* was examined by micro dilution plate assay as described previously[12]. *C. albicans* and *S. cerevisiae* was grown overnight on agar plates at 30^{o}C. Sabouraud dextrose agar was used for *C. albicans* growth, and YPD (2% yeast extract, 1% bacto-peptone and 2% glucose)[5] and YPL (2% yeast extract, 1% bacto-peptone and 2% lactate)[6] plates for *S. cerevisiae*. Following overnight growth, a single colony was diluted in 1 ml of 10 mM sodium phosphate buffer at pH 7.4. A hemocytometer was used to quantify the number of cells/ml and the cell concentration

was adjusted to 1.8×10^5 cells/ml. Cell suspensions (20 µl) were mixed with 20 µl of peptide dissolved in 10 mM sodium phosphate buffer at pH 7.4 and incubated for 2 h at 37°C with shaking at speeds of 550 rpm. The reactions were stopped by the addition of 360 µl yeast nitrogen base (3.4 g yeast nitrogen base without ammonium sulfate or amino acids and 10 g ammonium sulfate per liter) and 40 µl of cell suspension was spread on the appropriate growth medium plates and incubated for 24 h at 37°C. The number of colony-forming units (CFUs) was counted and each assay was repeated in triplicate. Loss of viability was calculated as [1-(colonies from suspension with peptide/colonies from suspension with no peptide)] × 100. KM-12 lethal dose $_{50}$ (LD_{50}) was estimated by performing linear regression analysis (the percent of viability versus the $\log_{10}$ concentration) and determining the *x* axis intercepts [14]. The LD_{50} was the concentration that killed 50% of *Candida albicans*. For examining the kinetics of fungicidal activity, the peptide was incubated with *Candida albicans* for different periods of time and the viability determined as described above.

Minimum inhibitory concentration (MIC) assay. MIC assays were carried following the CLSI M27-A2 standard with the modification as described below. Briefly, two-fold serial dilutions of the peptides were prepared with RPMI-1640 (Sigma R-7755) in 100 µl per well of a 96-well flat-bottom microtiter plates (Costar, Cambridge, MA). To demonstrate the effect of salt on peptide activity, different RPMI-1640 dilutions (1X, 0.5X, 0.25X, and 0.125X) were used. To each well of the microtiter plates containing the peptide, a 100 µl *C. albicans* cell suspension containing 1×10^4 cells /mL in a resazurin/water solution (0.01% w/v) was added. The final concentration of the peptides in the assay ranged from 0.2 to 100 µM (0.4 to 206.7 µg/ml, respectively). The microtiter plates were subsequently incubated at 35°C and examined at both 24 h and 48 h after exposure to peptide. Each assay plate contained a positive control of *Candida albicans* without

added peptide and the negative control of RPMI-1640 medium containing only the resazurin/water solution. For each peptide three independent assays were performed. The MIC assay results were interpreted visually using the criteria of the lowest peptide concentration that remained blue (indicating no growth) or the first dilution that changed from blue to slightly purple (equivalent to prominent growth inhibition). The peptide concentrations were determined based on the extinction coefficient of the four peptides.

The MIC values for fluconazole-sensitivity on *Candida spp.* were also determined using the same procedure.

Circular dichroism spectroscopy. CD spectra were obtained for KM-12 in 10 mM sodium phosphate buffer pH 7 as well as following incorporation into liposomes of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and micelles of sodium dodecyl sulfate (SDS) at 1:50 (mol/mol) peptide :lipid ratio. Preparation of samples for CD spectroscopy. Tubes containing 4.5 µL (0.1µmol) of KM-12 were vacuumed dry overnight, and the buffer and/or appropriate lipids were added separately. To study the structure in an aqueous solution, KM-12 was hydrated with 500 µl of 10mM of sodium phosphate buffer pH=7 to achieve a final concentration of 200 µM peptide. For DMPC liposomes, 678 µl of DMPC/chloroform stock (5 mg/ml) (Avanti, AL, USA) were added to KM-12, mixed and dried under nitrogen, and vacuumed dry for 48 hours. Following drying, 500 µl of 10mM of sodium phosphate buffer pH=7 was added and the sample treated with ultra-sonication for 1 h. For SDS micelles, 1.44 mg of SDS was dissolved in 500 µl of 10 mM of sodium phosphate buffer pH 7 to yield 10 mM SDS. Then SDS solubility was ensured by sonication in a water bath for 10 min. The SDS solution was then used to rehydrate KM-12, and the sample was mixed and subsequently sonicated for 30 min. All samples were centrifuged at 10,000 rpm for 5 min to remove any particulate material, and the

absorbance was measured on diode array (200 -300 nm) to confirm that the absorbance across the spectrum was below 1 absorbance unit. Circular dichroism measurements. CD spectra for KM-12 were generated using a Jasco-710 spectropolarimeter as described previously with some modifications[15]. The reading was made using a quartz cell with a 0.1cm path length at 25^{o} C. The spectra were recorded every 0.2 nm in the absorbance range of 190 to 250 nm with a 1.0 nm bandwidth and a scan speed of 20 nm/min. Six scans were performed and averaged. The background was subtracted from all spectra, and curve smoothing applied. The CD spectra are reported as the mean residue ellipticity ([Θ]) in degrees. cm^{2}. dmol^{-1}. The mean residue ellipticity ($[\theta]_{mrw)}$ was calculated by the following equation [15]:

$[\theta]_{mrw} = \theta / (10 \times c \times l \times N)$ where *c* is molar protein concentration, *l* is the cell path length in cm, and N is the number of amino acids in the peptide. CD data were further analyzed using the web-based K2D3 program (http://www.ogic.ca/projects/k2d2/)[16] and CDPro software[17].

Effect of sodium chloride, sodium azide and temperature on fungicidal activity. To study the effect of temperature on the killing activity, the fungicidal activity assay was performed using 2.5 µM KM-12 in 10 mM phosphate buffer pH 7 at two different temperatures, 4^{o}C and 37^{o}C. The effects of sodium chloride and sodium azide were studied using the fungicidal activity assays in 10 mM sodium phosphate buffer pH 7 at 37°C with the variable being the different concentrations of sodium chloride (5 mM,10 mM and 25 mM) , or 5 mM sodium azide.

Peptide Stability in Artificial Gastric Juice. The stability of KM-12 was tested using artificial gastric juice prepared as described previously with slight modifications [18]. The artificial gastric juice was prepared by dissolving 2 g NaCl and 3.2 g pepsin in 7.0 ml 0.085 M HCl and the volume was increased to 1000 ml with distilled water (pH 1.2). The peptide (5 µg in volume of 10 µl) was added to 90 µL of artificial gastric juice and incubated for different periods of time (0,

5, 10 and 20, 40 minutes) at 37^{o}C. The reaction was terminated by boiling for 10 min and the sample was centrifuged at 13,000 rpm for 10 min. in a microcentrifuge. The supernatant was collected, neutralized with NaOH, and analyzed by reverse phase HPLC. The peptides were analyzed by reverse phase HPLC using a PRP-360 column (Bio-Rad). A gradient elution was performed with mobile phase A (0.1% TFA in water) and mobile phase B (0.1% TFA in acetonitrile). The peptide was eluted with a gradient from 95:5 to 65:35 (mobile phase A: B) for 15 min at flow rate 1 ml/min. The HPLC total run time was 20 min and the injection volume was 50 µl. Chromatograms were recorded by UV detection at 220 nm and the data analyzed using CHROMULAN v0.79 software. Data were recorded from three independent assays to determine the half-life of the peptide. For the detection of cleavage sites, two samples (0 min and 60 min post exposure to gastric juice) of KM-12 were subjected to ESI mass spectrometry analysis, and then the spectra were overlapped and further analyzed.

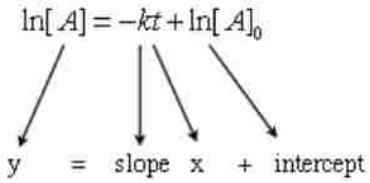
Peptide Stability in Human Saliva. Whole saliva samples (5 ml) were collected from three healthy donors ranging in age from 25 to 35 years in accordance with a protocol approved by the University of Arkansas Institutional Review Board. The stability of the peptides in human saliva was determined as described previously with slight modifications [28]. After the saliva was collected, it was immediately centrifuged at 13,000 rpm for 10 min at 4^{o}C in a microcentrifuge and the supernatant was aliquoted 1 ml portions and stored at -80^{o} C. One ml of saliva from each volunteer was pooled, mixed and filtered through a 0.45 µm membrane filter. The stability assays was performed using a constant ratio of peptide to saliva (1:9). Twenty µl of peptide in 10 mM sodium phosphate buffer (pH 7.4) was mixed with 180 µl of saliva to give a final peptide concentration of 0.5mg/ml. The peptide in saliva was incubated for different periods of time (0, 20, 40, and 60 min) at 37^{o}C, and the reaction terminated boiling the samples for 10 min. The

samples were subsequently filtered and analyzed by reversed phase HPLC as described above. The HPLC total run time was 20 min and the injection volume was 100 µl. The peptide dissolved in artificial saliva was used as a control. The sample was analyzed by mass spectrometry to identify the peptide fragments.

Calculation of peptide half-life. Following HPLC, the peak area of the KM-12 peak was examined (after subtraction of saliva and simulated gastric juice baseline) and correlated to the concentration. The area under the curve for KM-12 at time 0 (500 µg/ml) was considered as 100% and the change in the peak area was correlated to the concentration of KM-12. Natural logarithm of the peptide concentration (*ln*) was plotted versus incubation time, and fit into first-order decay equation [20].

$$\ln[A] = -kt + \ln[A]_0$$

Where $[A]$ the concentration of peptide at time t, k is the reaction rate coefficient, t is the time and $[A]_0$ is initial peptide concentration . The first order decay equation is a linear equation under natural logarithm:



The $t_{1/2}$ was then calculated using this formula:

$$t_{1/2} = \frac{0.693}{k1}$$

The stability and protein binding capacity of KM-12 in serum. Peptide stability and protein binding capacity was assayed in diluted serum as previously described with some modification [19]. Forty microliters of fetal bovine serum (Equitech-Bio) were added to 20 µl of KM-12 dissolved in distilled water at a concentration of 0.5 mg/ml. The volume was adjusted to 200 µl with 10 mM phosphate buffer (pH 7) and the samples incubated a 37^{o}C for 5 min. After incubation, 20 µl of trichloroacetic acid (TCA) was added and the mixture incubated at 4°C for 10 min and subsequently centrifuged at 13000 rpm for 10 min. The supernatant was subsequently neutralized with NaOH. The amount of KM-12 present in the supernatant was determined by reverse phase-HPLC as described in previous section with the controls of peptide alone and peptide in serum after 0 min incubation.

4.3 Results

Peptide synthesis and cleavage from the resin: The peptides used in this study are shown in Table 1. Due to the presence of cysteine in the peptide, the synthesis and preparation of the peptides required an optimization of the protocols for cleaving the peptide from the resin after synthesis. Three different cleavage protocols were evaluated to achieve the optimal yield. All three protocols contained the same percentage of TFA and the differences between them were the thiol scavengers and the precipitation procedure. Method A utilized non-thiol scavengers (5% triisopropylsilan, and 5% phenol), MTBE and 3 h incubation time that resulted in a complex HPLC profile where several byproduct peaks appearing along with KM-11 (Figure 1A). Moreover, the yield of KM-11 was low as the height of KM-11 peak was less than the byproducts. In the second approach, the thiol scavengers were changed to thioanisol, ethandithiol, and anisole (Figure 1B) to improve the yield of KM-11 and simplify the complexity of the HPLC profile, but some byproducts remained. The sizes of the two major byproducts were determined by mass spectrometry to be larger than KM-11 with 58 m/z and 288 m/z. Finally, using the same thiol scavengers as in the second approach, the precipitation step was performed using diethyl ether and this yielded an HPLC profile containing the single KM-11 peak (Figure 1C) that was confirmed by mass spectrometry.

To determine whether these conditions for cleavage and precipitation were unique to KM-11, the first and third approaches outlined above were reevaluated for the cleavage of the KM-13 peptide that also contains an N-terminal cysteine. As illustrated in Figure 2, the HPLC profile for the cleavage products using the non thiols scavengers and methyl-t-butyl ether/hexane precipitation was very complex as seen with KM-11, while the thiols scavengers and diethyl ether precipitation yielded a single KM-13 peak. These data demonstrate that the cleavage of

cysteine-containing peptides from the Rink resin requires nontraditional cleavage procedures, and the inclusion of thiol scavengers and diethyl ether precipitation was subsequently used for the preparation of all cysteine-containing peptides.

Peptide dimerization: All of the peptides used in this study (Table 1), except KM-17, were synthesized as a monomer and subsequently dimerized via disulfide bond formation. For KM-17, which contains both an N- and C- terminal cysteine a disulfide bond was formed to produce a circular peptide. The dimerization was accomplished by incubation of the peptides in the presence of 10% DMSO and dimerization monitored by Ellman reagent and HPLC. As illustrated in Figure 3, the oxidation is a time dependent process and 24 h of incubation at room temperature was the optimal time for 95% of the monomer to be converted to a dimer.

The efficiency of dimerization and the identity of the peptides purified by HPLC were confirmed by mass spectrometry. Unfortunately, MALDI-TOF was unable to confirm the effectiveness of the dimerization process. For example, the MALDI spectrum for KM-12 (the dimer of KM-11) contained two peaks at 885 m/z (KM-11) and 1767 m/z (KM-12) with equal intensity (Figure 4A); however, when the same sample was analyzed by ESI, the major peak was the dimer (Figure 4B). Since the HPLC elution profile and the Ellman reagent reaction also suggested the samples were dimers with no free cysteine residues, it was concluded that the MALDI-TOF analysis was inherently causing the reduction of the disulfide bonds. Thus, peptide samples containing a cysteine residue were confirmed using ESI spectrometry.

The circularized monomer of KM-17, referred to as KM-18, was dimerized at a concentration of 1mg/10 ml to promote intramolecular disulfide bond formation. The HPLC profile showed two additional peaks, besides KM-17, representing possible dimers (Figure 5). The peptide mixture

produced from KM-17 oxidation was also reduced by dithiothreitol to confirm the peaks in the HPLC profile were derived by the disulfide bond formation of KM-17 (Figure 5). Mass spectrometry was used to verify the identity of the KM-17-derived peptides (Figure 6).

