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Development of Novel Antifungal Peptides Based on a Natural Model of Histatin-5 Peptide

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ABSTRACT

Our research group is working toward the development of novel antifungal peptides based on a natural model of peptide histatin-5. Histatin-5 is found in human saliva and known to protect our body against oral infections by *Candida* species. Candidiasis, or an infection caused by *Candida* species, is considered one of the most medically important fungal infections worldwide. Blood stream infections caused by *Candida* species are the fourth leading cause of hospital-acquired fungal infections that is associated with high mortality rates and high costs of treatment. This study investigated the modes of action of histatin-5 with the use of one 16-mer derivative lacking eight amino acids from the N-terminus of the native histatin-5 and three enantio, retro and retroenantio 16-mer analogs. All four derivatives showed significant fungicidal activity with *Candida albicans* via mechanisms independent of SSA2p hypothesized to be the histatin-5 receptor on the plasma membrane of *C. albicans*. All four derivatives were shown to target artificial yeast phospholipid membranes and their killing activities involved the electron transport chain or respiration of the yeast cells. The data obtained from this study suggested the existence of a short five amino acid sequence within the 24-residue sequence of histatin-5 peptide that may have fungicidal properties. Several novel peptides were generated by the dimerization of the 5-mer amino acid sequence and these peptides were shown to have strong antifungal activities. *In vitro* assays were used to investigate potential toxicity of the new antifungal peptides to mammalian cells and these peptides were shown to be nontoxic to NIH3T3 murine fibroblasts and sheep red blood cells at concentrations up to 100μM. The testing of acute toxicity and immunogenicity of some of these peptides in mice was also performed to obtain data on the *in vivo* tolerance of animals. The results of this work are presented in the following chapters of this dissertation.

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Finally, I am thankful for my parents, my wife and my daughter. Their presence, support and sacrifices helped me very much in completing this dissertation.

DEDICATION

This dissertation is dedicated to my family, especially to my parents whose unconditional sacrifices and support for my endeavor in the field of science and medicine can hardly ever be repaid.

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LIST OF ABBREVIATIONS

5-FOA	5-Fluoroorotic acid
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ddH2O	double-distilled water
DNA	Deoxyribonucleic acid
DPPC	1, 2-dipalmitoyl-sn-glycero-3-phosphocholine
DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
ETC	Electron tranport chain
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaPB	Sodium phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDVF	Polyvinylidene fluoride
PI	L-α-phosphatidylinositol
SC-Ura	synthetic complete medium lacking uracil
WST	Water soluble tetrazolium salt
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

CHAPTER 1

INTRODUCTION

A. Infections caused by *Candida* species in humans

Candidiasis is one of the most common fungal infections in humans and it is caused by the *Candida* species. *Candida* species are commensal organisms normally found on the skin and in the cavities of the human body (Skinner & Fletcher, 1960); however, they can become pathogenic when the normal defense or flora of host subjects is disrupted by disease or immunosuppression. Among pathogenic *Candida* species, several entities are commonly implicated in human candidiasis such as *Candida albicans, Candida glabrata, Candida parapsilosis* and *Candida tropicalis* (Hazen, 1995, Lewis, 2009). The clinical settings of candidiasis can vary from mild to severe depending on the sites of infection and status of the host immune system. Several common forms of human candidiasis encountered in clinical practice are thrush (oropharyngeal/ esophageal candidiasis), vaginal yeast infections (genital/ vulvovaginal candidiasis) and invasive candidiasis (blood stream infections).

Thrush caused by *Candida* species is a form of mucosal infection. It can occur in very low- birth-weight (< 1000g) neonates (Faix *et al.*, 1989, Saiman *et al.*, 2000), elderly or immuno-compromised individuals such as those who are being treated with immunosuppressive drugs, cytotoxic chemotherapy or radiotherapy (Ellepola & Samaranayake, 2001). Clinical signs and symptoms of thrush caused by *Candida* species, especially *C.albicans*, include white plaques on oral or pharyngeal mucosa that are usually painless. However, fissures at the corners of the mouth could be found and those lesions could be very painful (Harrison, 2005). Oral thrush can spread to adjacent anatomical structures of the oral cavity such as the esophagus and cause difficulties in swallowing (Vazquez & Sobel, 2002). Mucosal candidiasis can progress into more invasive forms if the integrity of the affected mucosa is disrupted by trauma, surgery, procedures, etc. (Harrison, 2005)

Vaginal yeast infections are another common form of candidiasis in adults. *Candida* vaginitis is characterized by a local itching and burning sensation coupled with a white scanty vaginal discharge (Sobel, 2007). The condition is common among adult female population. It was estimated that approximately two-third of American adult females experienced at least one episode of vulvovaginal candidiasis during their life time (McCormack *et al.*, 1994). In one epidemiology study done via telephone interview of more than 2000 American women aged 18 year or older, 6.5 percent reported at least one episode of suspected *Candida* vaginitis in the previous 2 months period. The study estimated the annual cost for the treatment of *Candida* vaginitis to be approximately 1.3 billion USD (Foxman *et al.*, 2000).

Among the most severe forms of candidiasis is *Candida* blood stream infections which are a health problem in the United States and worldwide. *Candida* species were reported to be the fourth leading cause of nosocomial bloodstream infections in a group of 24,179 patients in 49 US hospitals during a 7-year study period (Wisplinghoff *et al.*, 2004). Other epidemiology studies reported the incidence of invasive candidiasis in the range of 6-24 cases/100,000 US populations/year during the period of 1998-2000 (Hajjeh *et al.*, 2004, Diekema *et al.*, 2002). At the worldwide level, the incidences of invasive candidiasis in European countries such as Iceland and Denmark were reported to be 4.9 and 11 cases/100,000 populations/year, respectively (Asmundsdottir *et al.*, 2002, Arendrup *et al.*, 2005). Invasive candidiasis was shown to be associated with mortality rate up to 40% (Gudlaugsson *et al.*, 2003, Bougnoux *et al.*, 2008) and a high cost of treatment. The annual treatment cost for blood stream candidiasis was estimated to be at least 200 million USD/year in the US (Rentz *et al.*, 1998) and 520 million AUD/5 years in Australia (Slavin *et al.*, 2004). For the above reasons, candidiasis is an important fungal infection in humans that should be dealt with seriously.

B. Structures of the cell wall and plasma membrane of *C.albicans*

     C. albicans is a diploid yeast (Jones *et al.*, 2004) that can exist in either the yeast or hyphal form. The latter is thought to be essential for the virulence of *C. albicans* via the up-regulation of virulence factors such as adhesin and protease (Lane *et al.*, 2001, Nantel *et al.*, 2002). *In vitro* studies suggested that the hyphal form was more invasive than the yeast form (Dalle *et al.*, 2010) and it was found in the epithelial cells of patients suffering from cutaneous candidiasis (Scherwitz, 1982). In terms of structure, both forms have a cell wall composed of chitin, β-glucans and mannoproteins, with 50-60% of the weight of the cell wall being composed of β-glucans while mannoproteins and chitins account for 30-40% and 0.6-3%, respectively (Chattaway *et al.*, 1968, Sullivan *et al.*, 1983). Current models suggest that the β-(1,3) glucan molecules are bound together by hydrogen bonds in the cell wall of *C. albicans* and that those molecules form a scaffold for mannoproteins and chitins to bind (Kapteyn *et al.*, 2000, Kapteyn *et al.*, 1995). Electron microscopy studies have suggested that the *C. albicans* cell wall has a fibrillar network outside its outermost layer and that this fibrillar network could play a role in the adhesion of *C. albicans* to the host cell (Tokunaga *et al.*, 1986)

     Like other eukaryotic cells, *C. albicans* has a phospholipid plasma membrane with 7-10% of the dry weight of the plasma membrane reported to be phospholipids and the phospholipid composition of the plasma membrane varied depending on its biological forms (Marriott, 1975). Thus far, the neutral phosphatidyl ethanolamine was reported to be among the most abundant phospholipids of *C. albicans* plasma membrane in both the yeast and hyphal forms (Marriott, 1975). The molar ratio of ergosterol to phospholipids in the plasma membrane of *C. albicans* has not been reported in the literature, however, this ratio may be close to the value of 0.5 currently known for other fungal organisms (van Meer *et al.*, 2008). *In vitro* study with artificial

membranes that contained ergosterol at different concentrations suggested that the presence of ergosterol improved the fluidity of phospholipid membranes (Hsueh *et al.*, 2005). Ergosterol is, therefore, an important component of the plasma membrane in fungi and is among the targets of some antifungal agents such as the polyene and azole drugs.

C. Mechanisms of action of current antifungal agents

The treatment of candidiasis and other fungal infections is currently based on the use of antifungal agents grouped as polyenes, azoles, pyrimidine analogues and echinocandins. These drugs have different targets on the *Candida* cells and they can act via the disruption of the yeast cell wall, the inhibition of ergosterol or β-glucan synthesis, or the disruption of RNA and DNA synthesis in the yeast cells. The use of these drugs separately or in combination has been effective in the treatment of many patients suffering from candidiasis.

Polyenes, such as amphoterin B or nystatin, are antifungal agents which kill fungi by forming pores in the plasma membrane after binding to ergosterol molecules on the phospholipid bilayer *(Figure 1)*. Data obtained from studies with artificial membranes suggested that amphotericin B and nystatin formed pores of approximately 4 angstroms in radius in the phospholipid bilayers (Holz & Finkelstein, 1970, Holz, 1974). One study with sterol-containing artificial phospholipid membranes suggested that the transition of amphotericin B from the monomeric to the aggregate state in the phospholipid bilayer occurred when its relative concentration to lipids reached a threshold and the transition coincided with the ion channel activity of the membranes (Fujii *et al.*, 1997). The formation of pores on target membranes ultimately results in the disruption of cellular integrity and death of yeast cells (Liao *et al.*, 1999, Phillips *et al.*, 2003).

Unlike polyenes, which work via pore formation, azole agents inhibit the 14-α demethylase enzyme involved in the synthesis of ergosterol, an important constituent of fungal biomembranes (Yoshida & Aoyama, 1987, Hitchcock *et al.*, 1990) *(Figure 1)*. These azole agents were shown to have a high affinity for cytochrome $P450_{14DM}$ (now called Erg11p), the enzyme responsible for the 14 α-demethylation reaction of lanosterol. Furthermore, the azoles were shown to form complexes with the heme iron of cytochrome $P450_{14DM}$ (Yoshida & Aoyama, 1987, Hitchcock et al., 1990). The azoles are the most commonly used antifungal agents and they have fungistatic effects on many fungi; however, they do display fungicidal activity on *Cryptococcus neoformans* and *Aspergillus fumigatus* (Klepser *et al.*, 1998, Lass-Florl *et al.*, 2001).

Beside the polyenes and azoles, the pyrimidine analogs, such as fluorocytosine, exert antifungal activities by disrupting RNA chain elongation and inhibiting DNA synthesis in susceptible fungal cells (*Figure 1*). Fluorocytosine synthesis was first reported in 1957 (Duschinsky, 1957) and the drug was used to treat candidiasis and cryptococcosis several years later (Tassel & Madoff, 1968). 5-Fluorocytosine is known to enter the yeast cells and be converted into 5-fluorouracil and then 5-fluorouridylic acid. 5-Fluorouridylic acid is then phosphorylated before being incorporated into RNA and results in the disruption of protein synthesis (Polak & Scholer, 1975). Another metabolite of 5-fluorouracil is 5-fluorodeoxyuridine monophosphate that is known to inhibit the enzyme thymidylate synthase (Diasio *et al.*, 1978). 5-Fluorocytosine is rarely used in monotherapy, rather it is usually employed in combination with other antifungal agents to achieve better treatment of fungal infections.

Recently added to the list of antifungal agents are the echinocandins that have a cyclic peptide core and a lipid side chain. Echinocandins are potent antifungal agents known to block

the formation of 1, 3 β-D glucan of fungal cell wall via noncompetitive inhibition of the enzyme 1, 3 β-D glucan synthetase (Sawistowska-Schroder *et al.*, 1984) *(Figure 1).* 1,3 β-D glucan is an important component of the yeast cell wall. Further studies identified *FKS1* as the gene that encoded for a transmembrane subunit involved in the enzymatic activity and susceptibility of the enzyme 1, 3 β-D glucan synthetase to echinocandins (Douglas *et al.*, 1994). Some *FKS1 Candida* mutants have been identified to have reduced susceptibility of the enzyme 1, 3 β-D glucan synthetase to echinocandins fungicidal activity (Park *et al.*, 2005). Three members of the echinocandin group, namely caspofungin, micafungin and anidulafungin, currently have the approval from the Federal Drug Agency for the treatment of human candidiasis. These echinocandins are currently being used in severe cases of candidiasis, fluconazole-resistant candidiasis or in cases of candidiasis in which the patients are intolerant to fluconazole (Pappas *et al.*, 2009).
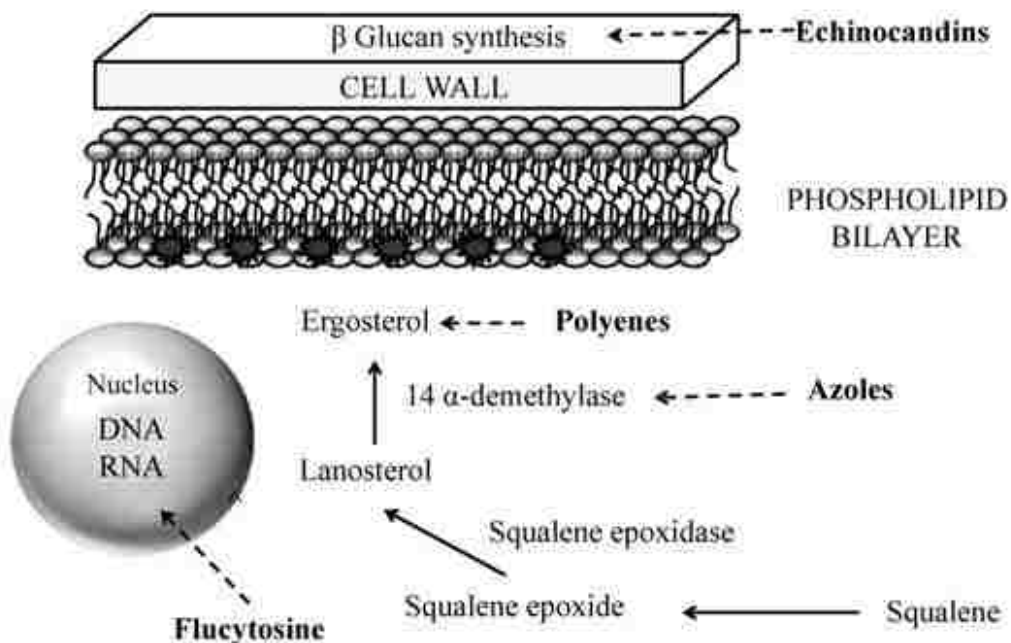

Figure 1. Modes of action of current antifungal. Schematic diagram of the mechanism-of-action of the Echinocandins, Polyenes, Azoles, and Flucytosine as described in the text.

D. The emergence of drug-resistant *Candida* species and the need to develop new antifungal agents

Despite the availability of current antifungal agents, the emergence of drug-resistant *Candida* strains has been an alarming finding. *Candida species*, like other medically important fungi, could develop mechanisms that help them evade the killing action of antifungal agents. Modifications in ergosterol biosynthesis, for example, were known to be used by *Candida lusitaniae* to escape the antifungal action of the polyenes such as amphotericin B (Young *et al.*, 2003). Some clinical isolates with very low ergosterol content in their phospholipid membranes were reported to have reduced susceptibility to amphotericin (Dick *et al.*, 1980, Nolte *et al.*, 1997). In terms of resistance to azoles agents, up-regulation of drug efflux transporter proteins encoded by *CDR1* and *CDR2* were detected in azole-resistant isolates of *C. albicans* (Sanglard et al., 1995, Sanglard et al., 1997). The increased expression of these efflux transporter proteins are believed to reduce intracellular accumulation of azole s and confer azole-resistance in some *Candida* species. In addition to the up-regulation of ATP binding cassette transporters, mutation of the *ERG11* gene, which encodes for the enzyme 14α-demethylase, is thought to mediate the resistance of some *Candida* strains to azoles (Marichal *et al.*, 1999, Perea *et al.*, 2001).

Resistance to the members of echinocandin drugs by *Candida* species has also been reported. Mutations in the *FKS1* gene which codes for one subunit of the enzyme 1,3 β-D glucan synthetase is thought to mediate the resistance to the antifungal activity of echinocandin drugs (Douglas *et al.*, 1997). Mutations of amino acids in a region from Phe 641 to Asp 648 of Fks1p was shown to correlate with reduced susceptibility to caspofungin in a study using site-directed mutagenesis of the Fks1p in *C. albicans.* In addition, some clinical isolates of *C. albicans* with reduced susceptibility to caspofungin were also shown to have Ser 645 of Fks1p replaced by another amino acid (Park et al., 2005). In contrast to what was found with azole antifungal

drugs, over-expression of ATP binding cassette transporters encoded by *CDR1, CDR2 or MDR1* was not shown to confer reduced susceptibility of *C. albicans* to echinocandins (Niimi *et al.*, 2006).

The emergence of new drug-resistant *C. albicans* strains demands the development of newer antifungal drugs for the fight against candidiasis and other fungal infections. One logical direction towards the development of fungal therapeutics is the adaptation of antifungal agents that already exist in the nature. Among those agents, antifungal peptides with their special modes of action seem to offer promising candidates.

E. The existence of antifungal peptides in nature

Antifungal peptides can be found in nature and represent a line of host defense in various species such as plants, insects, animals and humans. More than 500 naturally occurring peptides that possess antifungal activities have been registered in the Antimicrobial Peptide Database (Wang, 2003). Among those peptides, at least 50 entities are of human origin while the others have been found in various species such as plants, bees, frogs, pigs, etc. Natural antifungal peptides are believed to help protect host species against pathogenic fungi. One example of peptides with such protective actions is the magainins. Magainin peptides were found on the skin of African clawed frogs *Xenopus laevis* and are known to inhibit growth or induce lysis of bacteria, fungi and protozoa which exist in contaminated water where the species live (Zasloff, 1987). Antifungal peptides are enormously diverse in terms of amino acid sequences and antimicrobial spectrum, however, they share common features such as having an amphipathic amino acid sequence or a net cationic charge at physiologic pH (Yeaman & Yount, 2003). Furthermore, antifungal peptides usually possess specific structures such as α-helix (Lequin *et*

al., 2003), β-sheet (Fahrner *et al.*, 1996) or a combination of disulfide bonds (Huang *et al.*, 2004). Those secondary structures are currently being used to classify antimicrobial peptides in some registries (Wang, 2003, Sitaram & Nagaraj, 2002).