Fungicidal activity of the KM-12: To evaluate whether the KM-11 dimer, termed KM-12, resulted in the enhancement of the fungal killing activity against *Candida albicans* fungicidal activity assays were performed (Figure 7). The antifungal activity increased significantly by the dimerization of KM-11, as a 2.5 μM concentration of KM-12 killed close to 100 % of the cells while the KM-11 monomer killed only 55%. The monomeric form of KM-11 was maintained by performing the fungicidal activity assay in the presence of 5 mM DTT. In data not shown, it was determined that 5 mM DTT does not affect the viability of *C. albicans*.

To specifically define the LD_{50} of KM-12 with *C. albicans*, a dose dependent fungicidal assay was performed (Figure 8). It was found that the peptide reached 100% killing at a concentration of 2.5 μM and the LD_{50} was determined to be 0.308±0.035 μM. To determine the rate of fungicidal activity against *C. albicans*, the kinetics of killing was determined using 2.5 μM of KM-12 (Figure 9), and it was found that less than 20% of the cells were viable after 1h and KM-12 achieved nearly 100% killing in 2 h.

Characterization of fungicidal activity of KM-12. Since KM-12 was developed using the histatin 5 model, it is plausible that the mechanism of action may be the same. To determine whether the antifungal activity of KM-12 was similar to that of histatin 5, some of the known characteristics of histatin 5 were examined for KM-12; namely sensitivity to salt, sodium azide, low temperature, and the activity against *Saccharomyces cerevisiae*.

The salt sensitivity was evaluated by examining the effect of NaCl on the activity of KM-12 at different concentrations of NaCl (5mM, 25mM and 150mM). It was found that NaCl possessed an inhibitory effect that was dose dependent with a 50% reduction in the maximum antifungal activity achieved at 150 mM NaCl (Figure 10A). Although the KM-12 antifungal activity is sensitivity to NaCl concentration, it was less sensitive than the C-16 histatin 5 or KM5 peptide examined in Chapter 2 and 3. It was also observed that incubation at 4^{o}C exhibited an inhibitory effect on killing as shown in Figure 10B.

The role of cellular respiration in the activity of KM-12 was evaluated using two different approaches: killing activity in the presence of the respiratory inhibitor sodium azide and the killing activity of *S. cerevisiae* during fermentative growth. It was found that 5 mM sodium azide inhibited the fungicidal activity of KM-12 by 80% (Figure 10C). On the other hand, as illustrated in Figure 10D, KM-12 exhibited a similar potency of killing against *S. cerevisiae* versus *C. albicans,* where 2.5 µM of KM-12 killed 93% of *S. cerevisiae* cells while the same concentration killed 99% of the *C. albicans*. The explanation for the latter observation is unclear. Nevertheless, the results with sodium azide inhibition suggests that active respiration is important for the killing activity of KM-12, similar to the other histatin 5 peptide derivatives that have been examined (Chapter 2 and 3).

Activity of KM-12 in the minimum inhibitory concentration assay. The fungicidal activity of KM-12 was evaluated using a more clinically relevant assay, the minimum inhibitory concentration assay (MIC). This assay is performed in RPMI-1640 tissue culture medium, composed of several salts and cations, to more closely simulate the physiological condition. To evaluate the effect of ionic strength, an assay was performed at different concentration of RPMI-1640: 1X, 0.5 X, 0.25 X and 0.125 X, where X is the full ionic strength. Not surprisingly, it was

found that RPMI-1640 demonstrated an inhibitory effect and the activity of KM-12 was totally abolished in 1X RPMI-1640 (Table 2). Furthermore, the antifungal activity was improved by diluting the RPMI-1640, demonstrating the salt sensitivity of the peptide. For example, the MIC value decreased from >176 µg/ml in 1X RPMI to 5.5 µg/ml in 0.125X RPMI. Nevertheless, it is important to note that the MIC value for KM-12 was dramatically improved over that of the C-16 histatin 5 peptide which had virtually no killing activity even in 0.125X RPMI-1640 (Table 3 of Chapter 2).

Optimizing the KM12 fungicidal activity. KM-12 is a prototype peptide where the sequence could potentially be optimized to achieve increased fungicidal activity. Moreover, the optimization could assist in solving problems associated with KM-12, such as salt-sensitivity. To achieve that, several analogs were synthesized with slight modifications to the sequence. Two types of modifications were tried: mutating specific amino acid residues and changing the position of the cysteine residue to alter the structure of the peptide dimer. Following synthesis, preparation of the dimer and purification, the fungicidal activity of each peptide was evaluated by MIC assay against *Candida albicans* in 0.125X RPMI. The MIC activity of KM-12 and each of the analogs is shown in Table 3.

The effect of the hydrophobicity on fungicidal activity was examined by changing both the phenylalanine and tyrosine residues to tryptophan to produce the KM-14 peptide. This change did not improve the antifungal activity as the MIC value was 11.8 µg/ml. To improve the salt sensitivity, the lysine residues were replaced with histidine and arginine residues, generating KM-26 and KM-28, respectively. This change also did not improve either the activity or the salt sensitivity as the MIC values for KM-26 and KM-28 were 44.7 and 11.2 µg/ml, respectively. To evaluate whether the position of cysteine within the peptide influenced activity, the cysteine

residue was moved to the center of the peptide (KM-16), and this had an inhibitory effect on fungal cell killing as the MIC value for KM-16 was 44 µg/ml. The addition of an extra cysteine to the C-terminus to permit circularization of the monomer, generating KM-18 and two circular dimeric byproducts (KM-19 and KM-20) was evaluated. The activity of KM-18 was less potent than KM-12 with a MIC value ranging from of 8 to16 µg/ml, while the activity of the circular dimers (KM-19 and KM-20) was essentially abolished. It is important to note that the activity of KM-18 was near to that of KM-12, and if the circularization improves with the pharmacokinetic and stability properties of the peptide the slight reduction in activity may be a reasonable exchange for improved physiological properties. Therefore, the circular monomer remains a viable lead peptide worthy of further in vivo investigation in the future.

Spectrum of KM-12 fungicidal activity. To evaluate whether KM-12 exhibited a broad activity spectrum, the killing activity of the peptide was examined on the most common pathogenic *Candida* species (*C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. lusitaniae, C. kefyr and C. dubliniensis*) and MIC values were determined. First for each strain, the activity of a common therapeutic azole compound, fluconazole, was evaluated in parallel to determine whether KM-12 displayed a broader activity spectrum than current azole therapeutics. As shown in Table 4, three strains displayed resistance to fluconazole: *C. glabrata, C. krusei* and *C. tropicalis*. KM-12 exhibited a broader spectrum of activity against the fluconazole-resistant *Candida* species (C. *krusei and C. tropicalis*). *C. glabrata* was resistant to both fluconazole and KM-12. *C. glabrata* resistance to KM-12 may prove to be a useful observation in the future as we explore the mechanism-of-action of KM-12.

Structural analysis of KM-12. The secondary structure of KM-12 was evaluated using circular dichroism (CD) spectroscopy in aqueous solution as well as in the presence of charged lipids (neutral and negatively charged). An experiment was conducted to study the effect of charged lipids on the secondary structure of KM-12. In this study DMPC liposomes (1:50) were used to mimic neutral lipids, while SDS micelles (1:50) were used to simulate negatively charged lipids. As illustrated in Figure 11, KM-12 remained largely in a random coil structure in aqueous solution with some tendency to form a partial secondary structure, 5% α-helix and 11% β-turn. In the presence of DMPC liposomes the overall structure did not change as the majority of KM-12 remained in a random coil conformation, but the propensity to form a β-turn increased to 20%. In contrast, the peptide tendency to form β-turn reduced (12%) in SDS micelle and the peptide adopted a partial α-helix (23%), suggesting that negatively charged lipids on the membrane may be important for the formation of the secondary structure of KM-12.

Stability of KM-12 in human saliva. To evaluate the potential of using KM-12 in pharmaceutical applications for *Candida* infections of the oral cavity, the stability of KM-12 in human saliva was evaluated. The peptide was incubated in human saliva for various periods of time and the level of remaining peptide was determined using HPLC. In addition, the $T_{1/2}$ and k constant was calculated, and the proteolytic cleavage sites were identified using mass spectrometry. The chromatogram (Figure 12A) shows that KM-12 is susceptible to the enzyme degradation in saliva as the peak representing KM-12 decreased dramatically by extending the incubation time in saliva. The initial concentration of KM-12 was 500 µg/ml and after 60 min in human saliva, the peptide concentration was reduced to approximately100 µg/ml. The kinetics of degradation suggested that KM-12 proteolysis in human saliva is following a first order reaction in which the estimated $T_{1/2}$ was 26.35 ±2.45 minute and k constant was 0.0263 min^{-1} as shown in

Figure 12B. Mass spectrometry suggested one major fragment at 1321.8 m/z. This size is correlated with only one possible fragment that would occur if KM-12 has been cleaved after the first lysine from the N-terminus of one monomer as shown in Figure 13.

Stability of KM-12 in artificial gastric juice. In order to use KM-12 for systemic pharmaceutical applications via oral administration, the stability of KM-12 in simulated gastric juice was evaluated. Stability was examined at various times over a 60 min incubation period and the presence of the full length peptide was quantified using HPLC. The kinetics of KM-12 degradation in pepsin, including $T_{1/2}$ and k constant, were also determined. Mass spectrometry was also used to identify the cleavage sites. The HPLC data shows that the KM-12 peak decreased rapidly in the presence of simulated gastric juice and the peptide was completely degraded after a 40 min incubation period (Figure 14). The degradation of KM-12 by pepsin follows a first order reaction (Figure 14B) where the estimated $T_{1/2}$ was 7.83 ± 1.44 min and the k constant was 0.0946 min^{-1}. The comparison between the mass data of KM-12 exposed to artificial gastric juice for 0 min versus 60 min revealed that KM-12 is primarily cleaved after the phenylalanine leaving 1174.6 and 593 m/z peptides (Figure15).

Stability in Serum. Since oral medications will be absorbed into the blood stream or potentially delivered as an intravenous drug, the stability of KM-12 in serum as well as KM-12 binding to serum components was evaluated. KM-12 was incubated in 20% FBS for 0 and 5 minutes, and the serum proteins were precipitated with TCA before the supernatant was quantified by HPLC. Two important observations were seen during the HPLC analysis (Figure 16): a reduction in KM-12 peak and an increase in KM-11 monomer peak. Although the 0 min sample was mixed with serum and instantly precipitated with TCA, that was a sufficient time period to decrease the concentration of KM-12 by 40% and increase the concentration of KM-11 (monomer) by 50% .

After 5 min, approximately 80 % of KM-12 was either bound to serum proteins and precipitated or reduced to monomer.

4.4 Discussion:

In the last decade, the number of drug-resistant *Candida* strains has increased, emphasizing the need to develop novel drug models with unique mechanisms of action. Several researchers have shown histatin 5 is a good model for an antifungal drug and several attempts have been conducted to optimize and utilize histatin 5[21, 22]. Previously, we identified a short sequence (Chapter 3) within histatin 5 that contributes the majority of the antifungal activity, referred to as the KM motif. The KM motif is composed of five amino acids and retains strong antifungal activity against *C. albicans*.

In this study we took a step forward and tried to develop a more potent antifungal peptide utilizing the KM motif. The optimal goal of this study was to design peptides with the following properties: work via a mechanism similar to histatin 5, maintain a relatively small size, to have stronger fungicidal activity than histatin 5, to have broad spectrum activity against multiple Candida species, and finally to be stable in human fluids. Furthermore, this study defines the optimal conditions to synthesize these peptides efficiently.

Since KM-5 exhibited antifungal activity similar to histatin 5, a logic way to increase the activity without interfering with the mode of action was to dimerize KM-5 with the goal of additively increasing activity. To accomplish this objective, a cysteine residue was added to the N-terminus of KM-5, and the dimer generated by a disulfide bridge. To achieve the optimal production of these peptides several protocols were evaluated for the cleavage of the peptide from the Rink resin. The differences between the protocols were the type of scavengers and the precipitation strategy. The change in cleavage protocol was required because the use of nonthiol scavengers yielded various byproducts and poor cleavage, likely related to the presence of a cysteine residue. Our results showed that using thiol (Thioanisol, Ethandithiol, Anisole)

scavengers, and performing the precipitation in diethyl ether were the optimal conditions for obtaining the highest yield of pure peptide (Figure 1 and 2). The unexpected complexity of the cleavage product using standard conditions of peptide synthesis occurred because of the presence of cysteine, arginine, and tryptophan. The main function of scavengers is to reduce the amount of byproduct formed[23]. It has been published that thiol scavengers are essential for peptides containing cysteine-*tert*-butyl [24]. Although the *tert* group is easily removed during the cleavage, it tends to reattach to the free sulfhydryl if a thiol scavenger is not present [25]. Also *tert*-butyl can form carbocation counterparts (*tert*-butyl cations) which might interact with tryptophan, tyrosine and methionine [25].

Even though several protocols showed the use of thiol scavengers to be enough to reduce the byproducts, a large contaminating peak was still observed in the KM-11 cleavage product (Figure 1). The mass spectrometry showed this byproduct is 56 m/z larger than KM-11, which is equal to a *tert*-butyl group. So it is likely that the *tert*-butyl alkylation occurred in spite of the presence of thiols. It has been published that the use of MTBE in some peptides, especially aromatic-rich peptides, produces a significant level of *tert*-butyl-peptide byproduct [26]. The *tert*-butyl was not generated from the peptide protecting group; rather it came from *tert*-butyl-O cleavage of MTBE under the strongly acidic condition. This problem was alleviated by using diethyl ether for peptide precipitation.

After the peptide had been synthesized, the dimerization process was initiated. All peptides were dimerized successfully in the presence of 10 % DMSO at pH 8 (Figure 3 and 5). The dimerization was monitored using HPLC, Ellman reagent and mass spectrometry (MALDI-TOF and ESI). Interestingly, although HPLC and Ellman reagent confirmed the oxidation, MALDI-TOF failed to support the result as the spectrum showed the dimer and monomer at the same

intensity (Figure 4). In contrast, ESI confirmed that the dimer is the predominant species in the reaction consistent with the HPLC and Ellman data. This observation is not new as the ionization used in MALDI is capable of breaking the disulfide bonds and causing rearrangements unless a special matrix is used (2-(4-hydroxyphenylazo) benzoic acid: α-cyano-4-hydroxycinnamic acid (1:10)) [27] .

KM-12 was the first dimer generated and it contained two copies of the KM motif. The dimerization boosted the antifungal activity of the KM motif by almost 15 fold over the monomer as the LD_{50} decreased from 5 µM to 0.3 µM (Figure 8). Previously we measured the LD_{50} of the C-16 histatin 5 and KM-5 peptides with C. *albicans* using the fungicidal activity assay, and these peptides displayed an LD_{50} of 2.7 µM and 5µM, respectively. It has also been previously published that the LD_{50} of histatin 5 and P-113 (an active fragment of histatin 5) on *C. albicans* are 2.3 µM and 4.47 µM, respectively [28]. In the light of the reported data, KM-12 exhibited a significantly more potent fungicidal activity than KM-5, C-16, histatin-5, or P-113. This comparison may not be completely valid since all of these peptides were not directly compared in the same experiment. However, the C-16 peptide has been shown to retain the full activity of histatin-5 and the LD_{50} value obtained from our study is in agreement to histatin 5 from other studies, 2.7 µM and 2.3 µM respectively. It is important to emphasize that the *in vitro* fungicidal assay does not reflect the actual activity *in vivo*. For example, histatin 5 in the oral cavity is more active than in the fungicidal activity assay for multiple reasons. First, it is constantly produced in the oral cavity. The mean concentration of histatin 5 in submandibular and sublingual saliva at any giving time is 26 to 90 µg /ml[29]. Secondly, histatin 5 has an ability to bind with Zn^{+2} and this may enhance the antifungal activity [30]. Finally, histatin 5 is adsorbed

to the hydroxyapatite of the teeth[31], and this prolongs half-life of histatin 5 by protecting it from proteolysis enzymes [32].

Several examples of antimicrobial peptides (AMPs), both naturally occurring and synthetic, have been published utilizing disulfide dimerization to improve the activity [33]. For example, PAMP-36 , a cathelicidin-derived peptide produced naturally by pig leukocytes, is a homodimer composed of 36 amino acid in which the dimerization improved the activity as well as the ability to permeabilize the target membrane [34]. An example of a synthetic antimicrobial peptide is $(CKPV)_2$, a homodimer peptide developed from melanocortin hormone [35].

With some exceptions, most cationic antimicrobial peptides, including histatin 5, are inhibited by sodium chloride. As the nature of interaction between cationic AMPs and the negatively charged membrane is ionic, it can be weakened by monovalent and divalent cations, thus the fungicidal activity will be reduced[36, 37, 38-40]. KM-12 is also inhibited by sodium chloride but to a lesser extent than histatin 5 (Figure 10). The presence of 150 mM NaCl was enough to inhibit the activity of histatin 5 completely[37], while KM-12 was only 50% inhibited. This phenomenon might be related to the fact that KM-12 has more basic amino acids than in histatin 5, hence the interaction between KM-12 and the membrane is stronger; thereby a higher concentration of the salt is required. The effect of salts on the activity of KM-12 was also evaluated using RPMI-1640 tissue culture medium to more closely simulate a physiological environment in which multiple salts are present. Moreover, the MIC assay in RPMI-1640 is the standardized clinical assay used to evaluate fungal sensitivity to drugs; hence, it provides a platform for the analysis of the KM peptides consistent with that used in a clinical environment. The influence of RPMI ionic strength on KM-12 activity was obvious as the activity was decreased by increasing the concentration of RPMI (Table 2). In spite of the sensitivity to salt, the activity of KM-12 is

superior to histatin-5 and KM-5. The MIC value for KM-12 at 0.125X RPMI-1640 was 5.5-11 µg/ml, while histatin 5 C-16 peptide displayed a MIC value of 206.7µg/ml (Chapter 2). Moreover, histatin 5 did not demonstrate fungicidal activity at 0.25 X and 0.5 X RPMI while KM-12 did with MIC values of 44-88 and 176.8µg/ml, respectively. It's important to mention that MIC is also an *in vitro* assay and it does not necessarily correlate with the potential *in vivo* activity; however, it is an excellent assay for comparing the KM peptides to known antifungal compounds.

Although the exact mode of action of histatin 5 remains unclear, research has shown histatin 5 targets active mitochondria and subsequently depolarizes the mitochondrial membrane[43]. In this study, two assays were conducted to evaluate the role of respiration in KM-12 activity: sodium azide sensitivity and the fungicidal activity against *S. cerevisiae* grown by fermentation. Sodium azide, as a potent inhibitor of mitochondrial respiration, inactivates cytochrome c oxidase via intercalating between the heme a_3 iron and Cu_3 at the oxygen reduction site [44]. In addition, azide binds to the F1 catalytic domain within mitochondrial F-ATPases and inhibits the hydrolyase activity [45,46]. Multiple publications have shown that 5 mM sodium azide inhibits both the conventional and the alternative respiratory pathway in *Candida albicans*[43, 47]. The activity of KM-12 was inhibited severely by the presence of 5mM sodium azide. These data suggests that, similar to histatin 5, the fungicidal activity of KM-12 may be related to the cellular respiration (Figure 10).

Although *Saccharomyces* and *Candida* produce energy via respiration and fermentation, histatin 5 [48, 49], C-16 and KM-5 possess potent activities only against *Candida* species. This difference may be related to the fact that *S. cerevisiae* grows as a Crabtree-positive yeast that ferments glucose even in the presence of oxygen; whereas, *C. albicans* is a Crabtree negative yeast that

relies on respiration in the presence of oxygen. Consequently, *S. cerevisiae* mitochondria will be less active and histatin-5 will lose the ability to kill these cells. The ability of *S. cerevisiae* to hinder the translocation of histatin 5 to the cytoplasm is another possible explanation that has been suggested [49]. Surprisingly, KM-12 killed 92% of *S. cerevisiae* at a concentration of 2.5µM on a fermentable medium, while C-16 and KM-5 failed to achieve killing at 25 µM (Figure 10). This result suggests that KM-12 may work through a modified mechanism. This activity is not unique for KM-12 as several antimicrobial peptides have been published that exhibit potent activity on *S. cerevisiae*, such as MUC7 [50], Cecropin B, and Arasin 1[51] .

Another difference between the histatin 5 C-16 peptide and KM-12 is the kinetics of fungicidal activity. KM-12 attains the maximum activity within 1 hour while C-16 and the KM-5 peptides required two hours (Figure 9). This suggests that KM-12 is not only more potent that C-16 and KM-5, it also has a more rapid onset of action. Unfortunately, the kinetic results cannot be compared directly with other AMPs due to the use of different peptide concentrations and different incubation times.

After the killing activity of KM-12 had been characterized, several KM-12 analogs were generated in an effort to improve the fungicidal activity and to increase the tolerance to salt. The first KM-12 derivative involved replacing the tyrosine and phenylalanine with tryptophan to increase the hydrophobicity (KM-14). It has been published previously that tryptophan has a greater propensity to insert into membranes as well as to interfere with lipid polymorphism [52] which may lead to increase the activity . Although such an amino acid change has boosted the activity of other AMPs[53, 54], it did not improve the activity of KM-12 (Table 3). Therefore KM-14 was excluded from further studies. Other reasons that lead to the exclusion of KM-14 from further study are: tryptophan tends to increase red blood cell hemolysis activity[55] and it is less

stable in gastric juice[56]. The second replacement involved substituting lysine residues with histidine (KM-26) to improve the salt tolerance. The histidine positive charge is pH dependent, and at physiological pH it exhibits less positive charge than lysine. Accordingly, the hydrophobicity will be increased by this substitution as well retaining some of the positive charge. This amino acid replacement was shown to improve the activity of the histatin 5 related P-113 peptide[28]; however, it did not improve the activity or salt tolerance of KM-12 (Table 3).

Another analog was KM-28, in which lysine residues were substituted with arginine. It has been published that the arginine side chain interacts more strongly with lipid phosphates than the lysine side chain at physiological temperature [57]; therefore, this change was expected to improve the activity and salt tolerance . Unfortunately, this change did not improve fungicidal activity or salt tolerance. On the contrary, the MIC value (µg/ml) was higher than KM-12 (Table 3).

The final peptide modifications involved changing the cysteine location (KM-16) and the addition of an extra cysteine (KM-18 and KM-19). The influence of cysteine location within KM-12 was studied by shifting the cysteine to the center (FKCRKY) rather than being at the N-terminus (CFKRKY). The relocation of the cysteine was not a random selection; it was picked because a related sequence was found in another naturally occurring AMP which has potent antibacterial and antifungal activities, Lactoferricin B (FKCRRW) [58, 59]. The dimerization of KM-16 didn't improve the activity; in contrast it increased the MIC value. This result indicates the continuity of positive charges is essential for maintaining maximal antifungal activity (Table 3).

Several naturally occurring antimicrobial peptides contain multiple cysteine residues, where the disulfide bond is responsible for improving the rigidity of the peptide structure. Consequently, this enhances the activity and more importantly, the salt resistance [60, 61,62] . Theta-defensin is an example of an AMP in which multiple disulfide bonds play an essential role in salt sensitivity [63, 64], and the rigid structure may also enhance the activity through reducing the sensitivity to proteolytic cleavage [51, 65] . The addition of an extra cysteine to the C-terminus of KM-11, producing a cyclic monomer (KM-18) and cyclic dimers (KM-19 and KM20), did not increase the activity or the salt tolerance (Table 3. However it should be noted that KM-18, the cyclic monomer, had an MIC value similar to KM-12. Thus, KM-18 remains a viable candidate for further investigation, particularly since cyclization of peptides has been shown to increase the activity of antifungal peptides[64,66, 67, 68, 69].

Non-*albicans* species of Candida are responsible for 35-65% of systemic *Candida* infections in the general patient population[70]. They are more frequent in immune compromised patients including HIV/AIDS, cancer, and neonatal pediatric patients[70-72]. Some of these species also exhibit resistance to the most common fungal medications : *C.parapsilosis*[73, 74 75], *C. dubliniensis* [76], *C. kefyr* [72] and *C. lusitaniae* [70, 71] isolates have been found that are resistance to amphotericin B, while *C.glabrata*[77], *C.tropicalis*[78], *C.krusei*[79] are inherently resistant to fluconazole . The ultimate goal of our work is to design an antifungal peptide that is not only active against *C. albicans* but also on non-*albicans* strains. Excluding *C. glabrata*, KM-12 possesses a potent activity on all non-*albicans* species when assayed in 0.125X RPMI-1640 medium, while fluconazole failed to kill *C .tropicalis* and *C. krusei* (Table 4). Many strains of *C. glabrata* have been shown to be resistant to histatin 5 and other histatin family members [80], thus the resistance of *C. glabrata* to KM-12 was expected. The exact mechanism for this resistance is not fully

understood, however recent research has shown that it might be due to the reduction of histatin 5 uptakes into the cell[81]. This reduction may be explained by the fact that polyamine transporters (Dur3p and Dur31p) in *C. glabrata* are uncharacterized which are essential for histatin 5 translocation into the *C. albicans* cell. The overexpression of Dur3p and Dur31p in *C. glabrata* strains increased the susceptibility to histatin 5 by two-folds[81]. It's important to mention that although *C. glabrata* and *C. albicans* belong to the same genus, *C. glabrata* is more phylogenetically related to *S. cerevisiae* than *C. albicans*[82]. Also the composition of the cell wall is different as *C. glabrata* has higher surface levels of β-1,3-glucans as compared with *C. albicans*[81].

KM-12 primarily adopted a random coil structure; however there was some local structure. In the presence of DMPC liposome mimicking a neutral cell membrane, KM-12 gained a propensity to form an alpha helix, while in SDS micelles it showed a tendency to form a beta-turn structure (Figure 11). It is likely that the short length of KM-12 prevents the formation of a strong secondary structure. In all cases, KM-12 is too small to cross a yeast cell membrane in an α-helix structure, which requires at least 20 amino acids [28]. This result suggests that KM-12 as well as histatin 5 may use the carpet model to cross the membrane, where no secondary structure and specific size are required [83, 84]. In the carpet model, the peptides bind and align in parallel to the surface of the yeast membrane, and then the membrane is permeabilized and/or is disintegrated into micelle-like structures at high concentrations of peptides[85]. So how do KM-12 and histatin 5 differentiate between human and yeast cells? Until now the answer is unclear but the selectivity may be related to the fact that histatin 5 has the ability to bind to laminarins (beta-glucans), yeast cell wall polysaccharides, before translocation into the cytosol[86]. KM-12 may

also bind to the cell wall before it interacts with the cell membrane. Since humans lack cell walls, KM-12 selectively binds to fungal cells.

After characterizing the *in vitro* activity of KM-12, the stability in human fluids was evaluated. Because Candida infections can be local (oral and vaginal) or systemic (blood) and the route of drug administration varies (topical, mouth and intravenous), the stability of KM-12 was examined in saliva, gastric juice and serum.

Human saliva contains a pool of protease enzymes, more than 13, with various activities such as trypsin-like, chymotrypsin-like and histidine peptidases [87, 88]. These enzymes are mostly secreted from white blood cells and microflora, but some are produced by the salivary glands [89, 90]. The main target cleavage sites of salivary proteases are lysine and arginine residues for trypsin-like enzymes, aromatic amino acids for chymotrypsin-like enzymes, and histidine is the primary target for histidine proteases [87, 88]. KM-12 is unstable in human saliva with a $T_{1/2}$ of 26 minutes (Figure 12). The stability of histatin 5 in saliva has been evaluated in multiple publication and the kinetics of histatin proteolysis in saliva have also been measured[87, 91]. The rate of histatin 5 degradation in saliva was found to be 17.8 µg/ml/h and the $T_{1/2}$ was around 8 h [87]. However these studies did not use whole saliva, instead they used a 1:10 diluted version, so the data are not directly comparable. The difference in stability between KM-12 and histatin 5 may relate to the fact that histatin 5 can be adsorbed to the teeth [31]. This feature prolongs histatin 5 life via protecting it from proteolysis enzymes. The stability of other AMPs has been evaluated in saliva and in several cases they were degraded completely with less than 60 min[90, 92]. For example, the $T_{1/2}$ for the KSL peptide, a deca-AMP , in saliva was less than 5 minutes and the peptide was totally degraded within 10 minutes [18]. The problem of KM-12 stability can be solved using D-amino acids and this idea has been confirmed in multiple AMPs [18, 90, 92-95].