F. The selectivity of antifungal peptides in relation to common components of fungal cell walls or biological membranes

Why certain antifungal peptides specifically target pathogenic fungi, but not the host cells is not fully understood. However, the structure of fungal cells as compared to those of bacterial and mammalian cells may provide some answers. Unlike bacterial and mammalian cells, many fungi possess a cell wall rich in chitin, β (1, 3)-glucan and mannoproteins (Levitz, 2010) which are known to be among possible targets of antifungal peptides (De Lucca & Walsh, 1999, Makovitzki *et al.*, 2006). Chitin-binding property was reported in a number of antifungal peptides such as Ac-AMP1, Ac-AMP2 (Broekaert *et al.*, 1992), shrimp penaeidins (Destoumieux *et al.*, 2000), tachystatin (Fujitani *et al.*, 2002), Cy-AMP1 (Yokoyama *et al.*, 2009), hyastatin (Sperstad *et al.*, 2009), etc. With respect to the Cy-AMP1 peptide, its antifungal activity was markedly reduced by experimental mutations of its chitin-binding domain while its antibacterial activity was not affected by the changes (Yokoyama et al., 2009).

In terms of lipid compositions, fungal membranes contain ergosterol while those of mammalian cells contain cholesterol (van Meer et al., 2008). Sterols molecules could be a factor that helps antifungal peptides distinguish between yeast and mammalian biomembranes. One example of that argument was a study in which the antifungal peptide cateslytin was shown to induce changes in fluidity and the separation of artificial membranes that contained ergosterol but not cholesterol (Jean-Francois *et al.*, 2009). In another study, the incorporation of

cholesterols into unilamellar phospholipid vesicles was shown to inhibit the permeabilizing

effect of two class-L peptides on those vesicles (Tytler *et al.*, 1995).

G. Biological membranes as a main site of interaction between antifungal peptides and fungi

Current *in vitro* data support the concept that antifungal peptides can interact with the

phospholipid membranes of fungi. One common method used in such studies is the

measurement of fluorescent emission spectra of antifungal peptides labeled with 7-nitrobenz-2-

oxa-1, 3-diazole-4-yl (NBD) after the labeled peptides are allowed to interact with artificial

membranes. The presence of a blue-shift of the spectrum suggests the relocation of the labeled

peptides into a more apolar environment, i.e. the phospholipid layers of the biomembranes

(Pouny *et al.*, 1992). A number of antifungal peptides such as dermaseptin-b (Strahilevitz *et al.*,

1994), LL37 (Oren *et al.*, 1999), temporins (Domanov & Kinnunen, 2006), and pseudin-2 (Park

et al., 2011) were shown to interact with artificial phospholipid membranes via the utilization of

this labeling technique.

Freeze-fracture electron microscopy is another approach that has provided further

evidence of interaction between antifungal peptides and biological membranes. In this technique,

the cell membrane of fungal cells is fractured along the interface of its inner and outer leaflets

and a metal replica of the fracture surfaces is created. Information on the surface of the inner and

outer leaflets of the cell membrane together with the distribution of inter-membranous protein

particles is obtained by the examination of those replicas under a transmission electron

microscope. A study utilizing freeze-fracture electron microscopy in combination with immuno-

staining showed that treatment with LL37 peptide resulted in the relocation of the peptides into

the membrane of *C. albicans* as well as corresponding morphological changes to the fungal membrane (Den Hertog *et al.*, 2005).

In addition to those studies, neutron diffraction studies have suggested that some antifungal peptides could result in pore formation on phospholipid membranes. In those studies, the structural changes of the phospholipid membranes treated with antimicrobial peptides were detected by changes in the neutron scattering properties of the membranes. Data in terms of neutron in-plane and off-plane scattering were then analyzed for the detection of pores as well as the sizes of the pores formed on the membranes. The use of D_2O exchange helped alter the scattering properties of the pores and facilitate the acquisition of images with better contrast (He *et al.*, 1996, Ludtke *et al.*, 1996, Yang *et al.*, 1998, Yang *et al.*, 1999). Magainin-2 and protegrin-1 were showed by neutron diffraction technique to be among the antifungal peptides which resulted in pore formation in artificial phospholipid membranes (Yang *et al.*, 2000).

The formation of pores on target membranes by antifungal peptides can also be detected indirectly by the use of fluorescent dye-trapped vesicles. In this technique, fluorescent dyes such as fluorescein-dextran (Ladokhin *et al.*, 1997), carboxy fluorescein (Lee *et al.*, 2001) or calcein (Viejo-Diaz *et al.*, 2004) are loaded into phospholipid vesicles that are subsequently mixed with the peptides of interest. The release of the fluorescent dyes from the vesicles is monitored by a spectrofluorometer and indirectly suggests the formation of pores on the phospholipid layers of those vesicles. Melittin (Ladokhin et al., 1997), cecropin A (Kang *et al.*, 1998), PMAP-23 (Lee et al., 2001) and some other natural antifungal peptides were shown to permeabilize artificial phospholipid vesicles resulting in the release of trapped fluorescent dyes from phospholipid vesicles.

H. Proposed mechanisms of action for the interaction of antimicrobial peptides and target phospholipid membranes

Currently, there are three proposed mechanisms of action by which antimicrobial peptides interact with biological membranes. Studies using artificial membranes suggested that some antimicrobial peptides with antifungal properties could permeabilize those artificial membranes via different mechanisms termed the barrel-stave, the toroidal and the carpet models.

In barrel-stave model, antimicrobial peptide molecules are believed to interact among themselves and insert into target phospholipid membranes in a way that their hydrophobic segments face the phospholipid layers of the membranes while their hydrophilic segments face the lumen of transmembrane pores formed by the antimicrobial peptide molecules (Vogel & Jahnig, 1986). Symbolically, the peptide molecules function as the "staves" and the pores formed on the phospholipid membrane are "barrels". Antifungal peptides such as melittin (Vogel and Jahnig 1986) and perforin (Rosado *et al.*, 2008) may worked via this mode of action. .

Another mechanism used to explain peptide-membrane interactions is the toroidal model. This model suggests that antimicrobial peptides form amphipathic alpha-helices that orientate in a direction perpendicular to the plane of target membranes and form pores on the latter (Ludtke et al., 1996). The toroidal model is different from the barrel-stave mechanism in that it suggests some peptide molecules are always associated with the lipid head-groups of target membranes and that the wall of transmembrane pores are formed not only by peptide molecules but also by lipid head-groups. In addition, some lipid molecules of target phospholipid membranes bend on themselves and result in a configuration of the membrane like the inside of a torus that gives the model its name (Matsuzaki *et al.*, 1989, Ludtke et al., 1996, Yang *et al.*, 2001). A classic example of antifungal peptides thought to act via this model is a natural broad-spectrum antimicrobial

peptide named magainin-2 found in the skin of *Xenopus laevis* African clawed frogs (Ludtke et al., 1996, Zasloff, 1987). Other antifungal peptides also believed to act via this model are melittin and protegrin (Yang et al., 2001).

A third model of antimicrobial peptides-membrane interaction is the carpet mechanism proposed about two decades ago (Gazit *et al.*, 1996). The study used attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy to explore secondary structures and the orientation of cecropin P1 peptide molecules when the antimicrobial peptide interacted with the negative or zwitterionic artificial membranes. ATR-FTIR is a technique which utilizes infrared radiation to study secondary structures of peptides and proteins (PerkinElmer, 2005). Samples containing peptide or protein are exposed to infrared radiation and the absorption by the peptide or protein molecules in the samples will determine the infrared spectrum of the samples. The location of amide I and II bands in the infrared spectrum is related to secondary structures of the protein or peptide. Further deconvolutional and dichroic analyses of these bands provide information on the components and orientation of those secondary structures (Gazit et al., 1996). ATR-FTIR data from the above study suggested that cecropin P1 molecules are orientated parallel to the membrane, but did not enter negative or zwitterionic artificial membranes. That finding is part of what led to the proposal of the carpet model (Gazit et al., 1996). The current carpet model proposes that antimicrobial peptides form monomers or oligomers which orientate parallel to target membranes and these molecules will disrupt the integrity of the membranes when a certain threshold concentration of the peptides is reached (Shai, 2002, Wimley & Hristova, 2011). Among antifungal peptides, dermaseptin B2 was shown to act via this carpet mechanism on anionic phospholipid membranes (Galanth *et al.*, 2009).

The interaction between antimicrobial peptides and yeast cell membranes could eventually lead to the formation of not only pores but ion channels on target membranes. These pores or channels could result in the efflux of potassium, loss of mitochondrial transmembrane potential, ATP deficiency and cell death. Peptides of this group usually have α-helical or β-sheet secondary structures which can partition into and form channels in the phospholipid bilayers of biomembranes. Two examples of peptides with such a property are tritrpticin and protegrin-1 (Schibli *et al.*, 2002, Capone *et al.*, 2010). Tritrpticin is a peptide belongs to the family of cathelicidin peptides found in neutrophil granules. Experiments with bi-planar phospholipid membranes suggested that aromatic side chains of tritrpticin, especially those of tryptophan residues, partitioned into the artificial phospholipid membranes (Schibli *et al.*, 2002). Furthermore, tritrpticin was shown to induce ion conductance when a constant voltage was applied across a negatively charged or zwitterionic phospholipid membrane (Salay et al., 2004). Those findings suggested tritrpticin forms ion channels in phospholipid membranes. In addition to tritrpticin, protegrin-1 is another antifungal peptide thought to form ion channels in phospholipid bilayers (Capone *et al.*, 2010).

There were a small number of studies which suggested that antimicrobial peptides bound to a target site, especially lipids, on the membranes of fungal cells. Ergosterol or cholesterol molecules, widely known to be present on eukaryotic cell membranes, were shown to bind to cecropin or dermaseptin antifungal peptides when they were mixed together *in vitro* (De Lucca *et al.*, 1998). Other possible binding sites for antifungal peptides are lipid complexes containing sphingolipids. *Saccharomyces cerevisiae* mutants deficient in a membranous sphingolipid named mannosyldiinositolphosphorylceramide were less avid for and less susceptible to the *Dahlia merckii*-dervied antimicrobial peptide 1 (DmAMP1) compared to wild-type strains (Thevissen *et*

al., 2000). In addition to DmAMP1, another defensin peptide named RsAFP2 isolated from the radish seed of *Raphanus sativus* was also shown to interact with glycosylceramides present on the cell membrane of *Pichia pastoris* yeast cells. *Pichia pastoris* cells depleted in glycosylceramide were shown to be resistant to the antifungal peptide RsAFP2 (Thevissen *et al.*, 2004).

I. Intracellular targets of antifungal peptides

Some antifungal peptides are believed to be internalized into the intracellular space where they exert their killing effects on intracellular targets. One study using flow cytometry to explore the internalization of the antifungal tenacin-3 peptide suggested that the internalization process of tenacin-3 was energy and temperature dependent. In addition, the finding that the population of dead cells evaluated by propidium iodine was smaller than that of tenacin-3 positive cells in the same samples suggested that some cellular process was required for the killing effects of the peptide when the peptide was already inside the treated cells (Kim *et al.*, 2001).

Once inside the fungal cells, intracellular targets of antifungal peptides could be DNA and RNA molecules which bear negative charges. A truncated derivative of dermaseptin S3 peptide was found to result in nuclear DNA fragmentation and programmed cell death in *S. cerevisae*. Mutations of four genes known to be involved in yeast apoptosis conferred reduced susceptibility of these mutant strains to the truncated derivative. Furthermore, gel shift assay and *in vitro* DNAse protection assay suggested this peptide bound to DNA (Morton *et al.*, 2007). Another peptide named papiliocin found in the swallowtail butterfly *Papilio xuthus* was also believed to target nuclear DNA and mitochondria. *C. albicans* cells treated with papiliocin

showed increased loss of mitochondrial transmembrane potential, and the intracellular

accumulation of reactive oxygen species and apoptosis (Hwang *et al.*, 2011).

J. The model of natural peptide histatin-5 and the introduction of histatin-5-based

antimicrobial agent into clinical setting

Histatin-5 (DSHAKRHHGYKRKFHEKHHSHRGY, 24 amino acids) is a naturally-

occurring antimicrobial peptide present in saliva. It was isolated from human saliva more than

20 years ago and found to have killing activity on *C. albicans* (Oppenheim *et al.*, 1988). In

addition to *C. albicans*, other fungal species such as *Aspergillus fumigatus* and *Cryptococcus*

neoformans were also shown to be susceptible to histatin-5 (Helmerhorst *et al.*, 1999, Situ &

Bobek, 2000). The antimicrobial spectrum of histatin-5 also covers bacterial species such as

Streptococcus mitis (Murakami *et al.*, 1991). Known for its antifungal activities, histatin-5

insufficiency may be involved in the development of oropharyngeal candidiasis of patients who

have reduced salivary secretion as a result of head and neck radiation or Sjogren syndrome.

Efforts to identify functional domains or fragments of histatin-5 that have pharmaceutical

application have gained some promising results. A group of researchers identified a 12-mer

fragment of histatin-5 (P-113) that retained the antifungal activities of the parent peptide histatin-

5 (Rothstein *et al.*, 2001). An all-D amino acid isoform of P-113 named P-113D was shown to be

active against *Pseudomonas aeruginosa* in the sputum of cystic fibrosis patients (Sajjan *et al.*,

2001). A phase-2 clinical trial of a group of 106 healthy human subjects suggested that the 12-

mer fragment (P-113) in gel formulations might have protective effects against experimental

gingivitis evaluated by bleeding-on-probing criteria. The authors of the study concluded that the

gel formulation was well tolerated and no increase in side effects compared to placebo was

recorded (Paquette *et al.*, 2002). In brief, histatin-5 has been shown to be a good model of natural antimicrobial peptide that may have pharmaceutical values.

K. Objectives of this study

Given the promise of histatin-5 as a potential antifungal therapeutic, our studies focused on understanding the amino acid residues within histatin-5 that were critical for antifungal activity. With that knowledge in hand, we then sought to develop novel peptides with increased antifungal activity and to evaluate those peptides both *in vitro* and *in vivo* for potential toxicity to mammalian cells. Thus my research can be divided into three components: (1) Investigate the mode of interaction between histatin-5 and its cellular targets and further identify the antifungal fragment(s) of histatin-5 by the characterization of its 16-mer derivatives; (2) Develop novel antifungal peptides based on the core structure of the functional fragment(s) identified in objective 1 and evaluate possible toxicity of the newly developed peptides on mammalian cells *in vitro;* and (3) Evaluate potential acute toxicity and immunogenicity of some novel antifungal peptide candidates in mice.

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CHAPTER 2

CHARACTERIZATION OF FOUR 16MER DERIVATIVES OF HISTATIN-5 AND THE

IDENTIFICATION OF AN ACTIVE ANTIFUNGAL MOTIF FOR DEVELOPMENT OF

NOVEL ANTIFUNGAL PEPTIDES

A. Introduction

Histatin-5 (DSHAKRHHGYKRKFHEKHHSHRGY, 24 amino acids) is a naturally-occurring peptide present in the secretions of the parotid and submandibular salivary glands (Oppenheim *et al.*, 1988). It is coded for by the *HTN3* gene located on the short arm of chromosome 4 (GenBank, Gene ID: 3347) in humans. The gene is transcribed into a messenger RNA of 601 nucleotides and translated into a precursor protein of 51 amino acids. The precursor protein is converted via proteolysis into histatin-3 of 32 amino acids and subsequently histatin-5 of 24 amino acids in human saliva (Oppenheim et al., 1988, Castagnola et al., 2004). All three peptides were shown to possess antifungal activities against the yeast and hyphal forms of *Candida albicans* and the most active peptide among the three entities was identified as histatin-5 (Xu *et al.*, 1991). Histatin-5 is stable in unfiltered human saliva for 30-60 minutes and subjected to degradation by bacterial proteases present in the salivary fluid (Situ & Bobek, 2000).

Current data indicate that histatin-5 is effective against several medically important fungal pathogens. The antimicrobial spectrum of histatin-5 is known to cover several strains of *C. albicans, C. glabrata, Aspergillus fumigatus, Cryptococcus neoformans*, and even some strains of bacteria (Murakami et al., 1991, Tsai & Bobek, 1997, Helmerhorst et al., 1999b, Situ & Bobek, 2000). Furthermore, histatin-5 was shown to be effective against some *C. albicans* and *C. glabrata* strains that were resistant the azole antifungal agents (Tsai & Bobek, 1997)

Although the histatin-5 peptide was discovered more than 20 years ago, the mechanism of fungicidal activity has not been fully elucidated. The killing mechanism of histatin-5 was shown to involve its ability to permeabilize the membranes of *C. albicans* yeast cells (Edgerton et al., 1998). In addition, histatin-5 was shown to enter the intracellular space of the yeast cells

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treated with the peptide and the uptake of histatin-5 was attenuated in the presence of an electron transport chain inhibitor or a proton gradient uncoupler such as sodium azide or carbonyl cyanide m-chlorophenyl hydrazone, respectively (Gyurko et al., 2000, Jang et al., 2010). The finding that the intracellular uptake of histatin-5 required functional mitochondrial electron transport chain suggested the uptake process required cellular energy.

Once inside the yeast cells, fluorescently-labeled histatin-5 co-localized with mitochondria and resulted in mitochondrial transmembrane potential loss and cell death (Helmerhorst *et al.*, 1999a, Ruissen *et al.*, 2001). The targeting of histatin-5 to mitochondria was thought to be mediated by its structural similarities with some pre-sequences found in mitochondria-targeting peptides or proteins (Helmerhorst et al., 1999a, Nicolay et al., 1994) Another result of treatment with histatin-5, *C. albicans* cells displayed an increase in intracellular reactive oxygen species and this was thought to be responsible for the death of the yeast cells (Helmerhorst et al., 2001a). However, the postulated role of reactive oxygen species in the killing mechanism of histatin-5 was not supported by other authors (Veerman et al., 2004). The requirement of cellular respiration in the mode of action of histatin-5 on *C. albicans* was further supported by a study in which treatment of *C. albicans* with histatin-5 in anaerobic conditions resulted in less killing compared to that in aerobic conditions (Helmerhorst et al., 1999a). In addition, a petite mutant of *C. albicans* depleted in mitochondria was shown to be less susceptible to the antifungal effect of histatin-5 (Gyurko et al., 2000). All the above findings supported the involvement of mitochondrial function or respiration in the killing mechanisms of histatin-5.

Previous studies also suggested the involvement of several factors in the uptake of histatin-5 into the intracellular space of *C. albicans* cells. Histatin-5 was believed to bind to

Ssa2p on the cell membrane and in the cytosol of *C. albicans.* The Ssa2p is currently thought of as a cell membrane receptor for histatin-5 and believed to play an important role in the killing mechanism of histatin-5 (Li et al., 2003). *C. albicans* cells containing a homozygous gene knockout of *SSA2* were reported to have reduced susceptibility to histatin-5 (Li et al., 2006). However, more recent studies have suggested that histatin-5 is internalized via several members of the Dur polyamine transporters. *C. albicans* mutants depleted of certain Dur proteins were reported to have significantly reduced susceptibility to histatin-5 (Kumar et al., 2011). Polyamine transporters had been found previously in *Escherichia coli* and *Saccharomyces cerevisiae* (Igarashi & Kashiwagi, 1999). Furthermore, polyamines are organic cations known to be involved in the cellular synthesis of DNA and proteins and cell growth (Tabor & Tabor, 1984, Cohen, 1998).