We also examined the stability of KM-12 in simulated gastric juice which becomes important when considering oral administration of an antifungal compound. Pepsin, an enzyme found in simulated gastric juice, cleaves peptides and proteins before and after hydrophobic residues, such as phenylalanine, tryptophan, and tyrosine[18]. KM-12 is unstable in simulated gastric juice with a $T_{1/2}$ of 7.8 min (Figure 14). Using the mass spectrometry, we identified phenylalanine as the only cleavage site in KM-12 (Figure 15). Therefore, the stability in gastric juice could be solved by simply replacing the phenylalanine for D-phenylalanine. In fact, this idea was tested and the peptide containing D-phenylalanine was stable over for over 60 min in artificial gastric juice (Akkam, unpublished observation).

The stability of KM-12 in serum as well as the binding to serum proteins was evaluated to study the possibility of using KM-12 intravenously. These results showed KM-12 has a tendency to bind to serum proteins and the peptide was found to be unstable in serum (Figure 16). Although 20 % FBS was used in the assays, approximately 50% of KM-12 was either bound to serum proteins or was reduced to monomer. Binding to serum protein has benefits as it increases the volume of distribution and the duration of action. The serious problem was the reduction of KM-12 because the activity will be reduced. The only solution is to remove the cysteine and dimerize the peptide using a different method such as lysine-lysine covalent bond, or to produce a 10 amino acid peptide without dimerization.

4.5 Conclusions

KM-12 is a novel antimicrobial peptide designed utilizing two KM motifs dimerized via a disulfide bond. The activity of KM-12 on *C. albicans* is approximately fifteen times more potent than the monomer and ten times more active than histatin 5 or the C-16 peptide. KM-12 possesses potent antifungal activities on most common *Candida* species, including those resistant to fluconazole. The results of this study suggest that the mode of action for KM-12 may be similar to histatin 5 as the activity was inhibited in the presence of common histatin 5 inhibitors. Unlike histatin 5, C-16 and KM-5 peptide, KM-12 exhibits the ability to kill *S. cerevisiae* on fermentable medium. Structurally, KM-12 retains a random coil structure in aqueous solution, DMPC liposomes, and SDS micelles. KM-12 was evaluated for use as an oral, topical and intravenous compound. The $T_{1/2}$ was 26 and 7.8 minutes in saliva and simulated gastric juice, respectively. In serum, a portion of KM-12 was reduced to the monomeric form; however, KM-12 has shown a tendency to bind serum proteins. In conclusion, KM-12 is promising antifungal peptide and may serve as a candidate drug for pharmaceutical applications against Candida infections. KM-12 is a prototype design in which additional modifications may be required to achieve maximum benefits.

Table 1: KM peptides synthesized in this study.

Name	Sequence[1]	Molecular weight (g/mol)
KM-11	**C**FKRKY	855
KM-13	**C***W*KRK*W*	947
KM-15	FK**C**RKY	855
KM-17	**C**FKRKY**C**	988
KM-25	**C**F*H*RKY	894
KM-27	**C**F*RRR*Y	941

[1] *Underlined letter represents the cysteine and the italic represents the variable amino acid.*

Table 2: MIC assay for KM-12 activity against *Candida albicans*.

The concentration of RPMI	MIC value (µg/ml)
1X	>176.8
0.5X	176.8
0.25X	44-88
0.125X	5.5-11

Table 3: MIC assay of KM-12 analogs against *Candida albicans*.

Peptide name	Sequence[1]	Mean Hydrophobic[2] moment	MIC[3] µg/ml
KM-12	CFKRKY CFKRKY	0.40	5.5 - 11
KM-14	CWKRKW CWKRKW	0.12	118 – 23.6
KM-16	FKCRKY FKCRKY	0.40	44 - 88
KM-18	KRK F Y C C	0.35	8 - 16
KM-19 & KM-20	CFKRKYC CFKRKYC	0.35	197.2
KM-26	CFHRKY CFHRKY	0.37	44.65
KM-28	CFRRRY CFRRRY	0.39	11.16

[1]Red line indicates a disulfide bond.

[3]Mean hydrophobic moment was calculated using the Kyle and Doolittle scale[96] **.**

[3] MIC assay was performed in 0.125X RPMI-1640 medium.

Table 4: MIC assay for comparison of the activity of fluconazole and KM-12 on different *Candida* species.

Candida strain[1]	Fluconazole	KM-12[2]
C. albicans	1.4 -2.8	5.5 - 11
C. dublinesis	1.95 -3.9	11- 22
C. glabrata	37.5	88.4
C. kefyr	1.35 – 2.34	2.75-5.5
C. krusei	75-150	5.5-11
C. lucitaniae	0.29 – 0.58	1.38-2.75
C. tropicalis	>150	2.75-5.5
C. parapsilosis	15	5.5

[1] Specific clinical isolate and assay are described in the Materials and Methods

[2] Assay was performed in 0.125X RPMI-1640 medium

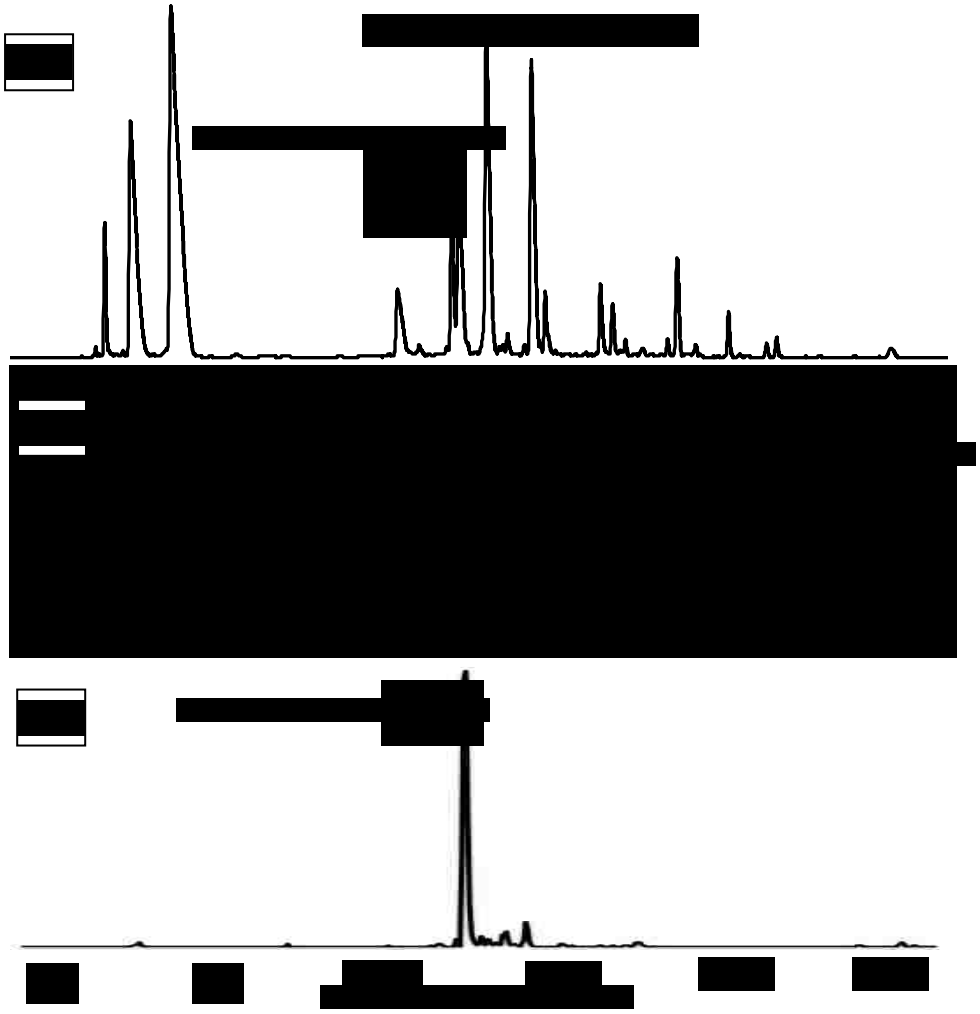


Figure 1: HPLC profiles for three different protocols of KM-11 cleavage from the Rink resin. (A) A cocktail composed of 85% TFA, 5% dH_2O, 5% triisopropylsilan, and 5% phenol was incubated with KM-11 for 3 h and peptides were precipitated in 1:1 v/v methyl-t-butyl ether/hexane. (B) A cocktail composed of 90% TFA, 5% thioanisol, 3% ethandithiol, and 2% anisole was incubated with KM-11 for 4 h and the peptide precipitated in 1:1 v/v methyl-t-butyl ether/hexane. (C) The same cocktail as in panel B but the peptide was precipitated in diethyl ether.

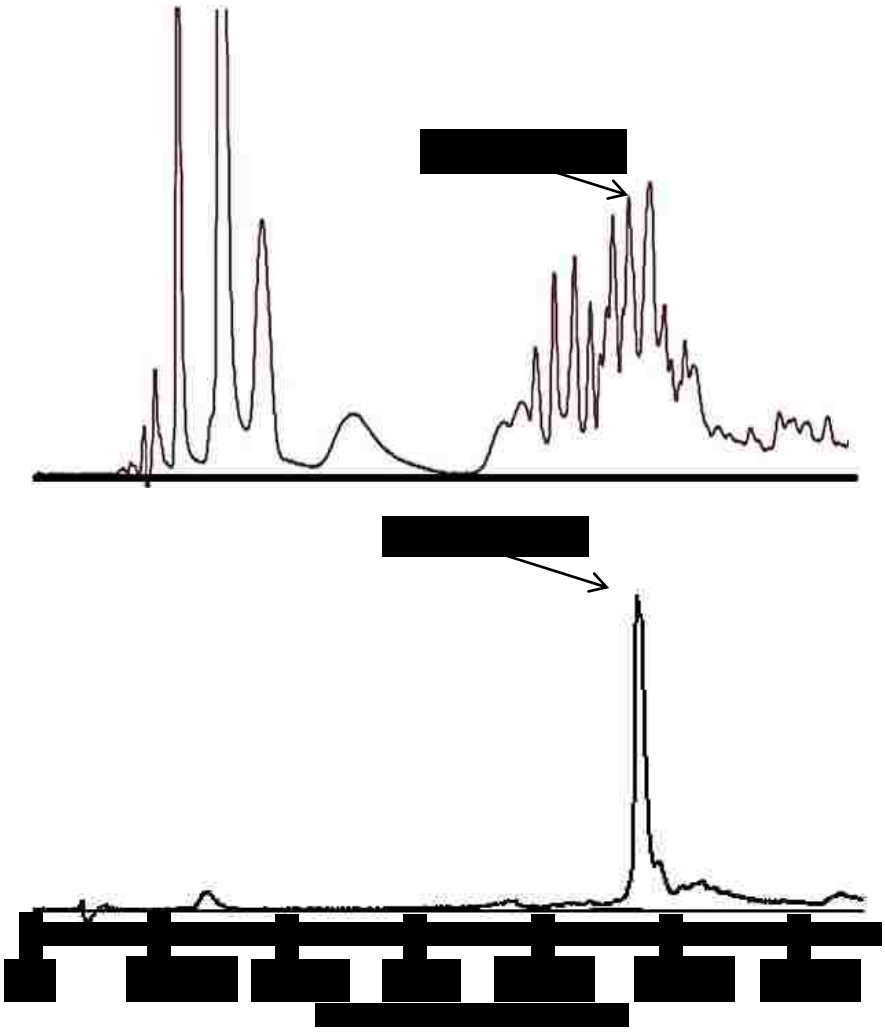


Figure 2: HPLC profiles for different protocols of KM-13 cleavage from the Rink resin. (Upper panel) A cocktail composed of 85% TFA, 5% dH2O, 5% triisopropylsilan, and 5% phenol was incubated with KM-11 for 3 h and peptides were precipitated in 1:1 v/v methyl-t-butyl ether/hexane. (Lower panel) A cocktail composed of 90% TFA, 5% thioanisol, 3% ethandithiol, and 2% anisole was incubated for 4 h and the peptides precipitated in diethyl ether.

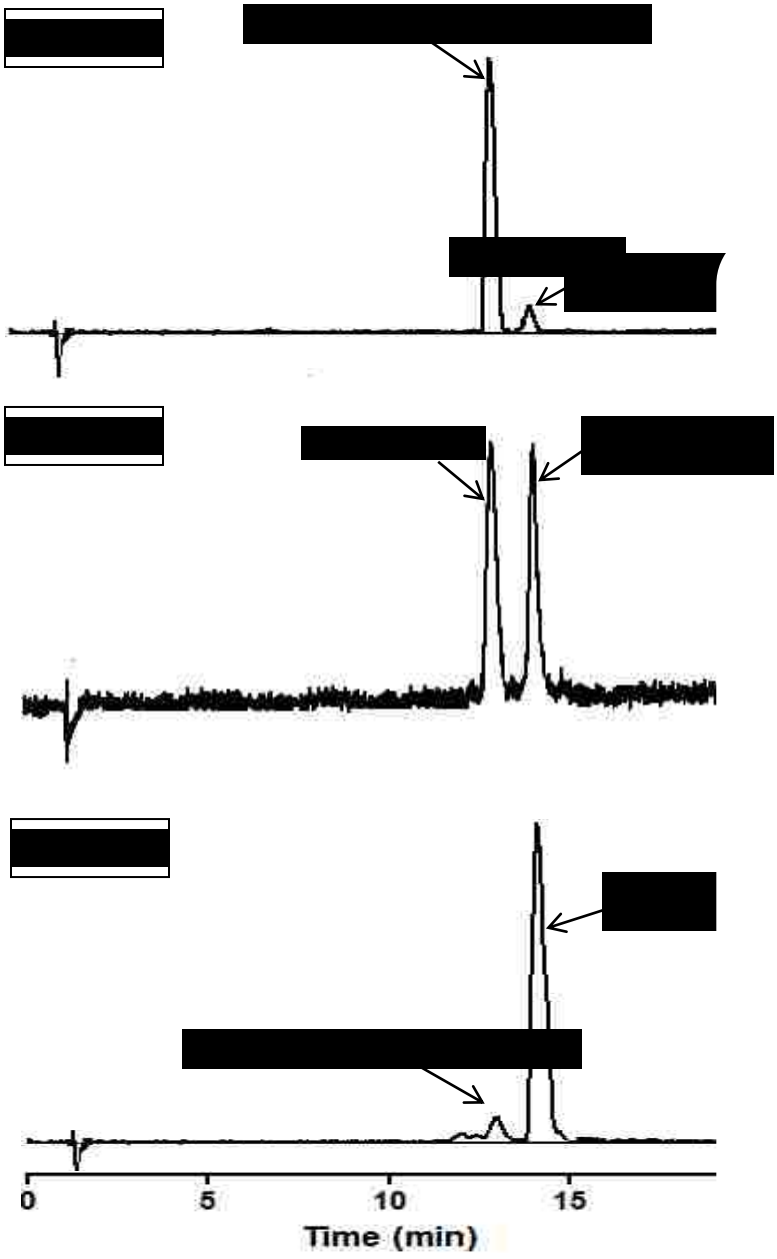


Figure 3: HPLC profile of the kinetics of KM-11 dimerization. The peaks containing the monomer (KM-11) and dimer (KM-12) are indicated. The procedure for dimerization and HPLC are outlined in the Materials and Methods.

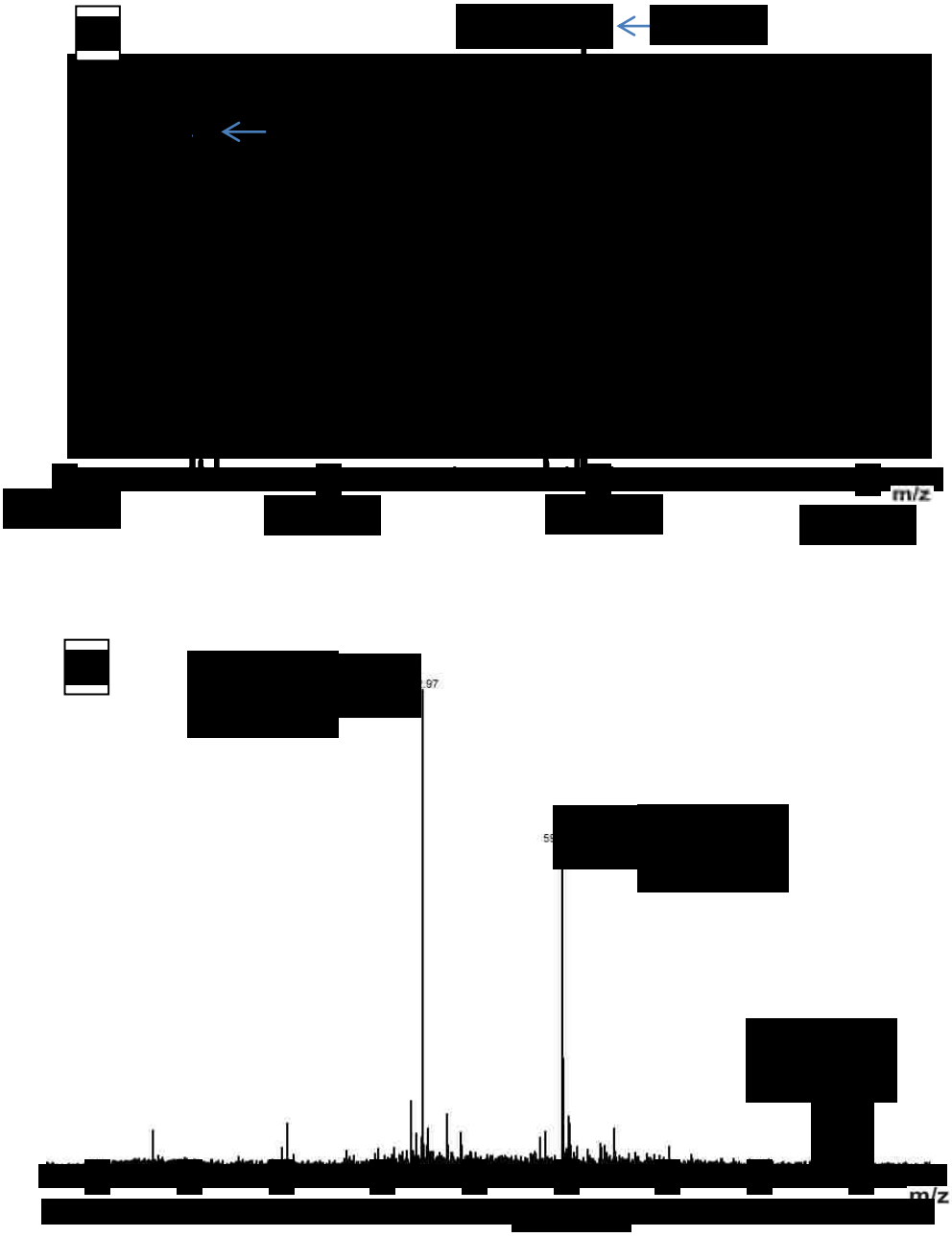


Figure 4: Mass spectrometry of KM-12 dimerization. Mass spectrometry was performed on the KM-12 peptide purified by HPLC as shown in Figure 3 using: (a) MALDI-TOF and (b) ESI spectrometry.

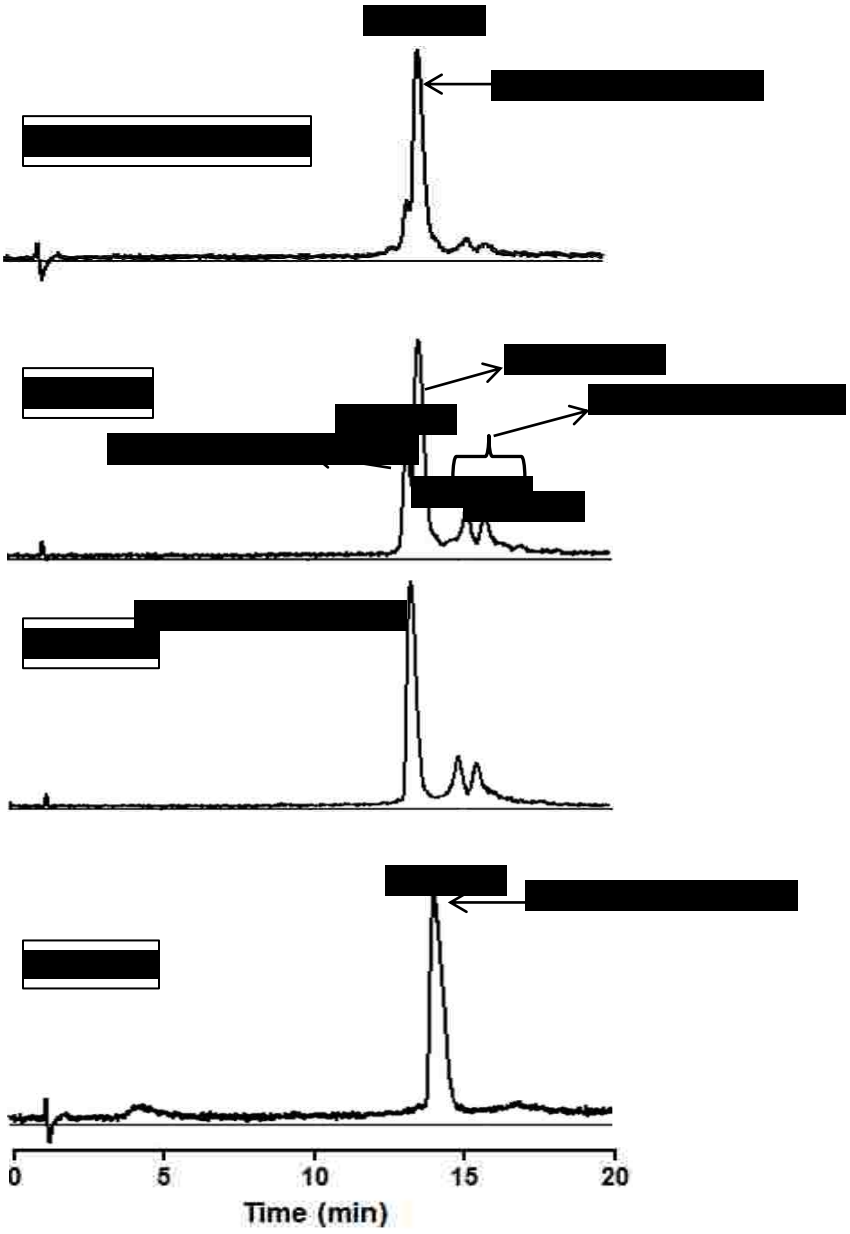


Figure 5: The HPLC profile of the kinetics of KM-17 dimerization. The peaks containing the linear monomer (KM-17), the circularized monomer (KM-18) and two dimer peptides (KM-19 and KM-20) are indicated. The procedure for dimerization and HPLC are outlined in the Materials and Methods. The addition of dithiothreitol (DTT) to the oxidized peptide sample was used to confirm the peaks were derived from KM-17 (Bottom panel).

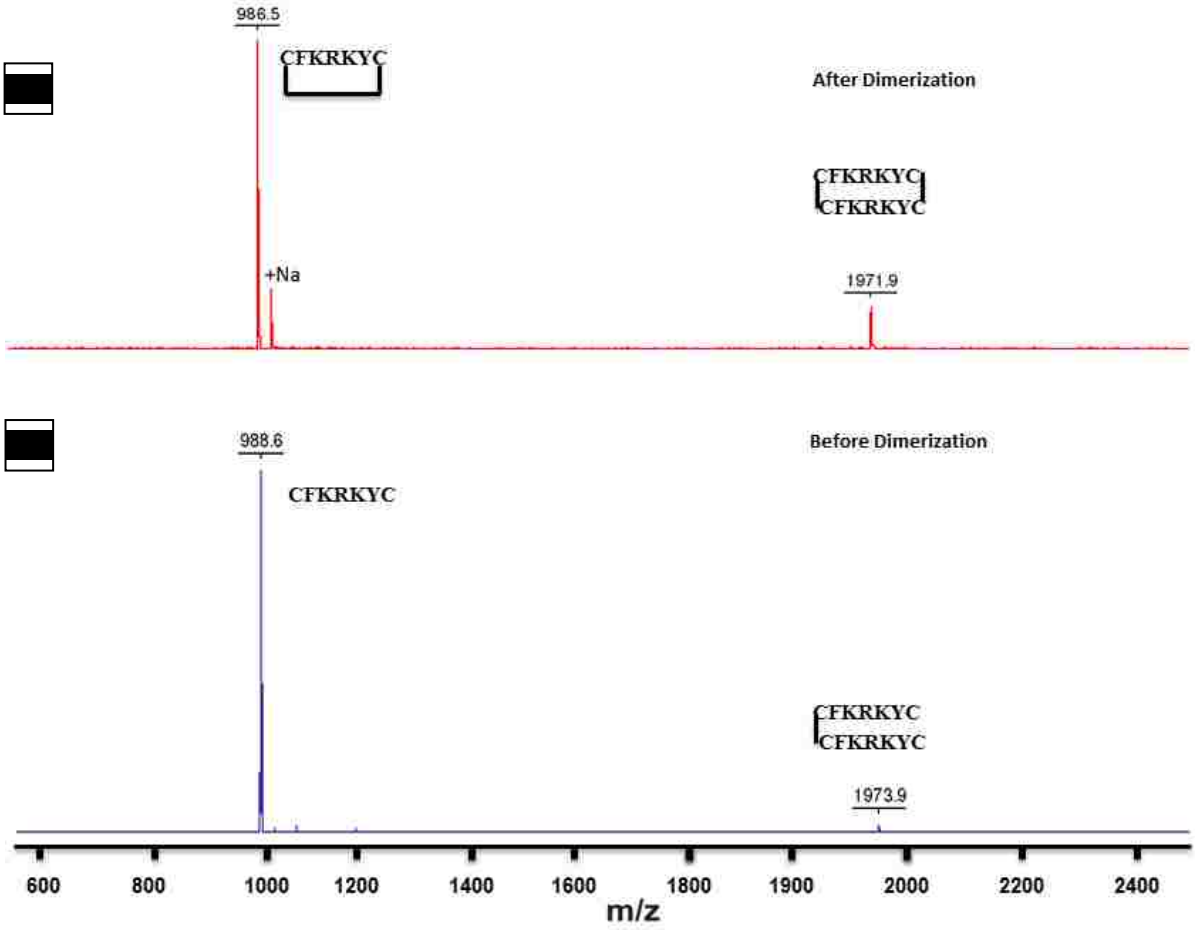


Figure 6: Mass spectrometry profile of KM-17 following oxidation of the peptide. MALDI data for the dimerization of KM-17 at initial concentration of 1mg/10 ml. (Panel A) After dimerization and (Panel B) before dimerization.

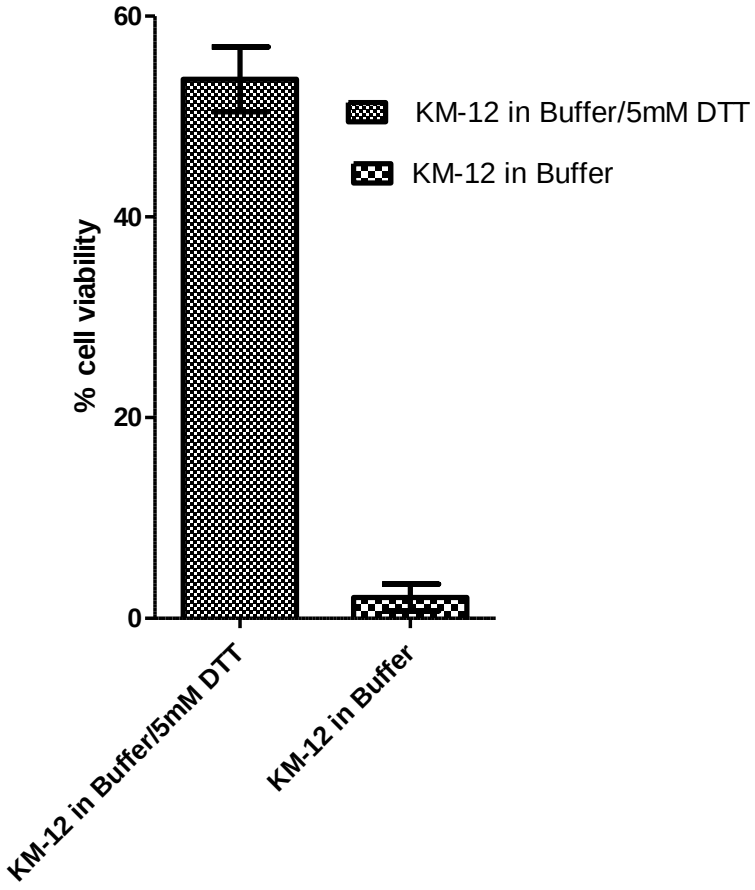


Figure 7: Effect of KM-11 dimerization on the antifungal activity. The KM-12 peptide (2.5 µM) was incubated with *C. albicans* (1.8×10^5 cells/ml) for 2 h at 37^oC in 10 mM sodium phosphate buffer pH 7.4 or in the same buffer containing 5 mM DTT. The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. The data represents the mean of three independent experiments with the error bars representing the standard deviation.