Efforts have been made to identify the functional domains within the 24-residue sequence of histatin-5 necessary for the antifungal activity of the peptide. One early attempt identified a 16mer derivative lacking eight amino acids from the N-terminus of the histatin-5. The derivative was reported to have antifungal activity comparable to histatin-5 against *C. albicans* (Raj et al., 1990). The smallest derivative reported to retain the antifungal activities of histatin-5 peptide was known to be comprised of 12 amino acids located around the center of the histatin-5 amino acid sequence (Rothstein et al., 2001). Since the oligomerization of a functional domain within an antimicrobial peptide could generate a much stronger antimicrobial peptide compared to its parental entities (Oppenheim et al., 2012), the determination of functional domain of a natural antimicrobial peptide is essential in the design of novel synthetic antimicrobial peptides.

Our research group is interested in the killing mechanism of histatin-5 and the application of that knowledge to the development of novel antifungal peptides. The initial goal was to

address the simple question of whether the linear amino acid sequence of histatin-5 was important for the antifungal activity or alternatively, whether the spatial arrangement of the amino acids relative to each other is more relevant. Secondly, we wanted to examine the role of stereochemistry in the antifungal activity of the histatin-5 peptide. For these studies, we chose to use a 16 amino acid derivative of histatin-5 lacking eight amino acids from the N-terminus, since this peptide was previously shown to possess all of the antifungal properties of the native peptide (Raj et al., 1990). In addition to the histatin-5 16mer (WL) peptide derivative, we generated three additional peptides termed the retro- (RL), enantio (WD) and retroenantio (RD) analogs to further explore the mechanism of action of histatin-5 *(Table 2.1)*. In this study, we investigated the mechanism of killing of the four 16-mer derivatives of histatin-5 with the use of several *C. albicans* strains and artificial phospholipid membranes. Our goal was to address whether the four histatin-5 derivatives possess equal antifungal activity or whether some of the peptides would display reduced activity relative to that of the others. If all four peptides displayed similar antifungal properties, this would suggest that the linear amino acid sequence of histatin-5 was less relevant than the spatial positioning of the amino acids relative to each other. Moreover, if the D-isomers displayed similar activity, this would suggest that stereochemistry is not relevant for fungicidal activity.

B. Materials and methods

Chemicals, enzymes and reagents. Enzymes, chemicals and reagents used in this study were purchased and stored as specified by the corresponding manufacturers. The enzymes BamHI, BglII, EcoRI, EcoRV, SalI, GoTaq polymerase, and Klenow DNA polymerase were purchased from Promega Corporation. Random primers were purchased from Invitrogen. BglII was also purchased from NEB and KOD polymerase from EMD Biosciences Incorporation. Yeast extract, peptone, Bacto ^{TM} agar, Sabouraud Dextrose Broth, and yeast nitrogen base were purchased from BD Biosciences. Glucose, sodium chloride, uridine and ammonium sulfate were obtained from EMD Millipore. Antimycin A, acridine orange, and sodium azide were purchased from Sigma-Aldrich. Sodium phosphate dibasic was purchased from J.T. Baker and sodium phosphate monobasic was obtained from ICN. 5-Fluoroorotic acid was purchased from US Biologicals and ^{32}P-ATP from MP Biomedicals. The lipids 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), soy PI, and 1μm polycarbonate membranes were purchased from Avanti Polar Lipids. Ergosterol was a gift from Dr. Daniel Fologea of Boise State University. PD-10 desalting columns were purchased from GE Health care Life Sciences. Hybridization transfer membranes were purchased from PerkinElmer Life Sciences. Alkali-Cation Yeast kit was purchased from MP Biomedicals.

Peptide synthesis. N-Fmoc protected amino acids and Wang resin was purchased from CalBiochem-NovaBiochem Corporation and Advanced Chemtech, respectively. All peptides were synthesized on a Model 433A solid-phase peptide synthesizer (Applied Biosystems Incorporation) 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% dH2O, 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/ddH2O and recovered by lyophilization 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. Stock solutions of the four 16-mer peptides of 330 µM were prepared in 10 mM sodium phosphate buffer pH 7.4 and diluted to the desired concentrations. All the peptide concentrations were based on the extinction coefficient of the peptides at 280nm. All peptide solutions were stored at -20°C until being used. The name and sequence of the peptides used in this study are given in *Table 2.1*.

Yeast strains and growth medium. The *C. albicans* yeasts strains SC5314 (wild-type clinical isolate) and BWP17 (*ura3Δ::imm434 /ura3Δ::imm434 his1Δ::hisG/ his1Δ::hisG arg4Δ::hisG/ arg4Δ::hisG*) were obtained from Fred Winston (Harvard Medical School, Boston, MA). *C. albicans* strains were routinely grown on Sabouraud Dextrose agar plates (30g of Sabouraud dextrose broth and 20g of agar per liter) and streaked on fresh plates every 3-4 days. For genetic manipulation, strains were grown on synthetic complete medium lacking the appropriate amino acids for auxotrophic marker selection (Guthrie, 2004).

Disruption of *SSA2* in *C. albicans*. The plasmid used for the disruption of *SSA2* alleles was constructed from pDM715 and *URA3*-blaster cassette *(Figure 2.1)*. The plasmid pDM715

was digested with BglII within *SSA2* and the BglII/BamHI digested *hisG-URA3-hisG* cassette was ligated into the plasmid to generate the disrupted copy of the gene. The correct insertion of the *hisG-URA3-hisG* cassette into *SSA2* was determined by restriction enzyme digestion and polymerase chain reaction (PCR) analysis and the plasmid designated pDM715UB. Using the *ssa2::hisG-URA3-hisG* cassette, *SSA2* was disrupted using the method previously described (Fonzi & Irwin, 1993). The pDM715UB plasmid was digested with BamHI and SalI to release the *ssa2::hisG-URA3-hisG* cassette and *C.albicans* transformation was performed with the use of Alkali-Cation Yeast kit. *C. albicans* transformants were selected on synthetic complete medium lacking uracil (SC-Ura). Colonies that grew on SC-Ura were isolated and genomic DNA was purified as described (Hoffman and Winston, 1987). The genomic DNA was subsequently used as a template for PCR to identify yeast mutants containing the disrupted *SSA2*. For the PCR confirmation of the gene disruption, primers oDM0587 (5'-CTG GAA TTA AGA GAA CAA CAA CATG-3') and oDM0536 (5'- CAG TAC CAG AAT CGA GCT GGC GCC AAG CGC-3') were employed. Following confirmation of the disruption, the correct strains were grown on synthetic medium containing 5-FOA to select for loss of *URA3* via recombination between the hisG cassette sequences (Fonzi & Irwin, 1993). The second allele of *SSA2* was subsequently disrupted in an identical manner and PCR employed to confirm the disruption of the second copy of the gene using oDM0587 (5'-CTG GAA TTA AGA GAA CAA CAA CATG-3') and oDM0369 (5'-GGT GAG GCA TGA GTT TCT GCT CTC TCA-3'). Final confirmation of the strain construction was determined by Southern blot analysis. After the *ssa2* null mutations were confirmed the *his1Δ::hisG* and *arg4Δ::hisG* auxotrophies were rescued to prototrophy. For this, the plasmid pDM583 containing *ARG4* was digested with Hpa1 to target integration of the wild-type gene to the *arg4::hisG* locus following transformation and subsequently selected on SC-Arg

medium. The *his1Δ::hisG* auxotrophy was rescued similarly using the plasmid pDM604 digested with Nru1 to target integration at the *his1::hisG* locus, and selected on SC-His medium. Thus, the strains used for the fungicidal activity assays were all prototrophic.

Southern blot analysis. The disruption of both alleles of *SSA2* was confirmed by Southern Blot. For the blot, 5μg of genomic DNA was isolated from the BWP17 parent strain, the *ssa2::hisG-URA3-hisG* heterozygous mutant, the 5-FOA selected *ssa2::hisG* heterozygous mutant and the *ssa2::hisG/ssa2:: hisG-URA3-hisG* homozygous mutant (Hoffman & Winston, 1987) and subsequently digested with BglII at 37°C overnight. The products of digestion were resolved on a 0.8% agarose gel and transferred to PDVF membranes for Southern blot analysis as previously described (Johnson *et al.*, 2005). A ^{32}P-ATP-labeled DNA probe was prepared for the hybridization by random priming method (Johnson *et al.*, 2005). The probe was obtained by digestion of pDM715 plasmid with EcoRI and EcoRV and a DNA fragment of 1.5 Kb was purified by agarose gel electrophoresis and radioactively labeled.

Microdilution fungicidal assay. To evaluate the fungicidal activity of peptides the appropriate *C. albicans* strains were grown overnight in YPD medium at 30°C. Following growth, 1 ml of the culture was centrifuged at 16,000 x g for 1 min. and the cells were washed twice in 10mM sodium phosphate buffer (pH 7.2) and resuspended in 1 ml of 10mM sodium phosphate buffer. Cell count was performed using a hemocytometer and number of cells per culture was adjusted to a concentration of 2.5 x10^5 cells/ml. In fungicidal activity assays, 20μl of peptides at appropriate concentrations or 10mM sodium phosphate buffer (pH 7.4) was added to 20 μl of *C. albicans* cells. The cultures were incubated at 37°C on a shaker at 170 rpm for 2 hours. Following the 2 h incubation, 360 μl of Yeast Nitrogen Base (3.4g of yeast nitrogen base and 10g ammonium sulfate per liter) was added to stop the killing reaction. The reactions were

mixed and 40μl of the cell suspension was spread on Sabouraud dextrose agar plates. The plates were incubated at 37°C overnight in a humidified incubator and the number of viable colonies was determined. To evaluate the effect of sodium azide or antimycin A on the killing activity of the antifungal peptides, the *C. albicans* cells were treated with 5 mM sodium azide or 5 μM antimycin A in sodium phosphate buffer (pH 7.4) during the killing assay.

Fluorescence leakage assays. Ergosterol-containing liposomes were prepared from phospholipids and ergosterol dissolved in an organic solvent that contained chloroform, methanol and water at a volume ratio of chloroform:methanol:H_2O of 65:35:8. Lipid mixtures were prepared at a concentration of 12 mg of lipids per 1 ml of organic solvent and had a weight ratio of DPPC:DPPE:soy PI:ergosterol of 5:4:1:2. This relative ratio was used to mimic *C.albicans* yeast membranes (Ratledge, 1988, Park & Lee, 2009). Lipid mixtures were thoroughly mixed, dried under a nitrogen stream for 20-30 minutes, and then evaporated in a vaccum evaporator overnight. Lipid cakes were hydrated with 1ml of 110 mM amonium sulfate during a period of 1 h at 72°C in a water bath with vigorous shaking. Non-ergosterol liposomes were prepared under the same solvent conditions except the lipid mixtures were prepared at a concentration of 10 mg of phospholipids per 1 ml of organic solvent and had a weight ratio of DPPC:DPPE: soy PI of 5:4:1. Lipid suspensions were then subjected to three additional free-thaw cycles and extruded through stacked 1μm polycarbonate membranes for at least 17 cycles to yield unilamellar vesicles. The extrusion was performed at 72-75°C using a thermo-controller. Buffer exchange was done after the extrusion step by gel filtration as per protocol provided with PD10 desalting columns. The buffer exchange step replaced the extra-liposomal buffer of 110 mM amonium sulfate with a 150 mM sodium chloride solution. The size distribution of unilamellar vesicles (liposomes) in the final lipid suspensions was investigated with the use of

Zeta Potential Analyzer Utilizing Phase Analysis Light Scattering Machine (Zetapals, Brookhaven Instruments Corp.). Liposomes in 150mM sodium chloride were stored at 4°C until use. For the fluorescence leakage assays, liposomes were loaded with 10 µM acridine orange in 10mM sodium phosphate buffer (pH 7.4) supplemented with 5% glucose. The method used for loading liposome with acridine orange was performed as described previously by Barenolz et al. (Barenolz, 1993). In brief, 60µl of liposome suspension was added into 1940 µl of 10µM acridine orange in 10mM sodium phosphate buffer/ 5% glucose and kept at room temperature in the dark for 4 hours. Extra-liposomal acridine orange was removed by gel filtration with the use of PD10 desalting columns. During this gel filtration step, extra-liposomal solution of 150mM sodium chloride was replaced with 10mM sodium phosphate buffer pH 7.4/ 5% glucose. Liposomes loaded with fluorescence have low fluorescent intensity because of the self-quenching phenomenon (Morrison, 2008). To evaluate the ability of the antimicrobial peptides to permeabilize the liposomes, the acridine-orange-loaded liposomes in 10mM sodium phosphate buffer/ 5% glucose were tested with the antimicrobial peptides in four-sided polystyrene cuvettes (Sarstedt, catalog number 67.754). The fluorescent intensity of the sample was monitored by Fluoromax 4P fluorospectrometer (Horiba Scientific). In more details, 120 µl of peptide was added into 1880 µl of liposome suspension following 300 s of fluorescence measurement to obtain a baseline value. The liposomes and varying concentrations of peptide were incubated form 1500 s and fluorescence measured. After 1500 s, Triton X-100 was added at a final concentration of 0.1% to lyse all liposomes. For control samples, 120 µl of 10mM sodium phosphate buffer was added into liposome suspensions instead of the peptides. The fluorescent intensity of the samples was monitored continuously during a 30 min period (excitation 490 nm, emission 525 nm) and plotted as percentage of acridine orange release compared to the total

release obtained with Triton X 100. The formula used to calculate percentage of release is as followed:

$$\text{Percentage of fluorescence release} = 100\% \times \frac{F_t - F_o}{F_{total} - F_o}$$

F_t: fluorescent intensity at time t

F_o: fluorescent intensity at time 0

F_{total}: fluorescent intensity obtained with triton X-100 at time 1800s

C. Results

Synthesis of four derivatives of histatin-5. It has previously been shown that a 16 amino acid derivative of histatin-5, lacking the N-terminal eight amino acids is functionally identical to the wild-type peptide in terms of fungicidal activity (Raj et al., 1990). Thus, we chose the 16mer peptide as the starting point for our studies. To evaluate the structural features of histatin-5 that were important for fungicidal activity, four histatin-5 16mer derivatives were generated: 1) the 16mer peptide with L-amino acids which is referred to as the wild-type peptide (WL); 2) the 16mer peptide with the L-amino acids in the reverse order of sequence which is referred to as the retro-peptide RL); 3) the wild-type 16mer synthesized with D-amino acids which is called the enantio peptide (WD); and 4) the retro-peptide synthesized with D-amino acids called the retroenantio peptide (RD) *(Table 2.1)*. Using these four peptides, we wanted to investigate whether the linear N- to C-terminal amino acid sequence was important for histatin-5 activity or whether it was merely the relative spatial positioning of the amino acids that was important for antifungal activity. Moreover, using the D-amino acid peptide derivatives, we wanted to evaluate whether the stereochemistry of the peptides was important for the antifungal properties of the histatin-5 derivatives.

Construction of an *ssa2/ssa2* homozygous mutant of *C. albicans*. Previous studies have suggested the Ssa2p is a cell surface receptor involved in the binding and uptake of histatin-5 (Li et al., 2003), prerequisite steps for fungicidal activity. Using the four peptide derivatives described above, we hypothesized that if Ssa2p was essential as a receptor for the uptake of histatin-5, then we should see distinct differences in the killing activity of the wild-type versus the retro peptide. As a corollary to this hypothesis, it was also predicted that the D-amino acid peptides should lack or have reduced activity since they would interact with a cell surface

receptor with lower affinity due to the chirality change. To further explore the role of Ssa2p in the fungicidal activity of the 16mer peptides, a *ssa2/ssa2* homozygous mutant of *C. albicans* was constructed as outlined in the Materials and Methods, and Southern blot analysis was used to confirm the successful homozygous knock-out of *SSA2 (Figure 2.2)*. A schematic of the predicted genomic structure following the gene knockout is shown *(Figure 2.2A)*, along with the probe using for Southern blotting. Compared to the wild-type parent strain, the heterozygous *ssa2::hisG-URA3-hisG* mutant showed two bands of 2.6 and 6.5Kb indicating the successful generation of the heterozygote *(Figure 2.2B, lane 2)*. Following the removal of the *URA3* gene via homologous recombination between the *hisG* repeats, the 6.5kb fragment was reduced to 3.8kb indicative of the *ssa2::hisG* allele structure (*Figure 2.2B, lane 3*). Following a second round of gene disruption, the *ssa2:hisG-URA3-hisG/ssa2:hisG* mutant was confirmed by the presence of the 6.5kb and 3.8kb DNA fragments (*Figure 2.2B, lane 4*).

Fungicidal activity of the histatin-5 16mer peptide derivatives with wild-type and *ssa2* homozygous mutants of *C. albicans*. To evaluate the killing activity of the four histatin-5 peptide derivatives against *C. albicans*, a microdilution fungicidal activity assay was performed as outlined in the Materials and Methods. As shown in *Figure 2.3* and *Figure 2.4*, both the L- and D- amino acid derivatives of histatin-5 (WL and WD, respectively) showed comparable killing activity on the wild-type and *ssa2/ssa2* mutant of *C. albicans* with an LD_{50} of ~1.5 μM. When the retro (RL) and retroenantio (RD) peptides were examined, it was found that they had a slightly reduced fungicidal activity against both the wild-type and the *ssa2/ssa2* mutant; however, the activity remained significant with an LD_{50} of ~7 μM. These data suggest that the wild-type and enantio peptides have identical fungicidal activity and a similar pattern was observed with the retro and retroenantio peptides, suggesting that the spatial positioning of the

amino acids within the peptide is more relevant for fungicidal activity than the specific N- to C-terminal amino acid sequence. Moreover, the identical fungicidal activity of the D-amino acid peptides indicates that the stereochemistry of the peptides is not relevant. Finally, when comparing the fungicidal activity of each peptide in the wild-type versus *ssa2/ssa2* mutant of *C. albicans,* there was no significant difference. Taken together, these data strongly suggest that Ssa2p is not the receptor for the histatin-5 16mer peptide derivatives.

It was plausible that the four 16mer peptides showed similar fungicidal activities, yet they were killing yeast cells via distinct mechanisms. Since prior studies have demonstrated that histatin-5 fungicidal activity was dependent on active respiratory metabolism in the yeast cell (Gyurko et al., 2000, Jang et al., 2010), we examined the fungicidal activity of the four histatin-5 peptide derivatives in the presence of electron transport chain inhibitors. Using the microdilution fungicidal assay, the fungicidal activity of the four 16mer peptides was evaluated in the presence of 5 mM sodium azide, an inhibitor of the electron transport chain. As a control, 5 mM sodium chloride was added instead of sodium azide. As shown in *Figure 2.5*, the antifungal activity of all four 16mer peptides was inhibited by the respiratory inhibitor, consistent with previously published results (Gyurko et al., 2000, Jang et al., 2010). It was also plausible that sodium azide was inhibiting through other mechanisms independent of respiratory metabolism. For example, the azide could be interacting directly with the peptide, inhibiting its ability to bind to cells. To rule out this possibility, a second respiratory inhibitor, antimycin A, was also evaluated using the microdilution assay. As shown in *Figure 2.6*, antimycin A also inhibited the antifungal activity of the four 16mer peptides, albeit, the wild-type L- and D- amino acid peptides (WL and WD, respectively) were less inhibited than the retro (RL) and retroenantio (RD) peptides.

Nevertheless, these data strongly suggest that the wild-type, enantio, retro, and retroenantio 16mer peptides were all functioning through a similar mechanism in killing *C. albicans* cells.

Liposome leakage assays. The microdilution fungicidal assays indicated the wild-type (WL), enantio (WD), retro (RL), and retroenantio (RD) 16mer peptides had similar fungicidal activity against *C. albicans*. To further evaluate the ability of these peptides to directly permeabilize membranes, an *in vitro* liposome leakage assay was developed. In this assay, liposomes were prepared with a membrane content similar to that of *C. albicans* having a weight ratio of DPPC:DPPE: soyPI: ergosterol equal to 5:4:1:2 (Ratledge, 1988, Park & Lee, 2009). The liposomal vesicles were subsequently loaded via passive transport with acridine orange, a fluorescent dye that can be detected by fluorometry. The release of the acridine orange from the liposome would then served as an indicator of membrane permeabilization.

Liposomes were initially evaluated for a baseline level of fluorescence for 300 s, and 20 µM of each peptide was evaluated for membrane permeabilization activity. As shown in *Figure 2.7*, the control sample containing only 10 mM sodium phosphate buffer did not result in the release of acridine orange fluorescence (excitation 490nm, emission 525nm) over the 1500 s course of the experiment. With the addition of 20 µM of the four 16mer peptides, all peptides showed equivalent permeabilization of the liposomal membranes and release of acridine orange, with approximately 60% vesicle lysis. Moreover, the kinetics of membrane permeabilization was similar for the four peptides. After 1500 s, 0.1% TritonX-100 was added to the reaction to permeabilize all liposomes. It is possible that the 20 µM peptide concentration was excessive, masking any small differences in the activity of the four peptides or the kinetics of their activity. To address this concern, the experiment was repeated using a 1 µM concentration of each of the four 16mer peptides. As shown in *Figure 2.8*, the results were identical in terms of kinetics of

permeabilization; however, only 20% of the liposomes were permeabilized. These data suggest that all four peptides are equally active in membrane lysis, but it is likely that a specific number of peptide molecules per liposome are required. Thus, reducing the concentration of the peptide decreased the overall membrane permeabilization.

With the liposome assay developed, it was possible to manipulate the lipid composition to determine which membrane components are important for the interaction of the histatin-5 derived peptides with the membrane. One of the obvious targets is ergosterol, a membrane sterol typically found in the membranes of yeast and fungi. Moreover, current azole drugs target ergosterol biosynthesis; therefore, it was important to address whether ergosterol was important for the activity of the histatin-5 derived peptides. Thus, liposomes were prepared as outlined above, except ergosterol was not included in the preparation. As shown in *Figure 2.9*, liposomes lacking ergosterol were equally susceptible to membrane lysis by the four histatin-5 derived peptides at a 20 µM concentration, with approximately 60% lysis of the membranes. These data suggests that the permeabilization of liposome membranes by the wild-type, enantio, retro, and retroenantio 16mer peptides is independent of membrane ergosterol content. Moreover, it should be noted that no proteins are present in these artificial membranes, suggesting the peptides can permeabilize membranes in a protein-independent manner.

Antifungal activity of novel fungicidal peptides. Through the study of the four histatin-5 derived peptides and by examination of the prior literature related to histatin-5, we identified a domain within histatin-5 that was hypothesized to possess antifungal activity (Yazan Akkam, Ph.D dissertation, University of Arkansas, 2013). That putative domain contained five amino acids (FKRKY), and close examination of the amino acid sequence revealed that this small peptide would be symmetrical with two aromatic amino acids flanking a symmetric

sequence with the dyad of symmetry at the arginine residue. If this were the domain that possessed the fungicidal activity, it would explain the reason the wild-type versus retro peptides were equally active in killing assays. To evaluate this hypothesis, a microdilution fungicidal assay was performed with the 5mer peptide (WL) and the retro sequence of the 5mer (RL) and both 5mers displayed fungicidal activity similar to the wild-type 16mer peptides at a concentration of 4 μM *(Figure 2.10).* These data suggested that the sequence FKRKY was responsible for the antifungal properties of histatin-5 and the fungicidal activity of this peptide is orientation independent.

With this information in hand, additional peptides were synthesized based on the sequence of the 5mer. The KM12 peptide was a dimer of FKRKY via cysteine disulfide formation. The KM14 peptide was similar to KM12 except the aromatic amino acids phenylalanine and tyrosine were changed to tryptophan with the goal of making the peptide more active against biological membranes. Previous studies showed that tryptophan residues preferred membrane interfacial region and could function as an anchor of a peptide to a phospholipid bilayer (de Planque *et al.*, 1999, de Planque *et al.*, 2003). Finally, KM23 and KM29 were 10mer and 9mer peptides, respectively, lacking the cysteine residues. As shown in *Figure 2.10*, the microdilution assay indicated that KM12, KM14, KM23, and KM29 were all equally active in killing *C. albicans*. More importantly, all four peptides were significantly more active than the 16mer peptides, indicating that multimerization of the FKRKY sequence enhanced fungicidal activity.

D. Discussion

In this study, it was shown that four 16mer derivatives of histatin-5 had antifungal activities against *C.albicans*. The wild-type 16-mer (WL) and the enantiomeric 16-mer (WD) showed comparable killing actions against *C.albicans*, suggesting that these peptide derivatives kill the yeast cells via chiral independent mechanisms. The use of all D- or L-isoforms of antimicrobial peptides to probe for clues of receptor-related killing actions of antimicrobial peptides had been reported in the literature (Bessalle et al., 1990, Wade et al., 1990). If both the D- and L-isoforms of a given peptide showed equivalent antimicrobial activities, it is very unlikely that the peptide would require chiral receptor-type interaction with its targets.

All four histatin-5 peptide derivatives used in this study showed a similar tendency to form α-helix in 50% trifluoroethanol (TFE) (Yazan Akkam, Ph.D dissertation, University of Arkansas, 2013). With the L-isoforms, the presence of a strong positive band at 194-197 nm and two strong negative bands at 206-210 and 220-224 nm on circular dicroism spectra suggested the existence of α-helical conformations of the L-isoforms in 50% TFE solution. With the D-isoforms, the presence of a strong negative band at 194-197 nm and two strong positive bands at 206-210 and 220-224 nm on circular dichroism spectra also suggested α-helical secondary structures of the D-isoforms in 50% TFE solution (*Figure 2.11)* (Yazan Akkam, Ph.D dissertation, University of Arkansas, 2013). CD spectra characteristics of α-helical conformations in aqueous and nonaqueous solutions had been reported in the literature (Jung & Dubischar, 1975). The four histatin-5 peptide derivatives, however, showed random coil structures in CD spectra if they were dissolved in 10mM sodium phosphate buffer (Yazan Akkam, personal communication) as histatin-5. The histatin-5 parent peptide was shown in previous studies to form α-helix in TFE solution but have random coil structures in aqueous solvents (Raj et al., 1998, Helmerhorst et al., 2001b). Interestingly, α-helical conformations was

determined previously to not have an important role in the mode of action of histatin-5 (Situ et al., 2000).

The data shown in this study also suggests that Ssa2p did not play an important role in the killing action of the four peptide derivatives. It was shown that the wild-type 16-mer and enantio isoforms of the peptide had comparable antifungal activites against *C.albicans*. This was also observed for the retro and retroenantio isoforms of the peptide, suggesting the mechanism of killing was unlikely to be protein receptor-dependent. Ssa2p is a heat shock protein present in the cell wall, cytosol (Lopez-Ribot et al., 1996) and cell membrane of *C.albicans* yeast cells (*Candida* genome database, ORF 19.1065) (Cabezon *et al.*, 2009). It was hypothesized that Ssa2p functions as a binding receptor for histatin-5 and that Ssa2p is required for the antifungal activity of the peptide in *Saccharomyces cerevisiae* and *C.albicans* (Li et al., 2003, Li et al., 2006). *C.albicans ssa2* mutants were reported to be less susceptible to histatin-5 compared to its parent wild-type strain (Li et al., 2006); however, this is in contrast to the results we observed with an *ssa2* homozygous mutant of *C. albicans*. Nevertheless, the mechanism(s) by which Ssa2p could affect the uptake of histatin-5 into the yeast cytoplasm remains unknown. The data of this study suggested that the four peptide derivatives killed *C.albicans* yeast cells via Ssa2p-independent mechanism(s), and that is supported by our results with the *ssa2*-null mutant which showed similar susceptibility to the four peptide derivatives as the wild-type strain.

Comparable killing actions of the L- and D-isoforms on *C. albicans* suggested that the four peptide derivatives did not need a chiral-receptor-typed interaction with its targets. With respect to the histatin-5 peptide, a recent study suggested that histatin-5 was translocated across the phospholipid membranes of *C. albicans* cells by members of the Dur polyamine transporters

(Kumar et al., 2011). The role of the Dur transporters will need further investigation in the future.

The four histatin-5 peptide derivatives were shown to require a functional electron tranport chains (ETC) for fungicial activity. Inhibition of the ETC in the yeast cells by sodium azide blocked the activities of the complex IV of the yeast ETC attenuated the killing effects of the four peptide derivatives. The use of antimycin A, which inihibited the oxidation of ubiquinol of the ETC, showed the same attenuating effects on the killing activities of the four peptide derivatives. It was postulated that energy (adenosine triphosphate) depletion would resulted in increased rigidity of the cell membranes of the *C.albicans* yeast cells and prevent the interaction between cationic peptides and their target membranes (Veerman et al., 2007). However, histatin-5 was also reported to result in mitochondrial transmembrane potential loss and cell death once being inside the yeast cells of *C.albicans* (Helmerhorst et al., 1999a). The data of this study suggested that the four peptide derivatives used a similar mode of action to target the yeast cells of *C.albicans* as histatin-5 did and that mode of action required a functional electron transport chain or respiration of the yeast cells to work efffectively.

The finding that the mode of action of the four peptide derivatives involved mitochondria corresponded with the data obtained from other studies that used cationic peptides with structural sequences similar to ours. The motif sequence of hydrophobic residue-K-R-K- hydrophobic residue present in our four 16mer derivatives and histatin-5 was shown to target to mitochondria which had negative phospholipid bilayers similar to those of bacteria (Yousif et al., 2009). The parent peptide histatin-5 was also shown to colocalized with mitochondria in fluorescent staining (Ruissen et al., 2001, Ruissen et al., 2002). It is very likely that the four 16mer peptides used in this study with the motif sequence of hydrophobic residue-K-R-K- hydrophobic residue in their

sequence could disrupt the normal structure of mitochondria and lead to the death of *C.albicans* yeast cells.

The ability to permeabilize the phospholipid membranes of the *C.albicans* yeast cells is likely to play a role in the mechanisms of action of the four 16mer derivatives. The four derivatives were shown to permeabilize the artificial yeast phopholipid membranes in liposome leakage assays. Even in the absence of ergosterol in the phospholipid bilayers, the release of acridine orange from fluorescence loaded liposomes was also detected when those liposomes were treated with the four peptides. The ability of the four peptide derivatives to permeabilize artificial yeast phospholipid membranes were comparable at the peptide concentrations of 20 μM and 1μM.

Many cationic peptides were known to disrupt the biomembranes of microbes. Several mechanisms such as barrel-stave, toroidal and carpet models have been used to explain the permeabilizing ability of those peptides (Yeaman & Yount, 2003). Histatin-5 was also shown to disrupt the membrane of *C.albicans* cells (Den Hertog et al., 2005); however, it was undetermined which model was really involved in the case of histatin-5. In this study, the changes from L- to D- isoforms and the inversion of the amino acid sequence did not affect the ability to permeabilize the artificial phospholipid bilayers of liposomes of the four 16-mer derivatives. This finding suggested that the spatial relationship and electrical charge of amino acid residues in the sequences of the histatin-5 derivatives are of importance in the mechanisms of action of the four peptides.

As the inversion of the amino acid sequence or the change from L-isoforms to D-isoforms did not have any dramatic effects on the killing and permabilizing activities of the 16-mer derivatives, it is logical to raise a question about the existence of a symmetrical functional

domain in the amino acid sequence of the four 16-mer derivatives. The existence of such a symmetrical sequence would help explain part of our data.

Functional domains of histatin-5 peptide had been proposed previously. An amino acid sequence of residues 9-24 within the sequence of histatin-5 was considered to comprise the fungicidal domain of histatin-5 while the first eight amino acids from the N-terminus was shown to not play an important role in the antifungal activity of histatin-5 (Raj et al., 1990, Helmerhorst *et al.*, 1997). A 12-mer peptide from amino acid 4 to 15 within the sequence of histatin-5 was reported to retain the antifungal activities of the histatin-5 parent peptide (Rothstein et al., 2001). Combining the data of these two study with ours, we identified a short amino acid sequence from amino acid 10 to 15 (GYKRKF) of histatin-5 that could contain antifungal domain of histatin-5. As the data from a previous study (Rothstein et al., 2001) also suggested that the phenylalanine residue present in this short sequence was essential for the activity of histatin-5, we suspected that the symmetric sequence of YKRKF could play an important role in the antifungal activity of histatin-5. Two 5mer peptides named 5mer WL (YKRKF) and 5mer RL (FKRKY) were synthesized and both showed antifungal activities against *C.albicans*. Interestingly, the dimerization of these amino acid sequence generated new peptides with very strong antifungal activities against *C.albicans* such as KM12, KM14, KM23 and KM29. These new antifungal peptides were shown to be much more active compared to their parent peptides of 5mer WL, 5mer RL or 16mer WL. We anticipate that the multimerization of this common functional domain could prove to be an effective way to generate new antifungal peptides with pharmaceutical value. Our next step will focus on the characterization of these new antifungal peptides and the investigation of potential *in vitro* and *in vivo* toxicity of these peptide candidates.

Table 2.1: Name and sequence of the peptides used in this study.