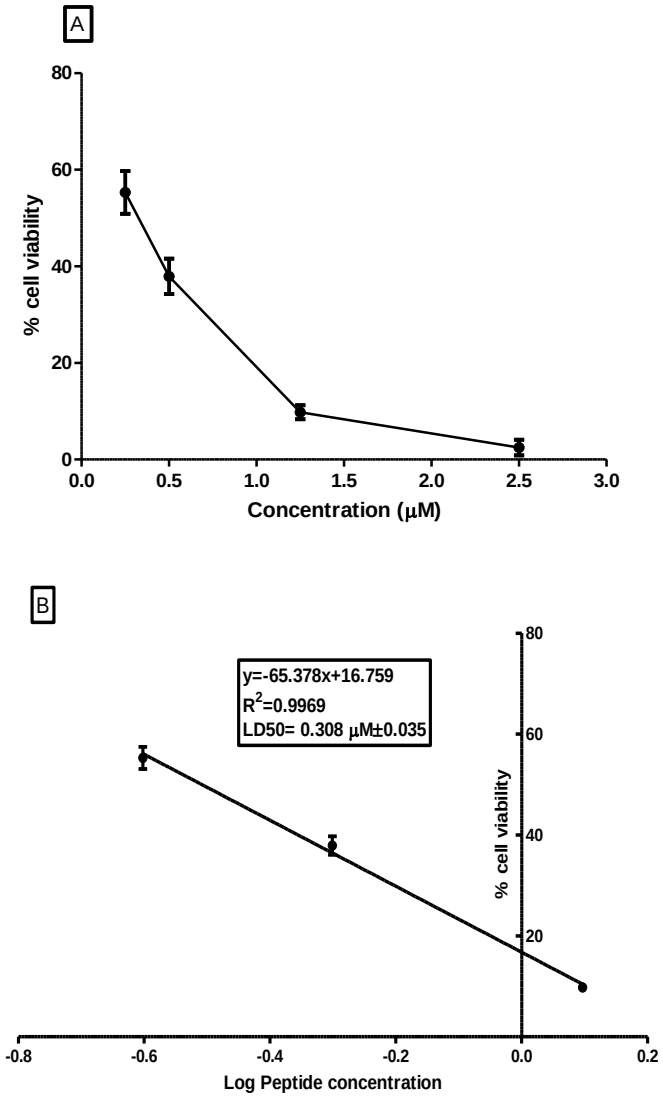


Figure 8: Dose-dependent fungicidal activity of KM-12. (A) Dose-dependent fungicidal activity of KM-12 against *C. albicans.* (B) Linear regression used for calculating the LD_{50} of KM-12 against *C. albicans*. Different concentrations of KM-12 were incubated with *C. albicans* (1.8×10^5 cells/ml) in 10 mM sodium phosphate buffer for 2 h at 37^oC. The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. The data represents the mean of three independent experiments with the error bars representing the standard deviation.

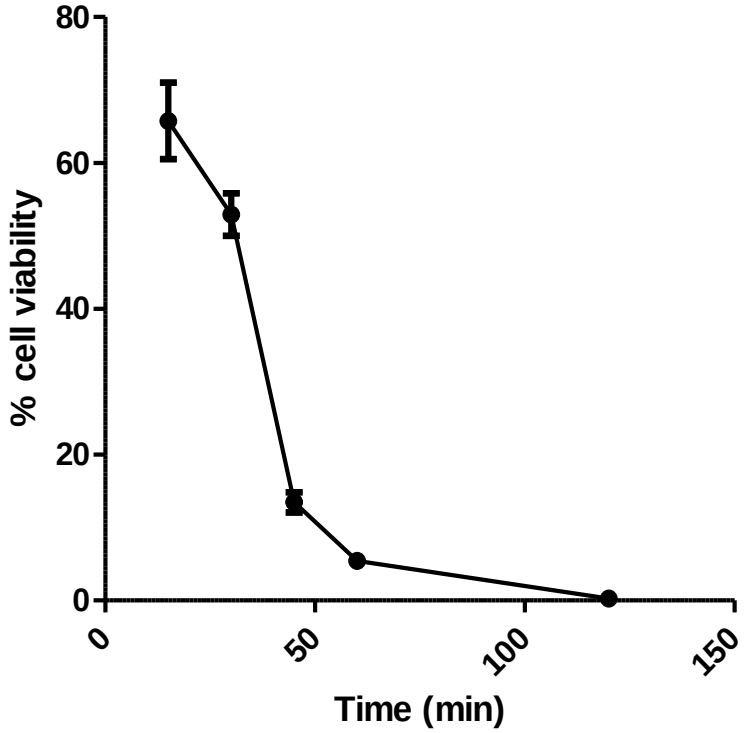


Figure 9: Kinetics of KM-12 fungicidal activity. The KM-12 peptide (2.5 µM) was incubated with *Candida albicans* (1.8×10^5 cells/ml) in 10 mM sodium phosphate buffer for different time periods (15, 30, 60 and 120 min). The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. The data represents the mean of three independent experiments with the error bars representing the standard deviation.

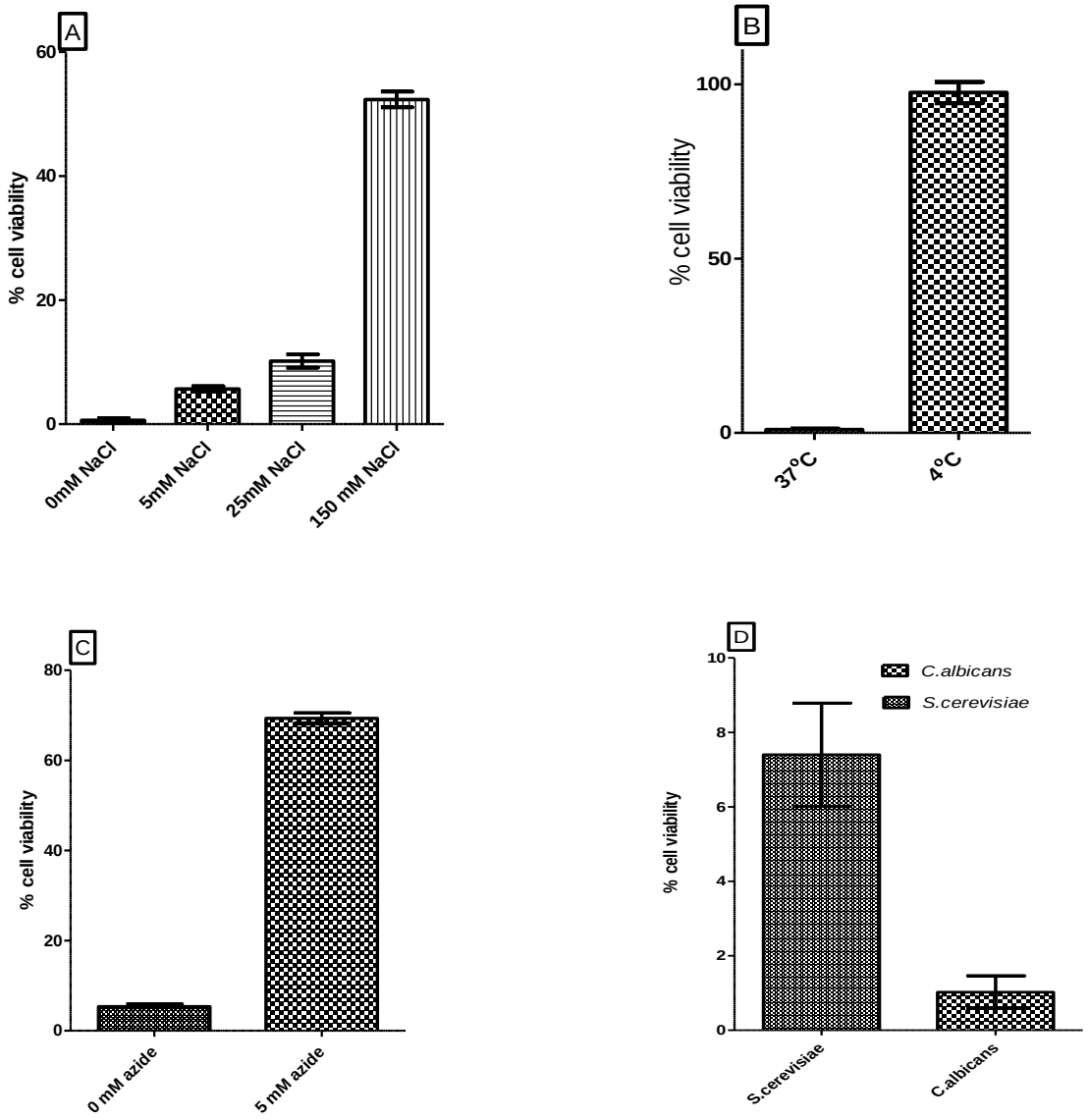


Figure 10: Characterization of KM-12 antifungal activity. The effect of: (A) Salt, (B) temperature, (C) respiratory activity, and (D) the fungicidal activity on *Saccharomyces cerevisiae* were examined by fungicidal activity assays. For each assay, 2.5 μM KM-12 was incubated with *C. albicans* or *S. cerevisiae* (1.8×10^5 cells/ml) in 10 mM sodium phosphate buffer for 2 h at 37°C. The percentage of viable cells was calculated as (viable colonies in the presence of peptide / viable colonies without peptide) × 100. The data represents three independent experiments and the error bar represents the standard deviation.

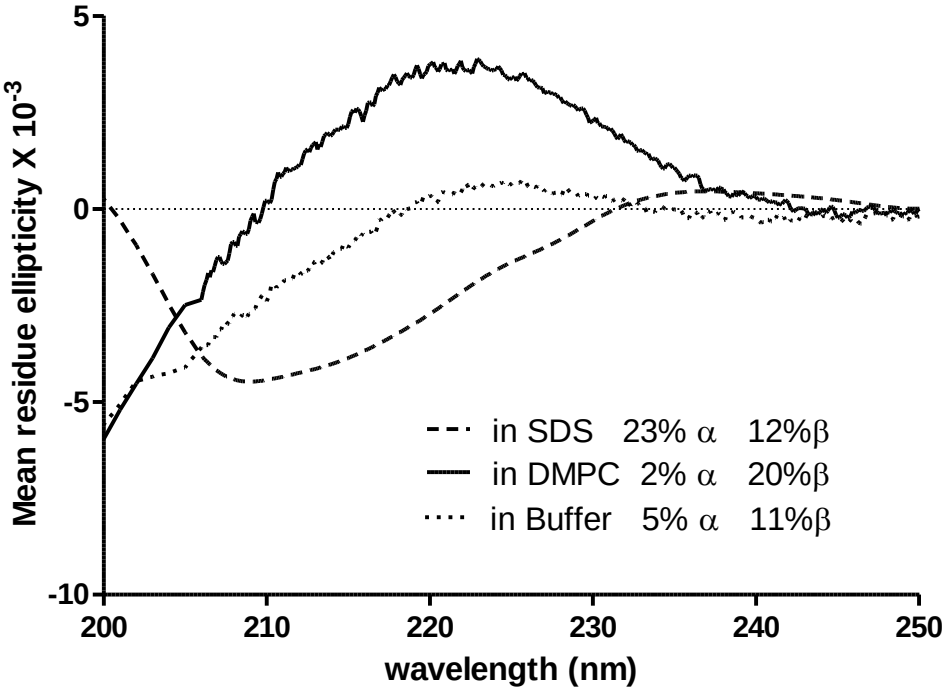


Figure 11: Circular dichroism spectroscopy of KM-12. CD spectra of KM-12 peptide in sodium phosphate buffer pH 7 as well as in the presence of SDS micelles and DMPC liposome were determined as outlined in the Materials and Methods. The calculated percentage of α-helix and β-turn is indicated. CD spectra are displayed in mean residue ellipticity [θ].