| Number	Name	Sequence	Isomeric form (L or D)	Acetylated and amidated
1	16mer WL	GYKRKFHEKHHSHRGY	L	No
2	16mer WD	GYKRKFHEKHHSHRGY	D	No
3	16mer RL	YGRHSHHKEHFKRKYG	L	No
4	16mer RD	YGRHSHHKEHFKRKYG	D	No
5	5mer RL	FKRKY	L	Yes
6	5mer WL	YKRKF	D	Yes
7	5mer RD	FKRKY	D	Yes
8	Monomer-6mer	CFKRKY	L	Yes
9	KM12	YKRKFC■CFKRKY	L	Yes
10	KM14	WKRKWC■CWKRKW	L	Yes
11	KM23	YKRKFFKRKY	L	Yes
12	KM29	YKRKFKRKY	L	Yes
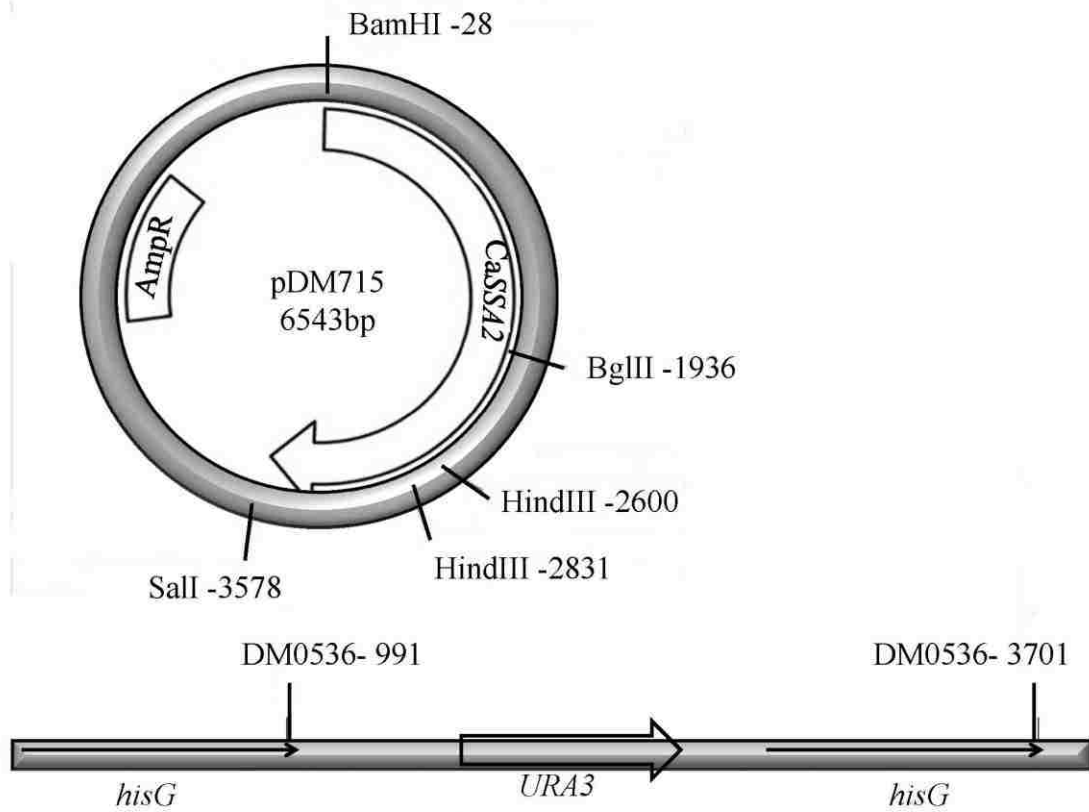
Figure 2.1: A schematic diagram of the plasmid pDM715 containing the *SSA2* gene and *hisG-URA3-hisG* cassette that was inserted into *SSA2* to generate the null allele used to knockout *SSA2* in *C. albicans*.
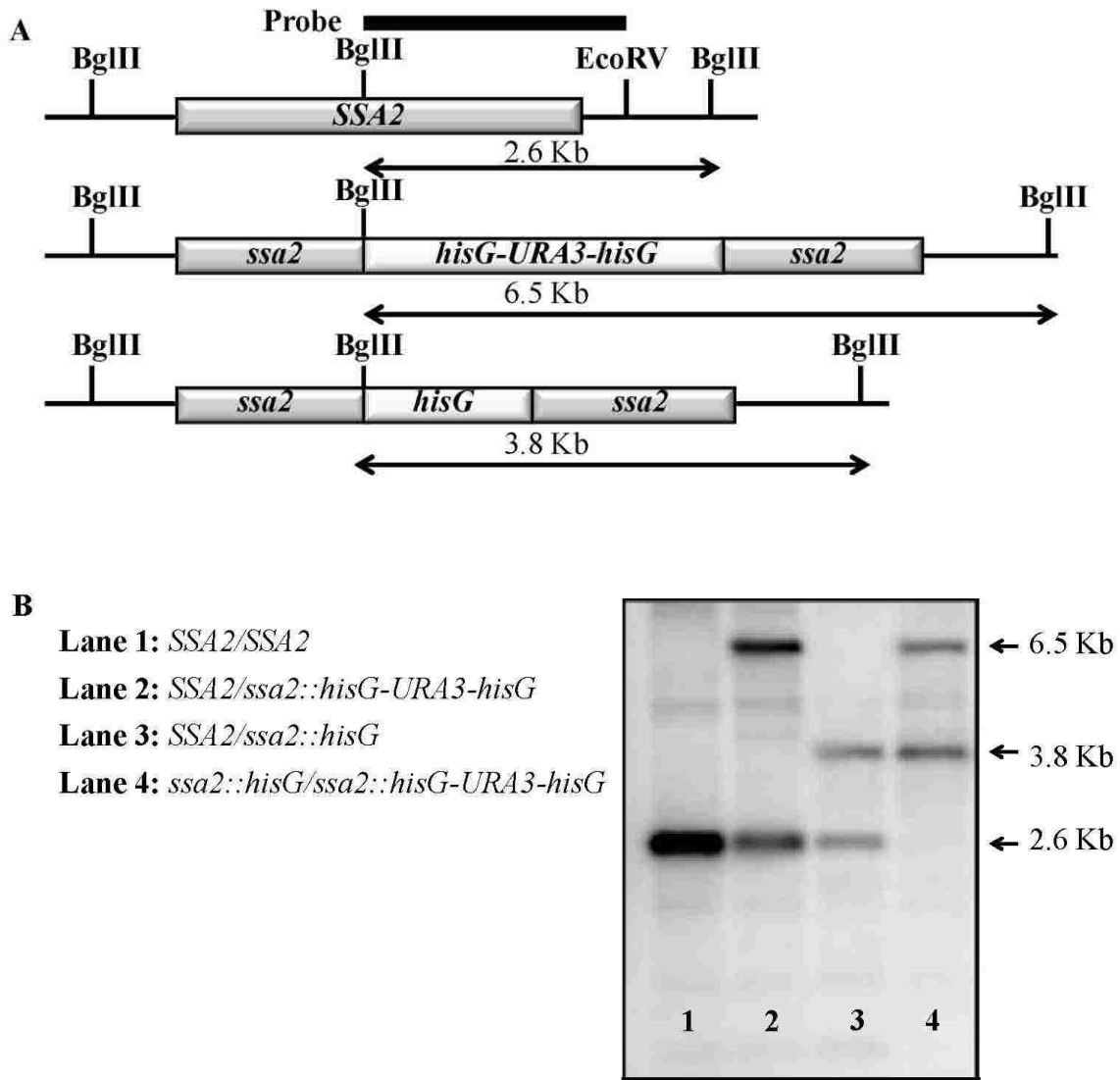
Figure 2.2: Confirmation of the *SSA2* disruption by Southern Blot. A: Schematic diagram of the SSA2 genomic locus and the predicted structure following gene disruption. The probe for *SSA2* in Southern blot is indicated. B: Southern Blot of four *C. albicans* strains. The genotypes of the strains are indicated on the left.
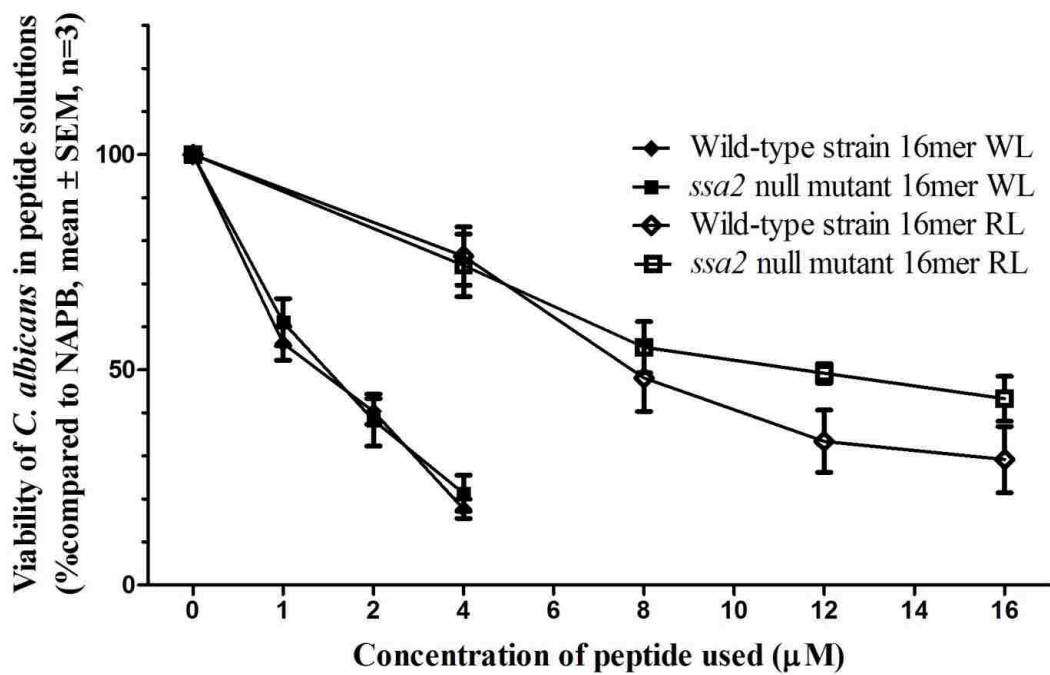
Figure 2.3: The susceptibility of the *C. albicans* wild-type and *ssa2* null mutant to the 16-mer WL and 16-mer RL peptides. *C. albicans* cells with the indicated genotype were incubated with varying concentrations of the 16-mer peptides in 10mM sodium phosphate buffer (NaPB) for 2h at 37°C as outlined in the Materials and Methods. The number of *C. albicans* colonies grown on Sabouraud without versus with peptide treatment were counted and compared among different treatment groups.
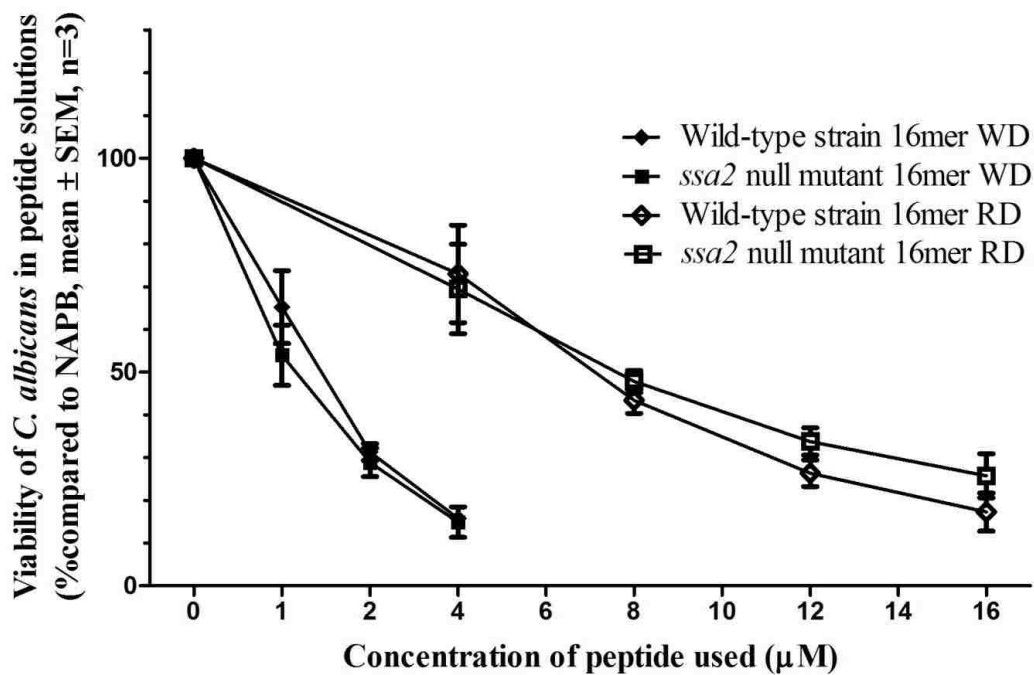
Figure 2.4: The susceptibility of the *C. albicans* wild-type and *ssa2* null mutant to the 16mer WD and 16mer RD peptides. *C. albicans* cells with the indicated genotype were incubated with varying concentrations of the 16-mer peptides in 10mM sodium phosphate buffer (NaPB) for 2h at 37°C as outlined in the Materials and Methods. The number of *C. albicans* colonies grown on Sabouraud without versus with peptide treatment were counted and compared among different treatment groups.
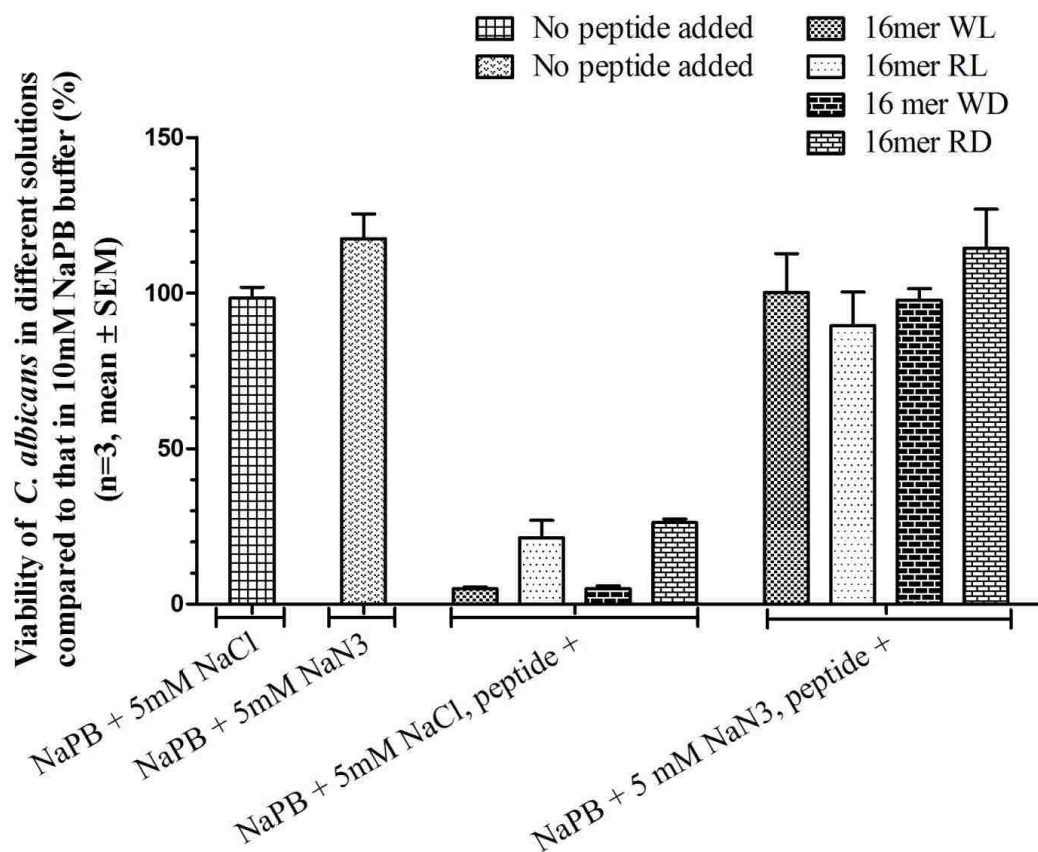
Figure 2.5: Inhibitory effect of sodium azide on the killing activity of four 16mer peptides.

Microdilution fungicidal activity assays were performed on *C. albicans* using 25 μM of the four 16mer histatin-5 peptide derivatives in the presence of 5 mM sodium azide. As controls, the cells were incubated with 5 mM sodium chloride. Data were expressed as mean ± SEM, n=3).
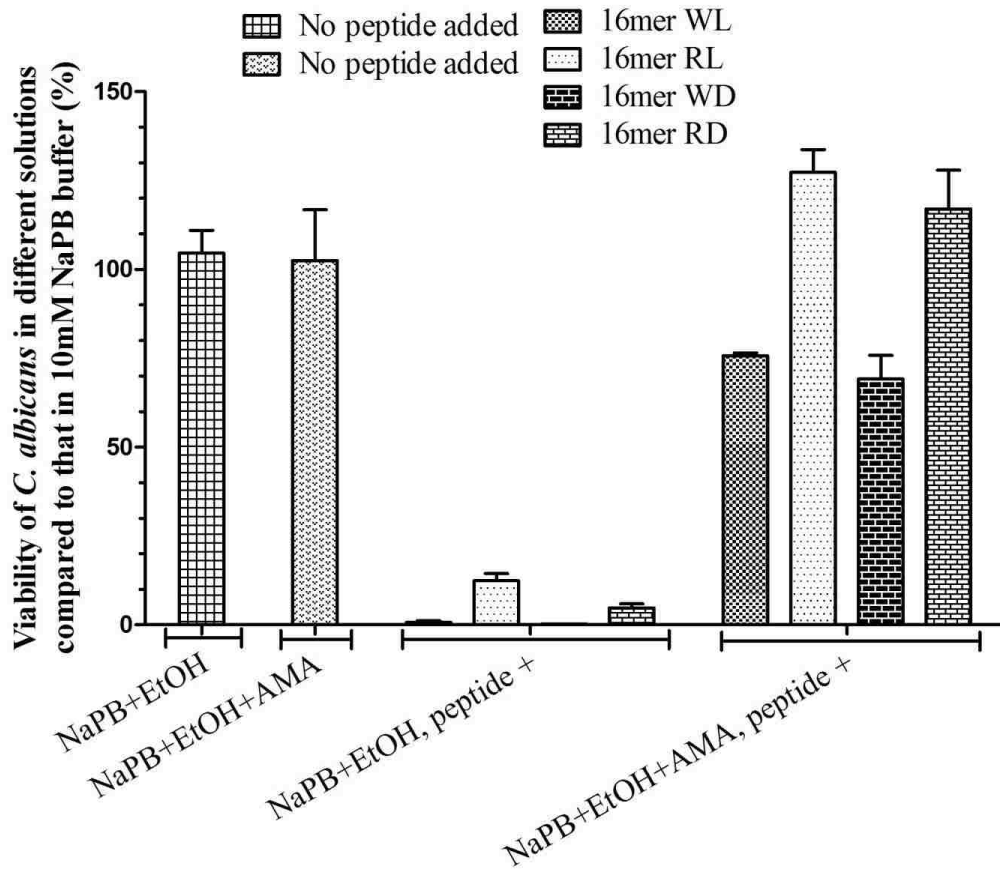
Figure 2.6: Inhibitory effect of antimycin A on the killing activity of four 16mer peptides.
Microdilution fungicidal activity assays were performed on *C. albicans* using 25 μM of the four 16mer histatin-5 peptide derivatives in the presence of 5 μM antimycin A. As controls, the cells were incubated with ethanol (EtOH) only. Data were expressed as mean ± SEM, n=3).
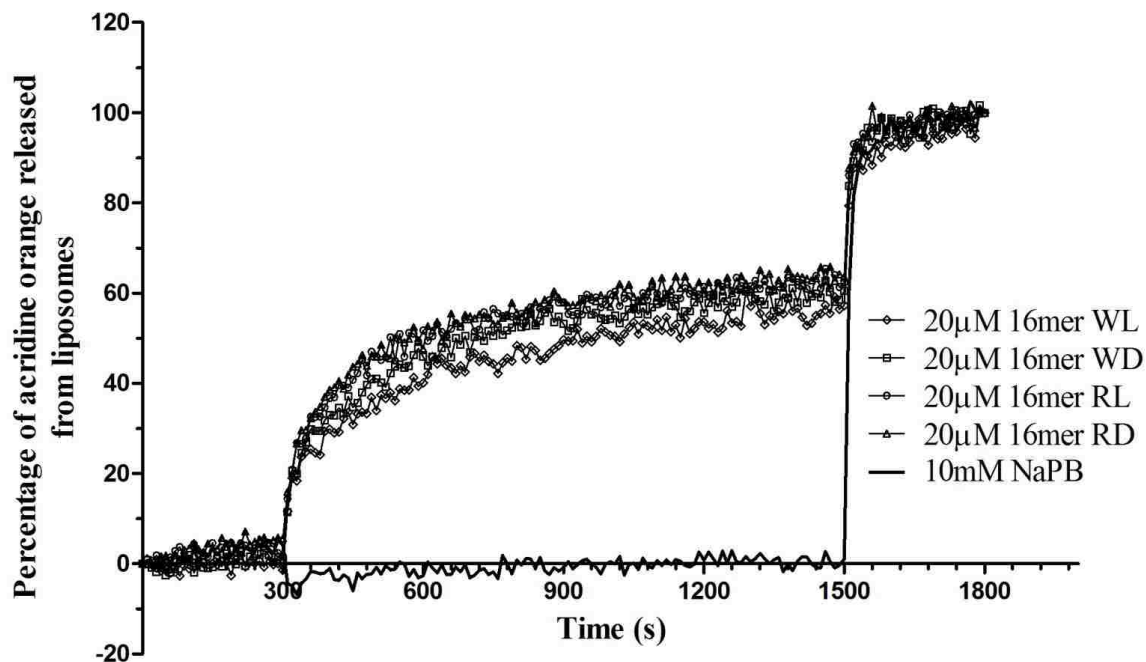
Figure 2.7: Liposome fluorescence leakage assays. The four 16mer histatin-5 derivative peptides at concentrations of 20 μM were incubated with acridine orange loaded ergosterol-containing liposomes. The percentage of fluorescent leakage from liposomes at time t (s) compared to total leakage obtained with Triton X-100 at time 1800 s. Peptides were added to the sample at 300 s. Control sample was treated with sodium phosphate buffer (NaPB) instead of peptides. Maximal fluorescent intensities were in the range of 80,000-220,000 counts per second).