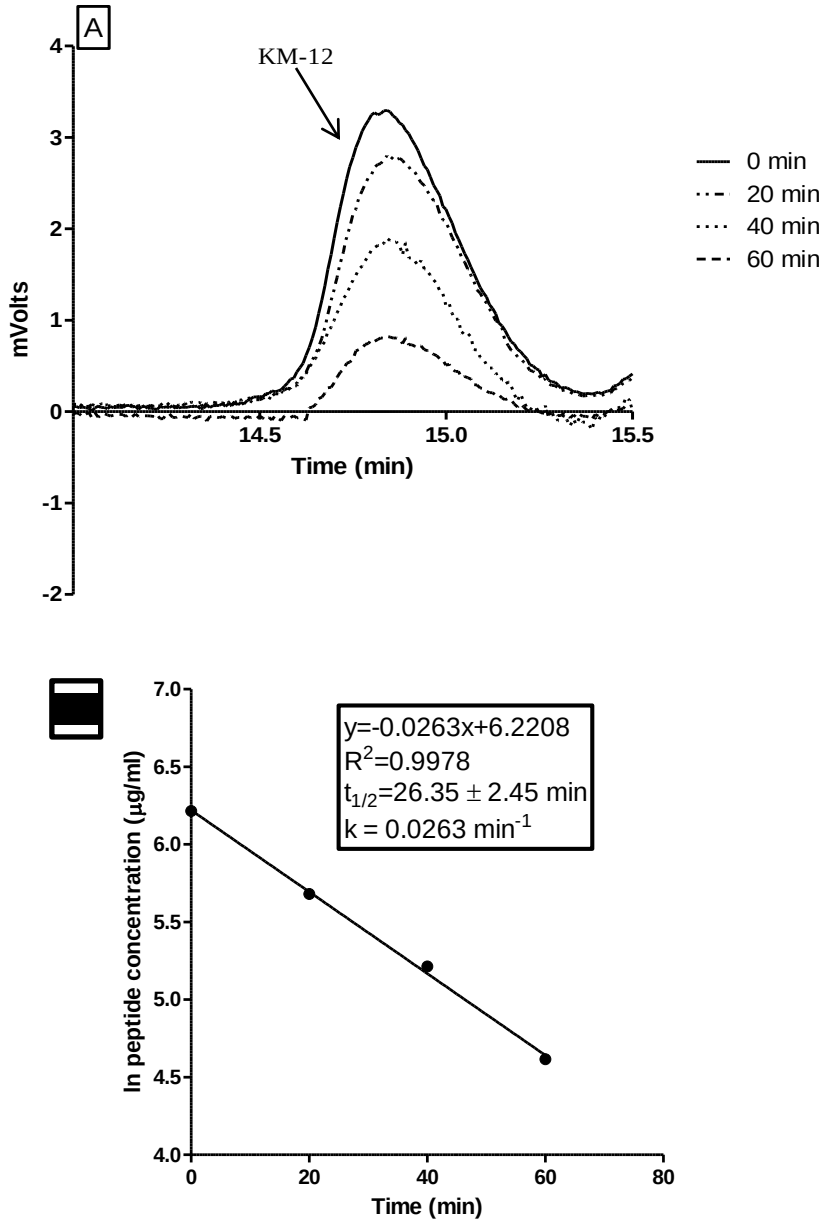


Figure 12: Stability of KM-12 in human saliva. A) HPLC profile of KM-12 following incubation with human saliva for the indicated times as outlined in the Materials and Methods. B) The determination of the half-life of KM-12 in human saliva.

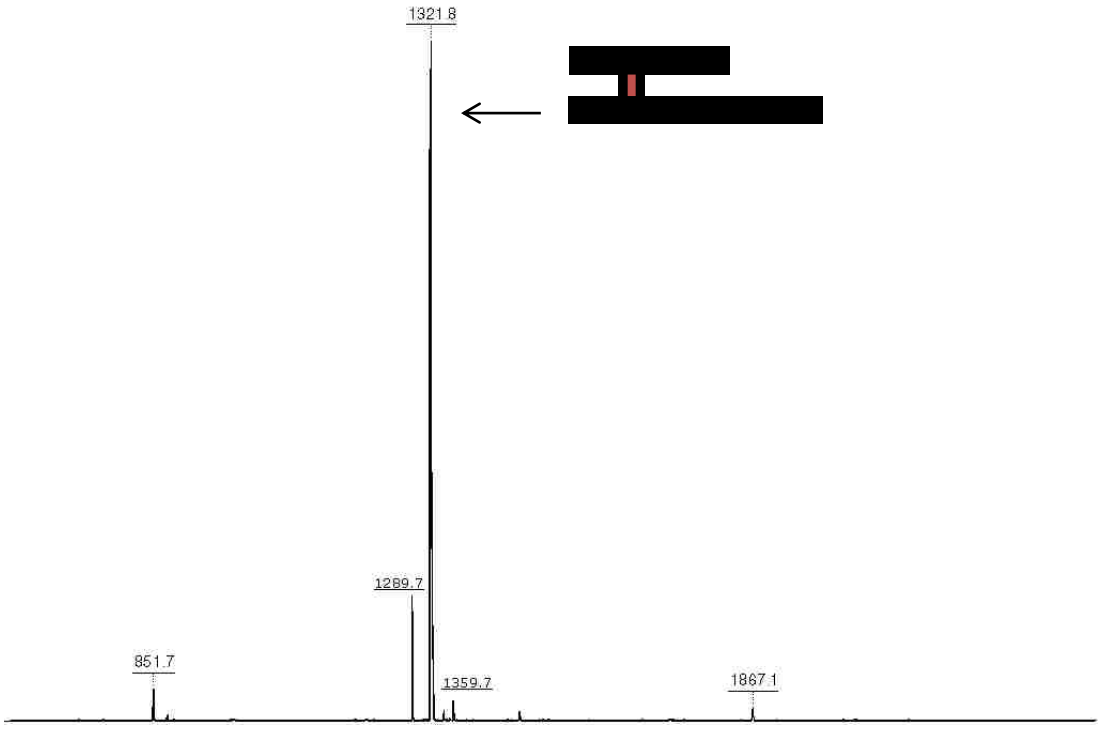


Figure 13: Mass spectrometry of KM-12 after exposure to human saliva. KM-12 was incubated with human saliva for 60 min, the reaction was terminated and subsequently mass spectrometry was performed to identify the cleavage site within the peptide. The putative cleavage product is shown.

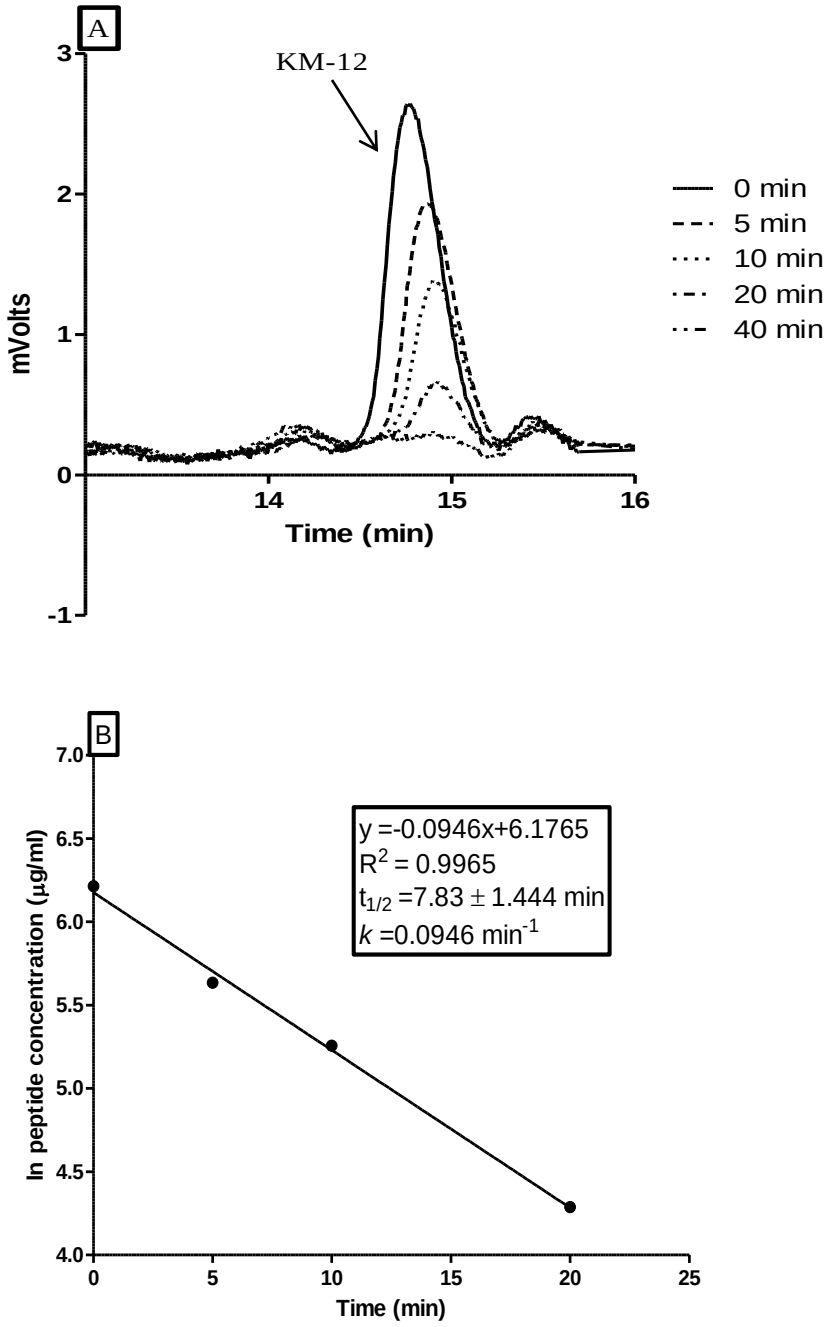


Figure 14: The stability of KM-12 in artificial gastric juice. A) HPLC profile of KM-12 following incubation with artificial gastric juice for the indicated times as outlined in the Materials and Methods. B) The determination of the half-life of KM-12 in artificial gastric juice.

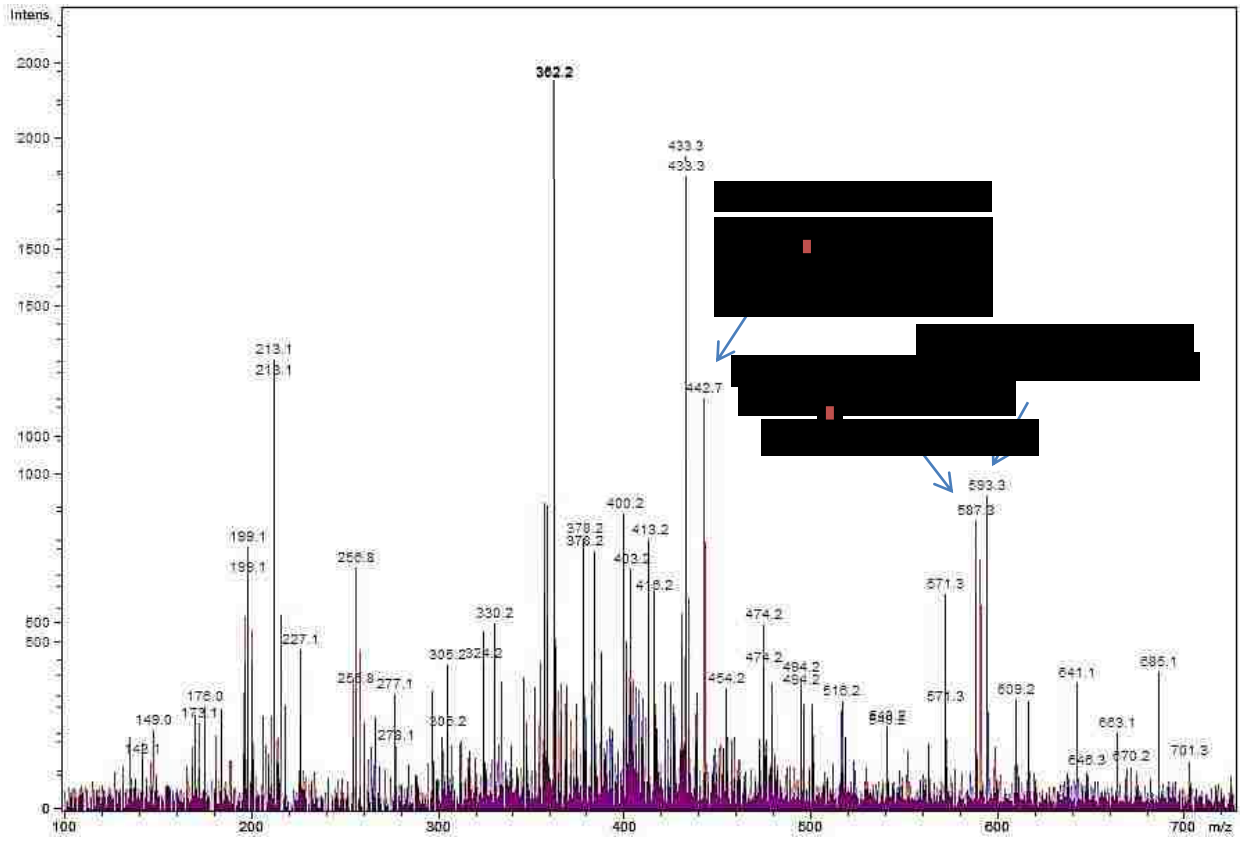


Figure 15: Mass spectrometry of KM-12 in artificial gastric juice. KM-12 was incubated with artificial gastric juice for 0 min and 60 min, the reaction was terminated and subsequently mass spectrometry was performed. The two samples were analyzed using ESI, and the data were merged together to identify the proteolytic fragments. The putative cleavage product is shown.

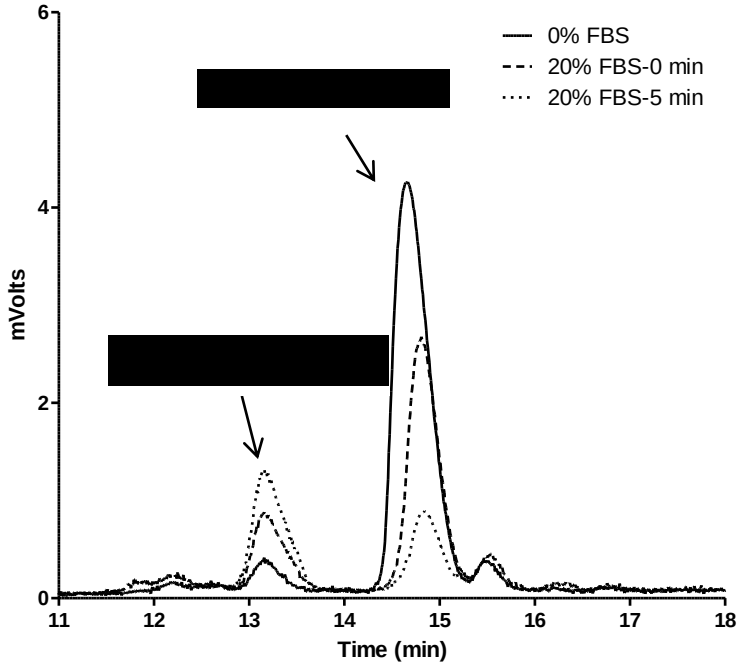


Figure 16: Stability of KM-12 in serum. KM-12 was incubated in 20% fetal bovine serum for 5 min, processed as described in Materials and Methods, and analyzed by HPLC. The KM-11 (monomer) and KM-12 (dimer) peaks are indicated.

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CHAPTER 5

Conclusions

Candida species are the most common fungal pathogens that affect humans [1]. Although *Candida* species are primarily considered commensal microorganisms of the gastrointestinal, urinary, and vaginal tracts of healthy individuals, they are also opportunistic pathogens and can be associated with high levels of mortality[2, 3]. The incidence of *Candida* infections are dramatically increased in immune compromised patients. For instance, research has shown 90% of HIV patients had oral candidiasis. In terms of the economic burden to the health care system, systemic *Candida* infections in the United States alone cost about 1.8 billion dollars and that accounts for 70% of the overall fungal infection costs [4]. Among all fungi, *Candida albicans* is the major cause of both mucosal and systemic human infections.