Figure 2.8: Liposome fluorescence leakage assays. The four 16mer histatin-5 derivative peptides at concentrations of 1 μM were incubated with acridine orange loaded ergosterol-containing liposomes. The percentage of fluorescent leakage from liposomes at time t (s) compared to total leakage obtained with Triton X-100 at time 1800 s. Peptides were added to the sample at 300 s. Control sample was treated with sodium phosphate buffer (NaPB) instead of peptides. Maximal fluorescent intensities were in the range of 80,000-220,000 counts per second).
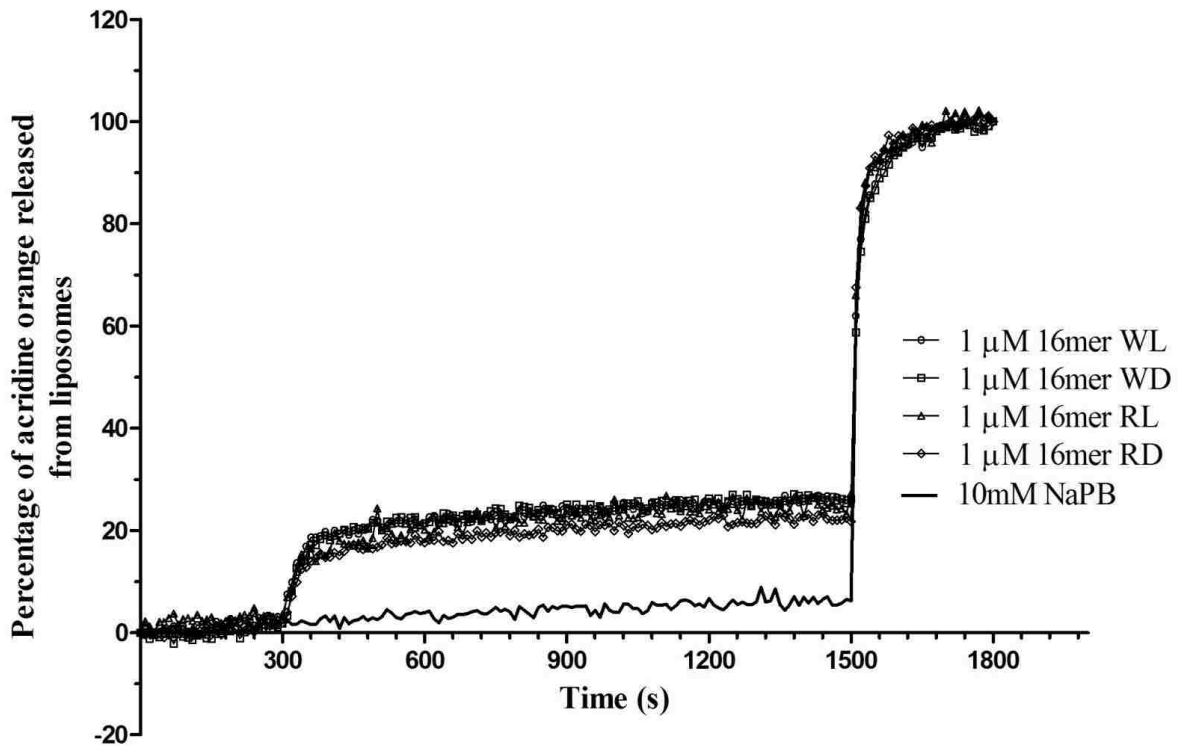
Figure 2.9 Fluorescent leakage assays with liposomes lacking ergosterol. The four 16mer histatin-5 derivative peptides at concentrations of 20 μM were incubated with acridine orange loaded liposomes lacking ergosterol. The percentage of fluorescent leakage from liposomes at time t (s) compared to total leakage obtained with Triton X-100 at time 1800 s. Peptides were added to the sample at 300 s. Control sample was treated with sodium phosphate buffer (NaPB) instead of peptides. Maximal fluorescent intensities were in the range of 80,000-220,000 counts per second).
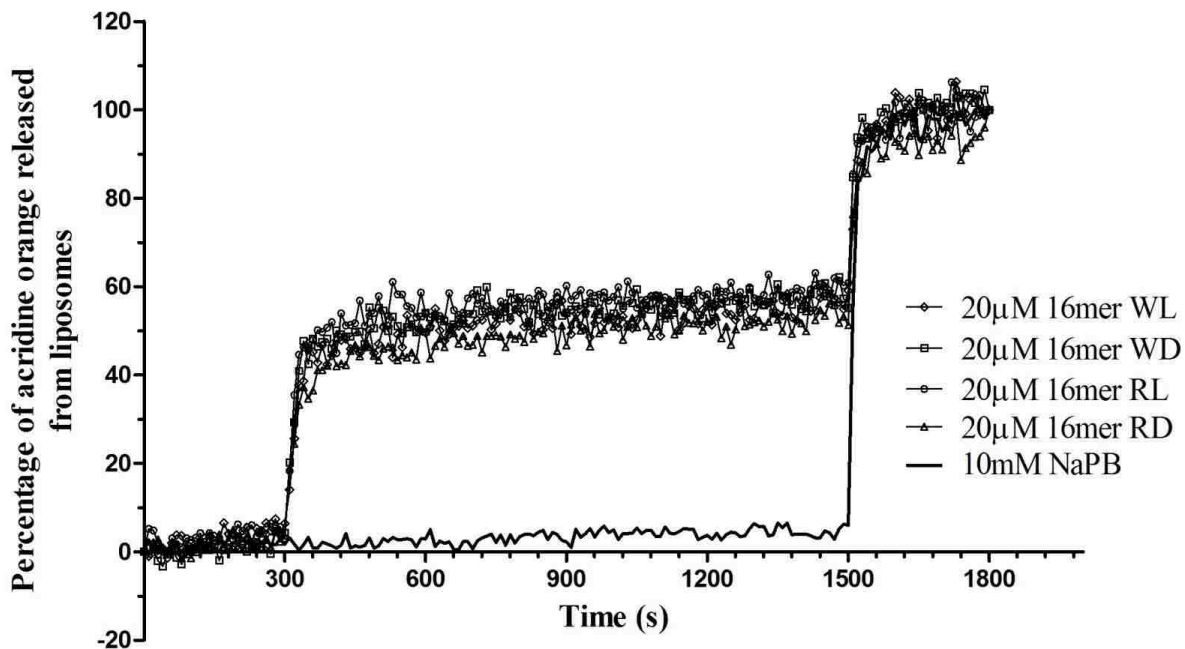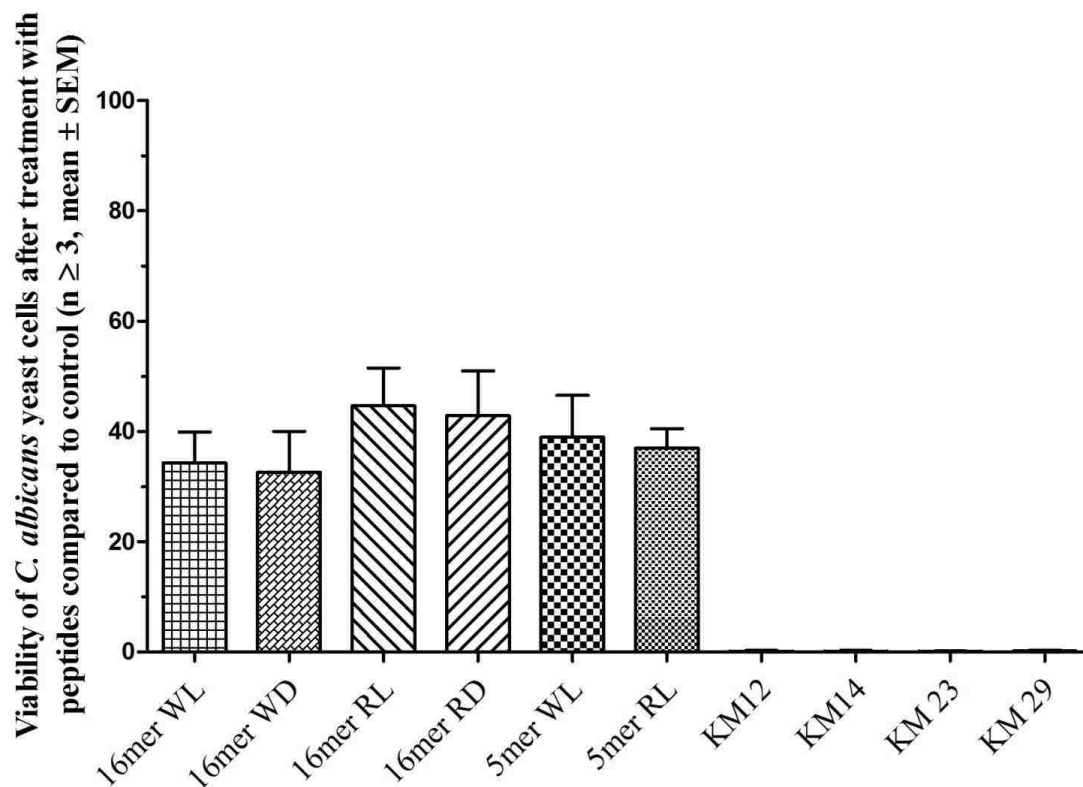
Figure 2.10: The antifungal activities of several novel peptide antifungals on *C. albicans*.
Microdilution fungicidal activity assay was used to compare the killing activity of KM 12, KM 14, KM 23 and KM 29 to the 16mer peptides and the five-amino-acid core peptide (5mer WL) or the retro version of the 5mer (RL). All peptides were tested using a 4 µM concentration. Data were expressed as mean ± SEM, n = 3).

Figure 2.11: The circular dichroism spectra of four 16-mer derivatives of histatin-5. The CD spectra were obtained in 50% trifluoroethanol (TFE) solution. Data provided by Dr.Yazan Akkam.

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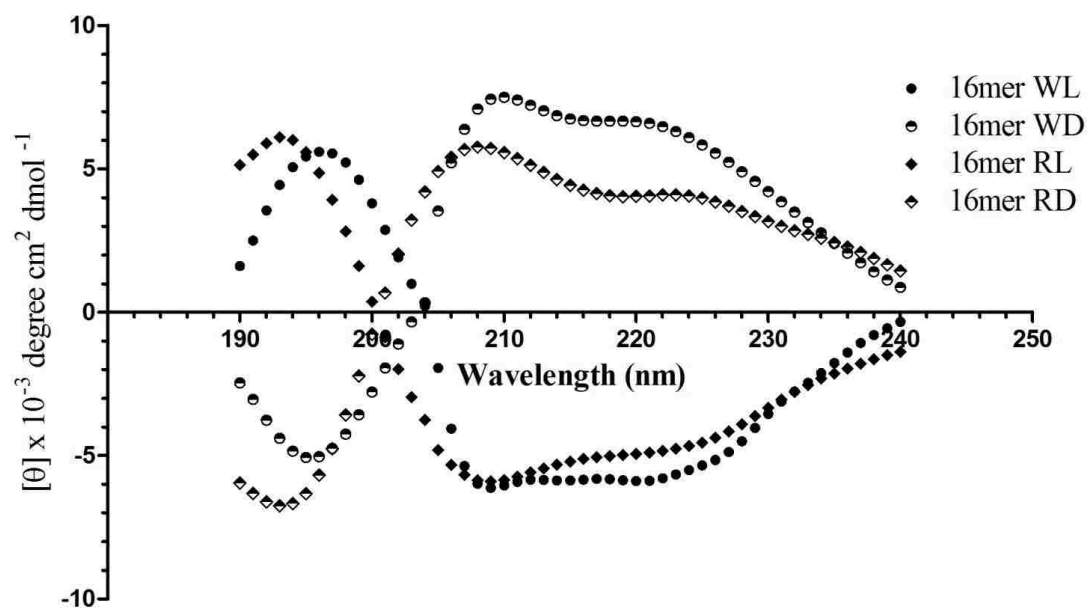Bessalle, R., A. Kapitkovsky, A. Gorea, I. Shalit & M. Fridkin, (1990) All-D-magainin: chirality, antimicrobial activity and proteolytic resistance. *FEBS Lett* **274**: 151-155.

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CHAPTER 3

**EVALUATION OF THE TOXICITY OF NEW ANTIFUNGAL PEPTIDES WITH THE
USE OF CULTURED MAMMALIAN CELLS**

A. Introduction

      The *in vitro* evaluation of toxicity of a newly developed drug is a mandatory step before the administration of that drug to an animal or human subject. The purpose of the step is to investigate its potential toxic effects on cultured mammalian cells before the drug is administered *in vivo*. Several mammalian cell lines commonly used for this step are fibroblasts, HeLa and hepatoma cells. Those cell lines are preferred to primary cells due to various factors such as culture feasibility and ability to obtain reproducible results (Bourdeau, 1990). Although *in vitro* toxicity assays cannot guarantee a drug will be non-toxic *in vivo*, those assays are considered a preliminary tool that helps screen for potential major cellular toxicity issues associated with a newly developed drug. There are also cell lines from specific organs such as the brain, lung, liver, reticular system that could be used to further evaluate potential organ-specific toxicity (Bourdeau, 1990).

      In vitro toxicity assays use a number of parameters to evaluate potential toxicity of a candidate drug to cultured mammalian cells. One of those parameters is morphological criteria which use a checklist to look at ultrastructural changes in the nucleus, nucleolus, cellular matrix and the plasma membrane of the cultured cells exposed to a drug of interest (Walton, 1975, Walton & Buckley, 1975). In addition to morphology, viability of the cells could be evaluated by dye exclusion assays (Strober, 2001) or by biochemical reagents that quantify metabolism parameters such as reductase activities and intracellular adenosine triphosphate levels (Lundin *et al.*, 1986, Maehara *et al.*, 1987). Also included in the list are cytotoxicity assays that quantify the release of lactate dehydrogenase into cell culture media (Korzeniewski & Callewaert, 1983) or activated caspases levels in cell lysates (Niles *et al.*, 2008).

      In terms of toxicity assays that quantify reductase activities, resazurin (Ahmed *et al.*, 1994) and tetrazolium salts such as MTT (Mosmann, 1983), XTT (Paull, 1988, Roehm *et al.*,

1991), MTS (Cory *et al.*, 1991) and WST-1 (Ishiyama, 1993) are commonly used. The general principle of viability assays with tetrazolium salts is that these salts are reduced by mitochondrial and cytosolic dehydrogenases into formazan that can be quantified by colorimetric means (Gonzalez & Tarloff, 2001). Unlike MTT, tetrazolium salts such as XTT, MTS and WST-1 are water soluble and could be added directly into culture media. The formazan products generated by these salts are also water soluble; therefore, a solubization step is not required in the assays that use these salts as compared to the assays with MTT. One noticeable advantage of toxicity assays with resazurin is that its metabolite of resorufin could be detected by fluorescent methods (Ahmed et al., 1994, Bueno *et al.*, 2002).

The validity of using ATP to evaluate mammalian biomasses had been studied and reported in the literature (Lundin et al., 1986). Adenosine triphosphate (ATP) is a form of energy of the cells and the combination of intracellular levels of adenosine monophosphate (AMP), adenosine diphosphate (ADP) and ATP could be used to calculate energy charge (Atkinson & Walton, 1967) which is an indicator of viability of the cell. Intracellular amount of ATP was shown to correlate well with the number of mammalian cells determined by cell count (Lundin et al., 1986, Hasenson *et al.*, 1985). The incorporation of luciferase enzyme from firefly into ATP assays makes those assays become more convenient for research usage (Lundin, 2000).

Dye exclusion assay is another method used to quantify viable cells. The dyes commonly used in this type of assay are trypan blue and propidium iodide. Trypan blue is a diazo dye with a negative charge and cannot penetrate the cell membrane of viable cells (Evans & Schulemann, 1914). When the cell is dead or there are pores on the cell membranes, trypan blue can cross the cell membrane and stains the cells with a blue color that can be detected with the use of a microscope (Tran *et al.*, 2011, Strober, 2001). Similar to trypan blue, propidium iodide cannot

penetrate the cell membrane of viable cells. When the cell is dead or the cell membranes are disrupted by hypotonic solution, propidium iodide can cross the cell membranes and intercalate nuclear DNA molecules. The intercalation of propidium iodide into DNA molecules emits fluorescence that can be detected by fluorescence microscope or flow cytometer (Krishan, 1975).

Unlike viability assays, cytotoxicity assays use biochemical agents to evaluate enzymes or substances involved in apoptosis and cell death. In terms of drug exposure, cytotoxicity assays require longer drug exposure time of up to 48 hours compared to that of 1-4 hours in viability assays (Riss 2004). Cytotoxicity assays are commonly used in the development of anticancer drugs. In cytotoxicity assays with lactate dehydrogenase, the release of cytosolic enzyme lactate dehydrogenase into culture medium is used to evaluate cell death (Korzeniewski & Callewaert, 1983, Decker & Lohmann-Matthes, 1988). In caspase cytotoxicity assays, activated initiator or effector caspases are detected by caspase-specific antibodies or by fluorochrome substrate that becomes fluorescent after being cleaved by caspase enzymes (Niles et al., 2008). Caspase enzymes were shown to correlate with cell death and apoptosis (Li & Yuan, 2008)

Hemolysis assays are a tool commonly used to investigate potential membrane disruption effects of antimicrobial peptides. Antimicrobial peptides are known to possess positive or negative charges which could interact with and disrupt the plasma membrane of target cells by mechanisms such as toroidal, barrel-starve and carpet models. Strong antimicrobial peptides such as melittin and alamethacin were known to cause hemolysis of red blood cells (Laine *et al.*, 1988, Dathe *et al.*, 1998). Hemolysis assay, therefore, is an indispensable part of any testing step of a newly developed antimicrobial candidate.

In this study, we incorporated several toxicity assays to investigate potential toxic effects of several new antifungal peptides developed at our lab. Viability assays were used to examine

the viable versus dead populations of NIH3T3 cells as a result of exposure to the peptides for 24h, 48h and 72 h. Hemolytic assay was utilized to explore potential lytic effects of the peptides on sheep red blood cells.

B. Materials and methods

Chemicals and reagents. Chemical reagents were purchased and stored at conditions specified by the corresponding manufacturers. Trypan blue 0.4%, Phosphate buffer saline pH 7.2, Dulbecco's Modified Eagle's Medium with phenol red, DMEM without phenol red, and Penicillin-Streptomycin were purchased from Gibco-BRL. WST-1 reagent was obtained from Roche Applied Science. Propidium iodide was purchased from MP Biomedicals. Newborn calf serum and L-glutamine were obtained from Sigma-Aldrich. Sheep red blood cells were purchased from Innovative Research Incorporation and stored at 4°C. Peptides were synthesized at our lab and dissolved in 10mM sodium phosphate buffer at millimolar concentrations in order to reduce the volume of peptide stock solutions needed for toxicity assays. The name and sequence of antifungal peptides used are provided in Chapter 2 in *Table 2.1.*

Culturing of cells. NIH3T3 cells (catalog number CRL-1658) were purchased from American Type Culture Collection and stored in liquid nitrogen. The cells were sub-cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% newborn calf serum, glutamine (8mM), penicillin (100 U/ml) and streptomycin (100µg/ml) before being used in toxicity assays.