Currently there are four categories of antifungal drugs: azoles, polyenes, echinocandins, and antimetabolites. Each class has some limitations, and multiple cases of drug-resistant *Candida* have been identified for each category. At therapeutic concentrations, azole compounds are fungistatic and only a few members of this family of drugs can be used to treat systemic infections. There are multiple genes that have been identified that affect resistance to azole compounds in *C. albicans* (*MDR1, CDR1, CDR2*) [5, 6], *C. glabrata* (*CDR1, CDR2*)[7, 8], and *C. dubliniensis* (*MDR1 , CDR1*)[9].

The polyene compounds, such as amphotericin B, while highly effective in killing fungi, have a narrow therapeutic index, limiting clinical use to only the most severe cases where other antifungals are not useful. Renal failure and nephrotoxicity are common consequences of polyene treatment, and it has been reported that the rates of acute renal failure in patients on polyenes are between 49% and 65% [10]. Although fungal resistance to polyenes has not been a major clinical problem, multiple cases have been reported in *Candida* species, including *C. albicans*[11], *C. krusei*[12], *C. lusitaniea*[13], *C. glabrata* [14] and *C. tropicalis*[15].

Echinocandins are the most recently discovered class of antifungal drugs introduced for clinical use. Because of the relative high risk of acquiring resistance, echinocandins are not recommended to be used as an initial therapy [16, 17]. Echinocandin resistant strains of *C. albicans* [18], *C. krusi* [19], *C. glabrata* [20], *C. lusitaniae* [21] and *C. parapsilosis* [22] have been observed in patients.

Antimetabolites, such as 5-flucytosine, are considered fungistatic and the acquisition of resistance occurs at a high rate. Thus, their use as a monotherapy is restricted, but can be combined with other antifungal agents such as amphotericin B [23] to effectively treat patients as needed. Again, several cases of resistant *Candida* species have been reported [24-26].

The limited arsenal of antifungal drugs coupled with the growing number of drug resistant clinical isolates of *Candida* species stresses the need for the development of new antifungal agents with new mechanisms of action [27, 28]. The resistance to current drugs has, in part, resulted from the excessive use of antifungals as an approach to prevent fungal infections in immune compromised patients [29]. In addition, some *Candida* species exhibit inherent resistance to the current fungal drugs. For example, the majority of *Candida glabrata* clinical isolates are resistant to fluconazole at therapeutically permissible concentrations, and *Candida lusitaniae* is known to be resistant to amphotericin B[30].

The average length of time for a drug to reach the market and get FDA approval is approximately 15 years[31]. Therefore, the current focus on developing new antifungal drugs is a logic step before our current arsenal of drugs fail. Additionally, designing new drugs with novel

mode of action is essential. Antifungal drugs with novel mechanisms of action are less likely to be affected by current resistance mechanisms and subject to the possibility of cross-resistance.

This research has focused on designing, developing and characterizing a novel family of antimicrobial peptides which may serve as a model for new antifungal drugs. This family, named Kumar-McNabb antimicrobial peptides (KM), was developed utilizing natural antimicrobial peptide models. The KM peptides were developed in a multistep process: selecting histatin 5 as a natural model, studying the structural requirement for histatin 5 activity, identification of KM motif as the minimum functional domain of histatin, and finally utilizing the KM motif to design a family of new peptides.

Histatin 5 as a model for a naturally occurring peptide. Besides their role in innate immunity, antimicrobial peptides (AMPs) serve as promising candidates for new therapeutics. AMPs are attractive models because they possess unique features such as broad activity, rapid action, low microbial resistance and high selectivity [32]. In general, the major source of new AMPs is natural [33]. Therefore, the search for a model peptide began by searching for AMPs that exist in a common infection site for *Candida* species such as mucosal tissues, especially the oral cavity [34]. In the oral cavity, five different AMPs were identified; however, only three of them exhibited significant antifungal activity: HNP1-4 , histatins and β-defensins [35]. The histatins are a family of histidine-rich cationic peptides produced by salivary glands [36, 37] and are considered to be the first line of defense against *Candida* infections of the oral cavity [36]. Histatin 5, a member of the histatin family, has the most potent fungicidal activity among oral AMPs, and has been studies extensively for several years [38, 39].

Structural requirement of histatin 5 fungicidal activity. Histatin 5 is a histidine-rich peptide composed of twenty amino acids. Several studies have shown that the full length peptide is not required for fungicidal activity since an N-terminal truncated peptide, termed C-16 (a 16 amino acid fragment of histatin 5), retains the full activity [40]. Since histidine is the most abundant amino acid in histatin, it would be presumed that the antimicrobial activity of the peptide would be strongly affected by the pH of the environment. In fact, changing the pH from acidic to basic is enough to shift the net charge from +14 to +5. Surprisingly, histatin 5 and C-16 retain the same activity against *C. albicans* over a wide range of pH (5 - 9) which strongly suggests that the histidine residues may not be involved in the antifungal activity.

The optimal number of positive charges and their role in histatin 5 fungicidal activities, as well as the structural requirements for the fungicidal activity (represented by C-16 fragment) were studied in this dissertation. Initially, four C-16 analogs were synthesized, referred to as C-16 (W), retro-C-16 (R) and two additional peptides containing only D-amino acids. The fungicidal activities of the four peptides were examined against *Candida albicans,* as well as the effects of temperature, EGTA (a chelator of divalet cations), sodium chloride, pH and sodium azide. The stability in saliva and artificial gastric juice were also evaluated.

Our results have shown that all four peptides exhibited the same activity against *C. albicans*, and the results of killing assays in the presence of common histatin 5 inhibitors (NaCl, sodium azide, EGTA and low temperature) support the hypothesis that the analog peptides have the possibility to work through the same pathway as histatin 5. Furthermore, the results suggested that the mode of action doesn't involve any receptor-mediated activity as the retro peptide and the D-amino acid-containing peptides exhibited the same activity. Surprisingly, the four peptides were also structurally similar and contained the same percentage of alpha helix in the presence of 50%

of TFE). The secondary structure studies of the retro and wild peptides using CD spectroscopy showed that the retro and wild peptide largely adapt a random coil structure in aqueous solution. The only difference observed between these peptides was the stability in gastric juice where the C16 peptide was more stable than the retro peptide.

These initial studies yielded several important results. First, the antifungal activity of histatin 5 is likely to be related to the spatial relationship of the amino acids within the peptide rather than the specific N-to C-terminal sequence or stereospecificity. Secondly, the antifungal activity was shown to be dependent on the positive charges that come from arginine and lysine residues, but not likely histidine. Thirdly, the characteristics of peptide's killing activity and mode of action imply that the four derivatives achieved the activity via same mechanisms pathways. Finally, our studies and other's indicate that a smaller region within histatin-5 derivatives may have potent antifungal activity.

Identification of the KM motif, the functional domain of histatin 5. One of the general problems utilizing therapeutic peptides as therapeutic agents is cost effectiveness. In order to utilize a peptide as a commercial drug it must economically feasible to prepare. Over the past two decades, several attempts have been carried out to optimize histatin 5 and attain the smallest active fragment. P-113, a12 amino acid peptide, is the most effective and smallest fragment of histatin 5-derived peptide developed to date.

After our studies with the retro C-16, we noted the presence of a small symmetrical sequence within C-16 peptide that would not be affected by the orientation of the amino acid sequence (retro or normal), and this fragment was (-YKRKF- , referred to as Kumar-McNabb or KM motif). Furthermore, this sequence contained three positively charged residues that were

required for the activity. The role of this KM fragment in histatin 5 anti-fungal activity was investigated by establishing a sequence comparison using published histatin 5 fragments and known analogs. The result revealed that the KM motif is found in every active fragment and any change in KM sequence lead to the abolishment or a decrease in the activity.

The function of the KM motif in antifungal activity was evaluated with two pentameric peptides, KM-5 and KM-6. Both peptides contained the same amino acid composition as the KM motif; however, KM-5 had the retro amino acid sequence (FKRKY) while KM-6 (YKRKF) had the normal sequence found in histatin 5. It is important to mention that those pentamer peptides were acetylated and amidated to improve the stability. The activity of the pentamers against *C. albicans* was evaluated and compared to C-16 peptide. Later, the similarities in the mechanism of action were compared to the histatin 5 C-16 peptide by evaluating the effect of sodium chloride, sodium azide, low temperature, as well as the activity against *S. cerevisiae*. Finally, the general characteristics of KM-5 activity were measured by determining the LD_{50}, the kinetics of fungicidal activity, and the effect of pH.

Our results show that the KM motif is the shortest active fragment in histatin 5, and is less than half the size of P-113. The activity of KM-5 is not affected in the retro orientation (KM-6) and that may explain why the retro C-16 peptide retained full activity as C-16. The characteristics of KM-5 fungicidal activity were similar to C-16 peptide and histatin 5. Moreover, like histatin 5, active respiration is also required for optimal KM-5 activity. By synthesizing several KM-5 analogs, it was found that any interference with the positively charged amino acid residues reduces the anti-fungal activity.

Although KM-5 (LD_{50}=5.5 µM) is less active than the C-16 peptide (LD_{50}=3 µM), it is much smaller and would be more economically efficient. Our results, supported by published data, suggest that the KM motif might be the functional motif of histatin 5 and the C-16 peptide. This study is not the first to report the presence of a common sequence in some histatin 5 fragments. KRKFHE has been reported as a common motif in the active fragments but its activity has not been evaluated [41]. Also KM-5 is not the only antimicrobial peptide with five amino acid in sequence , several small anti-fungal[42-44], antibacterial[45, 46]and antiviral [47]peptides have been reported including the antifungal drug family, the echinocandins.

Utilizing KM motif to design a new family of antimicrobial peptides. In this study, we developed the KM-12 peptide, a potent antifungal peptide, utilizing KM-5 as a model. KM-12 is a homodimeric peptide composed of two KM motifs, where a cysteine residue was introduced to the N-terminus of KM-5 to facilitate dimerization through disulfide bond formation. It is important to mention that the addition of cysteine didn't interfere with activity as the monomer, which retained the same activity as KM-5. With dimerization we achieved several targets. Firstly, KM-12 peptide has a high probability to work via the same mechanism like histatin 5 as the dimerization did not interfere with KM motif sequence. Secondly, from a cost effectiveness point of view, KM-12 is cheaper to make than any other 12mer peptide because only a six amino acid peptide (monomer) is required. Finally, the dimerization improved the activity approximately 10-fold over the naturally occurring histatin 5.

Although several analogues were synthesized to improve fungicidal activity and salt tolerance, it was found that the KM-12 sequence was the optimal. The studies of KM-12 antifungal characteristics support the theory that KM-12 might work through the same mechanism of action as histatin 5. KM-12 activity was inhibited by histatin 5 inhibitors such as sodium azide, sodium

chloride and low temperature. However, there were some differences noted. The kinetics of yeast killing is faster with KM-12 than histatin 5 as KM-12 achieved 95% loss of viability in one hour while histatin 5 achieved the same percentage killing after two hours. Furthermore, 2.5 µM KM-12 had the ability to kill 95 % of *S. cerevisiae* on fermentable media while histatin 5 has failed, as it required 25µM histatin 5 C-16 peptide to achieve 50% killing activity.

Focusing on the potential of KM-12 in the fungal drug market, the spectrum of KM-12 activity against various *Candida* species was evaluated. KM-12 was tested against *C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. lusitaniae, C. kefyr and C. dubliniensis*. Except for *C. glabrata*, KM-12 exhibited a potent activity against all Candida species, including those that displayed fluconazole resistance (*C. tropicalis* and *C. krusei*).

Regarding the possibility of manufacturing KM-12 peptides on a large scale, the synthesis protocol and dimerization procedure were optimized. The optimal resin cleavage conditions were achieved using a cleavage cocktail of 90% TFA, 5% Thioanisol, 3% Ethandithiol, and 2% anisole. The optimal incubation time for cleavage was 4 hours and the optimum precipitation buffer after cleavage was diethyl ether.

Because we sought an antimicrobial peptide for pharmaceutical application, the stability in human fluids was evaluated using KM-12. As all AMPs, KM-12 was unstable in simulated gastric juice and saliva. The $t_{1/2}$ of KM-12 in gastric juice and saliva was 7.8 and 26 minutes, respectively. We used mass spectrometry to identify the cleavage sites within KM-12 when exposed to saliva and gastric enzymes, creating the possibility of resolving this problem via the use of D-amino acids at the cleavage sites.

The negative aspects of KM-12 are the fact that the disulfide bond linking the dimer is liable in serum and the peptide is highly sensitive to salt concentration. One solution for resolving the instability of the peptide in serum is to remove the cysteine residues and generate a ten amino acid peptide. Salt sensitivity is a general problem in all cationic antimicrobial peptides. The effect of salt was obvious using RPMI-1640 medium to simulate physiological conditions. The fungicidal activity of KM-12 was improved by diluting the RPMI-1640 medium to reduce the salt content. Although KM-12 is salt sensitive, it's still more active in the presence of salt than histatin 5. The salt sensitivity and instability in serum may narrow the usage of KM-12 to topical application.

After we designed the KM-11 peptide (CFKRKY), a search on the database using the sequence revealed that a similar sequence is found in multiple peptides, such as the C-terminus of MtDef4 peptide (FRRRCF) and the N-terminus of Lactoferricin B (FKCRRW). In spite of MtDef4 containing 46 amino acid, any interference with the C-terminal region inhibits the activity against plant fungi *Fusarium graminearum* [48]. Research on Lactoferricin B has shown that the N-terminal sequence has the ability to suppress *Candida* cell growth [49] . Interestingly in both peptides, the disulfide bond is not essential for the activity.

In summary, this study focused on developing a new family of antimicrobial peptides, later referred to as Kumar-McNabb (KM) peptides, utilizing the histatin 5 peptide model. This family is designed to serve as a model for the development of additional peptides that serve as therapeutic agents for the treatment of Candida infections. Although a promising prototype peptide was designed and evaluated, future research is needed to achieve maximum benefits and solve problems associated with the prototypic peptide, KM-12.

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