Cellular toxicity evaluated by morphological changes. NIH3T3 cells were seeded on 96 tissue culture plates (BD Falcon) or 8-well culture slides (BD Falcon) at a density of 15,600 cells/cm^2 and allowed to attach to the bottom of the wells overnight in a humidified incubator at 37oC/7% CO^2. Overnight culture media were replaced with phenol red-free DMEM supplemented with peptides or buffer and the cells were incubated in the new culture media for 24 h or 48 h. At the end of designated treatments, the cultured cells were observed with a light microscope for the detection of morphological changes.

Cell viability evaluated by trypan blue. NIH3T3 were seeded to 96-well tissue culture plates as described in morphological assays. For trypan blue dye exclusion assays, NIH3T3 cells in 96-well plates were stained with trypan blue 0.4% (1:10 final dilution ratio) at 24 h after peptide treatment with peptide at final concentration of 100 μM (based on dry weight) or buffer only. Viable and dead cells were counted from a population of at least 200 cells per well with the use of a light microscope.

Cellular viability evaluated by WST-1. NIH3T3 cells were seeded on 96 tissue culture plates as described in morphological assays. The cells were designated to be treated with buffer or antifungal peptides at final concentrations up to 100μM in phenol red-free DMEM during a period of 24, 48 and 72 h. If the treatment period lasted for 72 h, culture media were replaced at 48h in order to ensure that the cells were provided with sufficient nutrients during the treatment period. 10μL of WST-1 was added into the wells at the end of the treatments and the plates were further incubated at 37°C for 45 min in a humidified incubator. Absorbance was measured at 450nm with the use of a microplate reader (EL808, Bio-Tek Instrument, Inc.).

Cellular viability evaluated by flow cytometry. NIH3T3 cells were seeded on 24-well tissue culture plates at a density of ~ 28,000 cells/well and allowed to attach to the bottom of the wells overnight in a humidified incubator at 37°C/7% CO_2. Normal DMEM culture medium was replaced with culture media that contained antifungal peptide at final concentration of 50 μM. Cells were designated to be treated with peptides or with buffer alone for 48 h. At 48 h, cells were harvested with trypsin and washed with phosphate-buffered saline. Cells were centrifuged at 149 x g for 10 min and cell pellets were resuspended in 0.25 ml of propidium iodide staining buffer (8μg/ml of propidium iodide in phosphate-buffered saline). Cells were incubated in propidium iodide staining buffer at room temperature for 15 min and then put on ice for

immediate analysis by flow cytometry (Becton Dickinson, FACSort[TM]). Maximal absorbance wavelength for propidium iodide is 535nm and maximal emission wavelength is 617nm.

Hemolysis assays. Hemolysis assays were carried out using sheep red blood cells in 96-well plates. 100μl of peptide in phosphate-buffered saline at appropriate concentration was prepared by serial dilution in the wells of 96-well plates. The maximal final concentration of peptides was 100 μM. Positive control wells used Triton X-100 at final concentration of 1% instead of peptides and negative control wells used phosphate buffered saline. 100 μl of 1% sheep red blood cells in phosphate-buffered saline were added into the wells prepared as described above and the cells were then incubated at 37°C for an hour in an automatic shaker at 170 rpm. The plates were centrifuged at 1000g for 5 minutes and 100 μl of supernatants per well were collected for the measurement of absorbance at 405nm by a microplate reader (EL808, Bio-Tek Instruments, Inc.). The percentages of hemolysis were calculated by the following equation:

$$\text{Percentage of hemolysis} = 100\% \times \frac{\text{Abs (sample)} - \text{Abs(negative control)}}{\text{Abs (positive control)} - \text{Abs(negative control)}}$$

Where:

Abs (sample) is the absorbance of supernatant obtained from the samples treated with peptides

Abs (negative control) is the absorbance of supernatant obtained from the samples treated with phosphate buffered saline

Abs (positive control) is the absorbance of supernatant obtained from the samples treated with 1% Triton X-100.

C. Results

Morphological evaluation of antimicrobial peptide toxicity. To evaluate the morphological effects of the antimicrobial peptides on mammalian cells, we chose to examine the peptides KM12 and KM14 that were synthesized in our lab. KM12 peptide sequence is YKRKFC-CFKRKY with the 5mer domain of FKRKY that was shown in Chapter 2 to have antifungal activity comparable to histatin-5. The 5mer domain was dimerized via disulfide bonds generated through artificially added cysteine residues. The KM14 peptide sequence is WKRKWC-CWKRKW. The aromatic residues phenylalanine and tyrosine were changed to tryptophan with the goal of enhancing membrane interaction and possibly antifungal activity. These peptides were shown to have potent antifungal activity (see Chapter 2), and they were considered our "lead compounds" in the development of novel antifungals. Thus, 50 µM of these peptides were incubated with NIH3T3 cells for either 24 h or 48 h in DMEM containing 10% newborn calf serum. As shown in *Figure 3.1*, no significant morphological abnormalities were observed at 24 h or 48 h post-exposure with either KM12 or KM14.

Viability assay for antimicrobial peptide toxicity. Although no morphological changes in cells were observed by the addition of KM12 and KM14 peptides, the next step was to evaluate the metabolic activity of the mammalian cells in the presence of the antifungal peptides. For these studies, the WST-1 reagent was employed. NIH3T3 cells were incubated with various concentrations of the peptide and the metabolic activity of the cells was determined at 24, 48, and 72 h post-exposure to the peptide.

As a starting point for the development of these assays, the 5mer peptide (FKRKY) synthesized with D-amino acids was used (termed the R5D peptide). The NIH3T3 cells were incubated with increasing concentrations of the peptide for 24, 48, or 72 h and the cells were

examined for viability by the WST-1 assay as outlined in the Materials and Methods. As shown in *Figure 3.2*, no significant difference in the metabolic activity of the NIH3T3 cells was observed, even at 200 µM peptide concentrations. One potential caveat to this experiment is the fact that the NIH3T3 cells are grown in DMEM containing 10% newborn calf serum. It was possible that the R5D 5mer peptide was bound by serum proteins and unable to interact with the cells; hence, no toxicity observed. To address this concern, NIH3T3 cells were initially grown in DMEM containing 10% calf serum, and switched to DMEM containing 0.5% calf serum for evaluating the peptide toxicity. While NIH3T3 cells will not proliferate significantly at 0.5% calf serum, they can be maintained in culture for 24 h. When the NIH3T3 cells in 0.5% serum were exposed to R5D peptide for 24 h at concentrations as high as 200 µM, no significant difference in metabolic activity was observed *(Figure 3.3)*. These data indicate that the core 5mer peptide (FKRKY) does not display any cytotoxicity as determined by the WST-1 assay.

When the KM12 and KM14 peptides were evaluated using the same WST-1 assay at peptide concentration up to 50 µM, no significant decrease in cell viability was observed (*Figure 3.4 and Figure 3.5*). In fact, there was an increase in the absorbance at 450nm (absorbance of the WST-1 reagent following reduction to formazan) in the controls as well as the KM12 and KM14 treated groups when the values were compared at 24h, 48h and 72h. This indicated that the NIH3T3 cells continued to proliferate in the presence of the peptides, suggesting KM12 and KM14 did not cause cytotoxicity.

Evaluation of cell toxicity by trypan blue and FACS. Thus far, none of the assays performed suggested KM12 or KM14 were toxic to cells. Nevertheless, we wanted to examine individual cells more closely to determine whether the prior assays simply lacked the precision to detect low level toxicity. For these experiments, we chose to examine cell viability via trypan

blue staining and propidium iodide uptake. Both trypan blue and propidium iodide are excluded from live cells, but they are taken into dead cells. Thus, NIH3T3 cells were exposed to 50 µM KM12 for 24 h and the cell viability was determined by trypan blue staining *(Figure 3.6)*. The percentage of live cells at 24 h in trypan blue exclusion assays was 93.7% for the control group and 94.1% for the KM12 treated group. Thus, no significant difference in cell viability was observed. Additionally, NIH3T3 cells were exposed to 50 µM KM12 or KM14 for 48 h and FACS analysis performed to evaluate propidium iodide uptake. These data showed the percentages of live cells in the control, KM12 and KM14 treated groups were 94.2%, 95.6% and 91.7%, respectively *(Figure 3.7)*. Therefore, no significant difference in cell viability was observed following exposure of NIH3T3 cells to either KM12 or KM14, reinforcing the results of the prior methods of toxicity evaluation.

Evaluation of red blood cell hemolysis. As a final *in vitro* step in evaluating the antifungal peptides for cytotoxicity, we employed red blood cell hemolysis. As noted in the introduction, RBC hemolysis is a gold standard for evaluating peptide toxicity. Thus, various peptides synthesized in our lab were evaluated for their ability to lyse sheep red blood cells using the protocol described in the Materials and Methods. As shown in *Figure 3.8,* the four 16mer peptides and the two 5mer peptides discussed in Chapter 2 showed no significant hemolysis activity with approximately 3% hemolysis at concentrations of 100 µM, which is considered negligible. The KM12, KM14, KM23, and KM29 peptides also displayed negligible hemolysis activity at concentrations up to 100 µM with a maximum hemolysis of ~3% observed (*Figure 3.9*).

D. Discussion

       In this study, several different cytotoxicity assays were employed to investigate potential

toxic effects of our newly developed antifungal peptides on mammalian cultured cells. As each

assay has its own limitations, the combination of different assays was used to achieve a better

conclusion in terms of potential toxicity of the new peptides. We evaluated morphological

changes of NIH3T3 cells after treatment with the antifungal peptides. Viability assays with the

use of WST-1, trypan blue and propidium iodide were also used to evaluate viability of the cells

after treatment with the peptides. Finally, hemolysis assays were used to evaluate potential

disruption of the plasma cell membrane of red blood cells as a result of treatment with the novel

antifungal peptides.

       In morphological assays, the morphology of NIH3T3 cells treated with R5D, KM12 and

KM14 peptides at a concentrations up to 50 µM were identical to those treated with buffer alone.

One obvious limitation of our morphological assays was that we did not perform electron

microscopy for the evaluation of cell morphology based on a checklist proposed by Walton et al

(Walton 1975). For that reason, the conclusion that our peptides did not induce morphological

changes of cultured NIH3T3 should be interpreted with prudence. However, we were able to

conclude that the antifungal peptides did not affect the ability of the cultured cell to form

monolayer on the bottom of culture plates or slides.

       Cell viability assays using WST-1 showed a trend increase in absorbance at 450nm with

time in all the control groups and the groups treated with the antifungal peptides. This finding

supported the concept that WST-1 is a valid assay to detect viable cell mass. A practical point

worth mentioning in WST-1 assay is that the cell density during seeding step must be determined

appropriately so that the adhered cells do not reach 100% confluence before the addition of

WST-1. Otherwise, non-adhered cells floating in cultured media as a result of over-confluence will die and the absorbance will not accurately reflect the proliferation of the cells in the culture wells. The absorbance of WST-1 metabolites in our assays increased with time from 24 h to 48 h 72 h in the control groups and the peptide-treated groups. This finding suggested that the cell density used was appropriate for the WST-1 assay and the absorbance, therefore, correlated with the cell mass present in the corresponding cultured well. Compared with the control groups, viability of NIH3T3 cells treated with KM12 and KM14 peptides was not less and could be even better, suggesting the mammalian cells continued to proliferate in the presence of the antifungal peptides.

Although WST-1 assay is a valid tool to detect viable biomass, it is not designed for the detection of non-respiring or dead cells. Dye exclusion assays with trypan blue and propidium iodide, therefore, were used to evaluate both the populations of dead and live cells in the samples treated with the three new antifungal peptides of 5-mer RD (R5D), KM12 and KM14. Propidium iodide has an advantage over trypan blue due to the fact that it can be used in combination with flow cytometry to inspect a population of thousands of cells in a matter of seconds to minutes. The results in this study showed that treatment of NIH3T3 did not significantly affect the viability of NIH3T3 after 48 h of peptide exposure. The percentages of live and cells in all the samples were greater than 91% and less than 9% , respectively, even after a step of harvesting by trysinization.

With respect to hemolysis assays, the percentages of hemolysis in the samples treated with new antifungal peptides developed at our lab at concentrations up to 50 µM were comparable to those in the negative control groups. Increasing the concentration of antifungal peptides to 100µM resulted in a slight increase of hemolysis in the samples treated with peptides

compared to those in the negative control groups. These mild increases in hemolysis of red blood cells could be explained by the carpet model which suggests that antimicrobial peptides at their threshold concentration could act like a detergent and disrupt phospholipid membranes. However, it is important to note that these concentrations are much greater than those needed *in vitro* to kill fungal cells. Nevertheless, data are helpful in determining the appropriate concentrations of antifungal peptide to be used in *in vivo* toxicity testing.

In summary, several different toxicity assays were used in this study to investigate potential toxicity of 5-mer RD, KM12, KM14, KM23 and KM29 peptides on mammalian cells such as murine fibroblasts and sheep red blood cells. Prototype antifungal peptides developed at our lab under the names of KM12 and KM14 were shown to be non-toxic to mammalian cells at concentrations up to 100μM in these toxicity assays. Our next step will focus on the investigation of potential acute toxicity and immunogenicity of these peptides in mice.
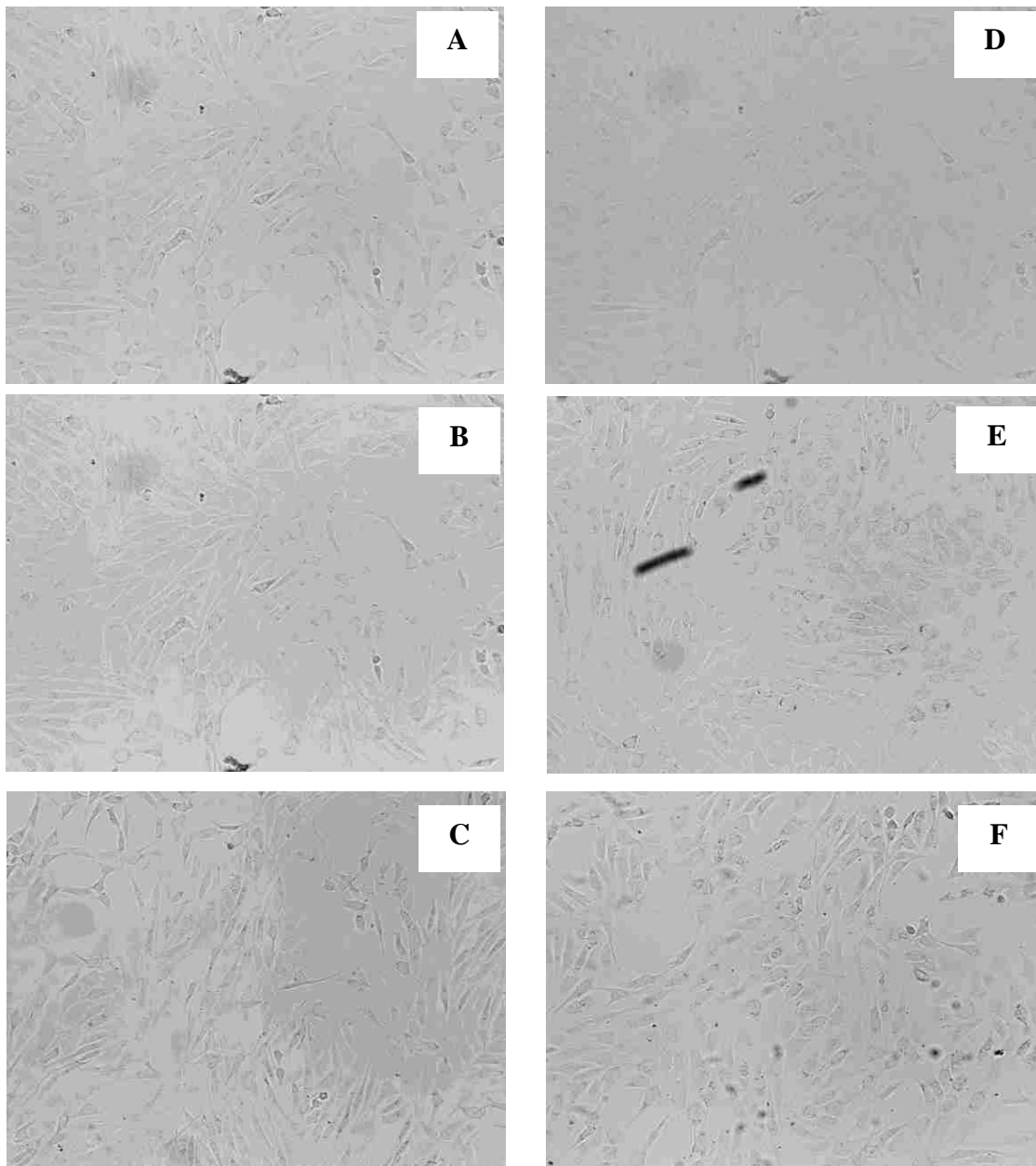
Figure 3.1: NIH3T3 morphology following exposure to antifungal peptides. A: cells in culture media alone for 24 h, B and C: cells treated with KM12 and KM 14 at a concentration of 50 μM for 24 h. D: cells in culture media alone for 48 h, E and F: cells treated with KM12 and KM14 at a concentration of 50 μM for 48 h. All peptide concentrations were determined based on extinction coefficients.
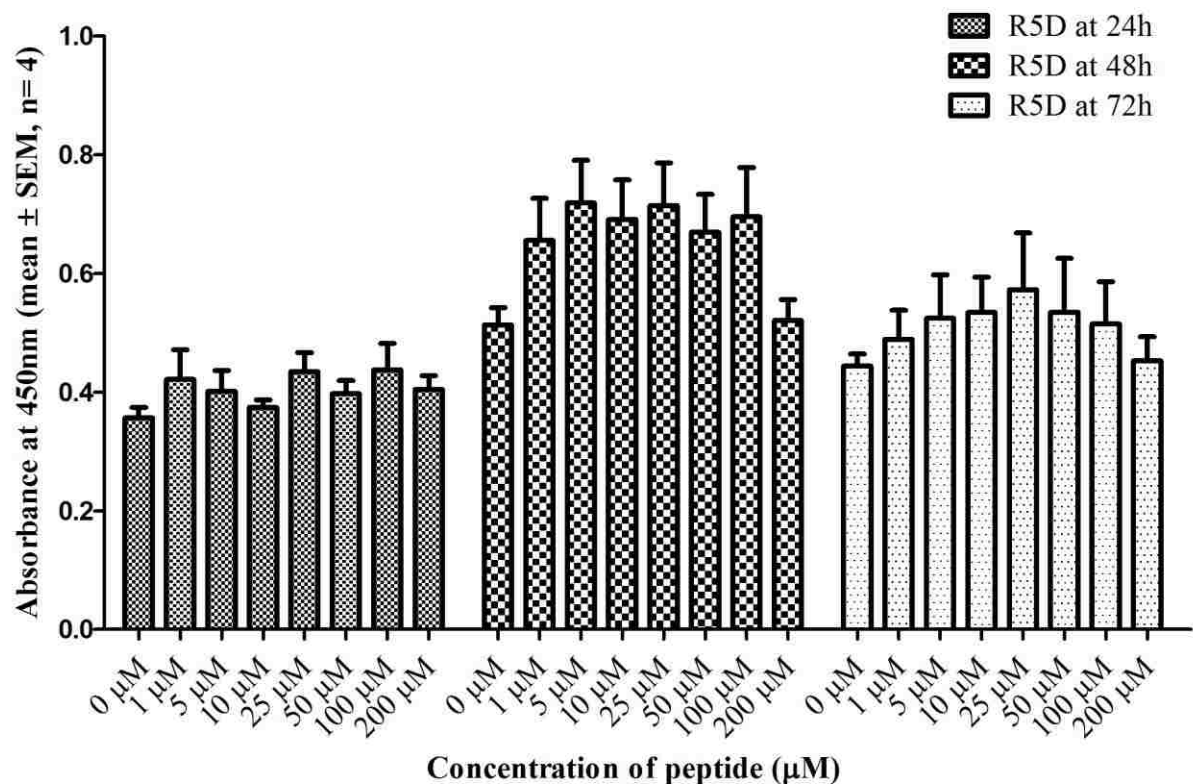
Figure 3.2: WST-1 viability assays with R5D peptide. NIH3T3 cells which formed a monolayer on the bottom of the wells of 96-well plates were treated with different concentrations of the R5D (5mer) peptide in 10% newborn calf serum DMEM medium for 24 h, 48 h and 72 h. The cells were incubated at $37^{\circ}C/7\%CO_2$. WST-1 was added to culture medium at the end of treatment period and absorbance was measured at 450 nm by a microplate reader. Data were expressed as mean $\pm$ SEM, n = 4.

Figure 3.3: WST-1 viability assays with R5D peptide in reduced serum. NIH3T3 cells which formed a monolayer on the bottom of the wells of 96-well plates were treated with different concentrations of R5D peptide in DMEM with 0.5% newborn calf serum (starving media) for 24 h. WST-1 was added into culture media at the end of treatment period and absorbance was measured at 450 nm by a microplate reader. Data were expressed as mean ± SEM, n = 4. Peptide concentrations were based on dry weight.

Figure 3.4: WST-1 viability assays with KM12 peptide. NIH3T3 cells which formed a monolayer on the bottom of the wells of 96-well plates were treated with different concentrations of KM12 peptide in DMEM plus 10% newborn calf serum for 24 h, 48 h and 72 h at $37^{\circ}C/7\%CO_2$. WST-1 was added into culture media at the end of treatment period and absorbance was measured at 450 nm by a microplate reader. Data were expressed as mean $\pm$ SEM, n = 4.
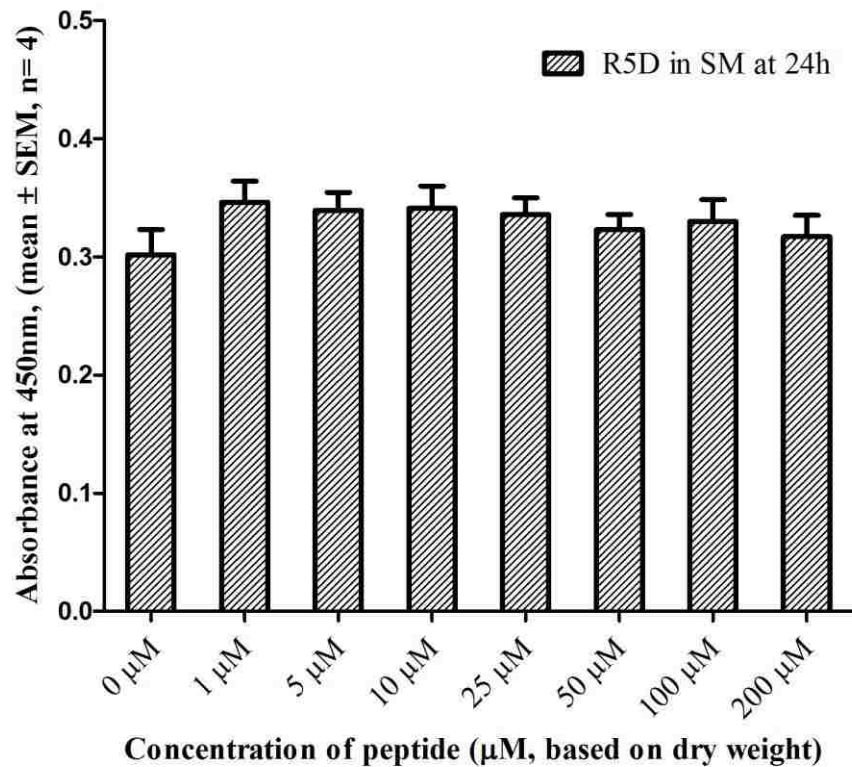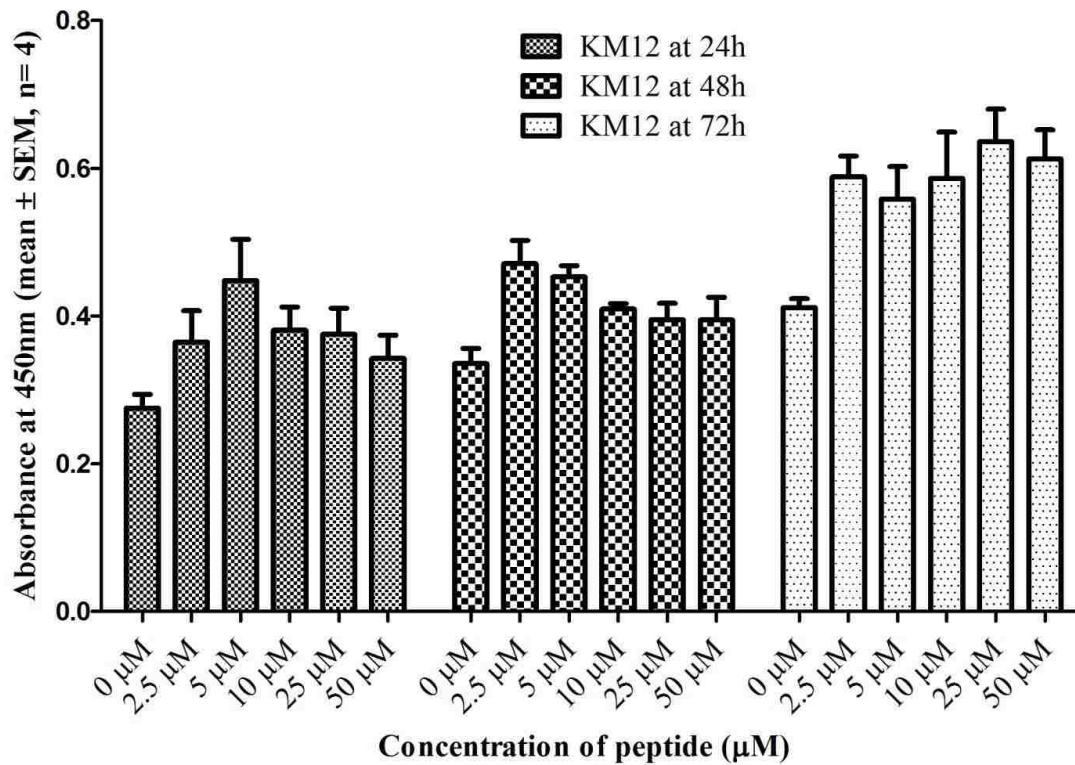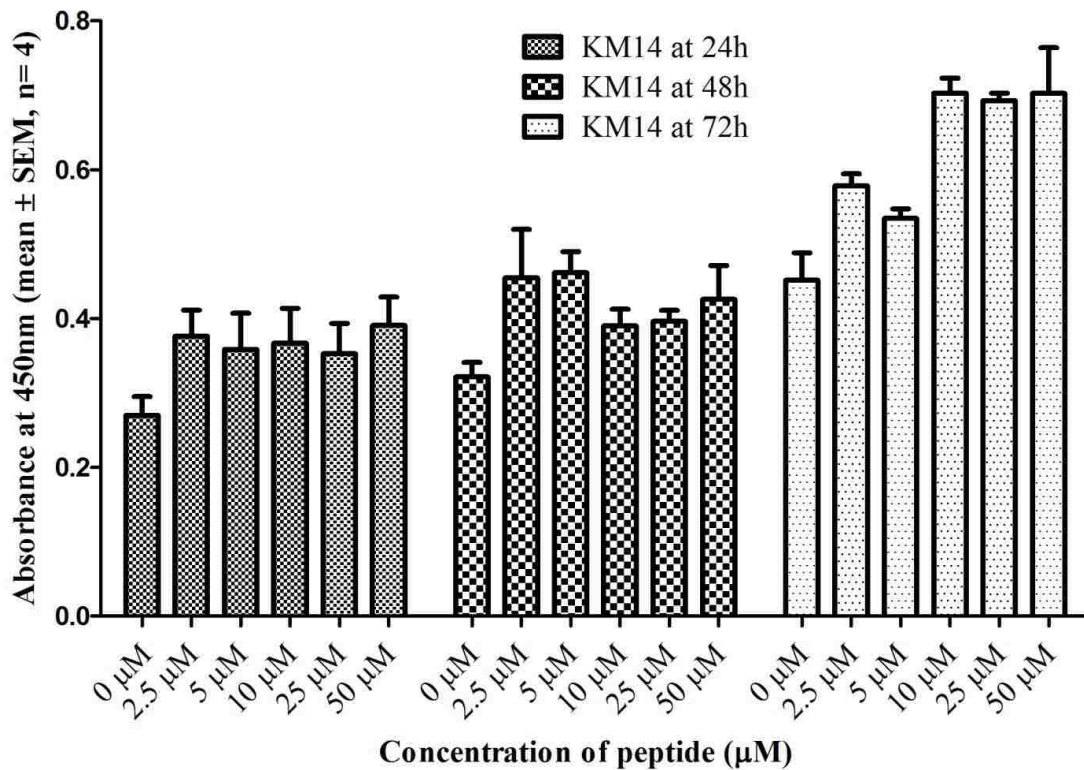
Figure 3.5: WST-1 viability assays with KM14 peptide. NIH3T3 cells which formed a monolayer on the bottom of the wells of 96-well plates were treated with different concentrations of KM14 peptide in DMEM plus 10% newborn calf serum for 24 h, 48 h and 72 h at $37^oC/7\%CO_2$. WST-1 was added into culture media at the end of treatment period and absorbance was measured at 450 nm by a microplate reader. Data were expressed as mean $\pm$ SEM, n = 4.

TRYPAN BLUE EXCLUSION ASSAY

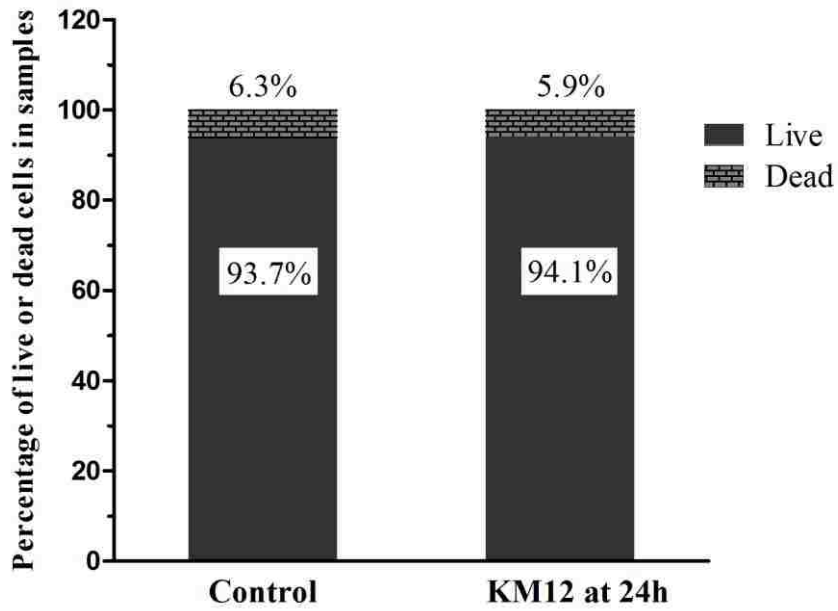|	CONTROL wells						KM12- treated wells					
	Well 1	Well 2	Well 3	Well 4	total	% total	Well 1	Well 2	Well 3	Well 4	total	% total
Live	239	204	245	204	892	93.7	201	280	277	255	1013	94.1
Dead	15	8	10	27	60	6.3	7	17	8	31	63	5.9

Figure 3.6: Trypan blue dye exclusion assay of NIH3T3 cells after treatment with KM12 peptide. NIH3T3 cells grown in 10% newborn calf serum DMEM were treated with 50μM of KM12 peptide for 24 h at $37^oC/7\%CO_2$. At the end of treatment period, the cells were stained with trypan blue. The percentage of live cells and dead cells in the control and KM12- treated groups were shown.

PROPIDIUM IODIDE STAIN IN COMBINATION WITH FLOW CYTOMETRY									
	Control samples					KM12 treated samples			
	Sample 1	Sample 2	Sample 3	total	% total	Sample 1	Sample 2	Sample 3	total	% total
Live	3590	3707	3511	10808	94.2	3572	3827	3798	11197	95.6
Dead	332	143	191	666	5.8	281	132	104	517	4.4

	KM14 treated samples				
	Sample 1	Sample 2	Sample 3	total	% total
Live	2229	4165	3976	10370	91.7
Dead	462	293	178	933	8.3
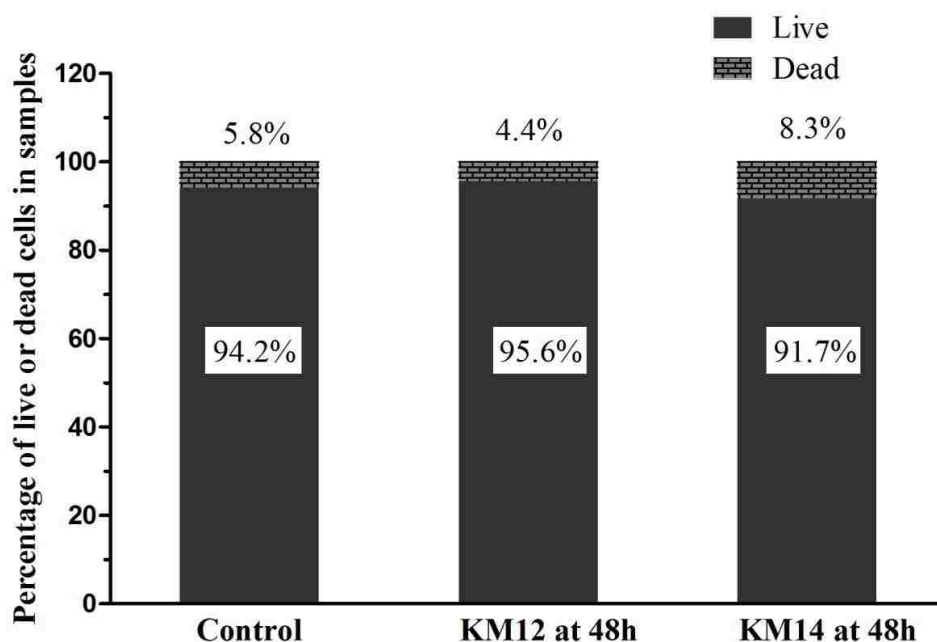
Figure 3.7: Post-treatment viability of NIH3T3 cells evaluated by propidium iodide staining and flow cytometry. NIH3T3 cells grown in 10% newborn calf serum DMEM were treated with 50μM of KM12 or KM14 peptide for 48 h at $37^{o}C/7\%CO_2$. At the end of treatment period, the cells were stained with propidium iodide and analyzed by flow cytometry. The percentage of live and dead cells in the control and peptide-treated groups were shown.
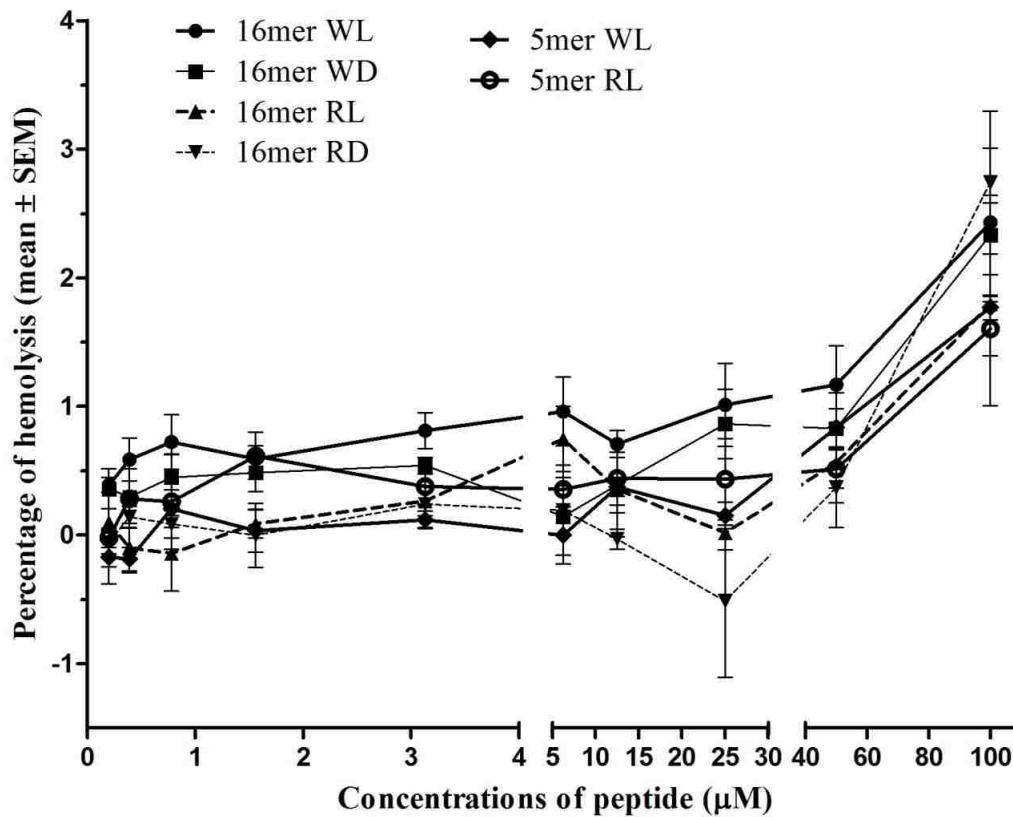
Figure 3.8: Hemolysis of sheep red blood cells treated with 16mer derivatives, 5mer WL and 5mer RL peptides. Sheep red blood cells in phosphate-buffered saline were incubated with the indicated peptides at concentrations ranging from 0.2 -100 µM. The supernatants were obtained after 1 h incubation at 37°C and absorbance was measured at 405 nm. The percentages of hemolysis were expressed as mean ± SEM, n = 3.

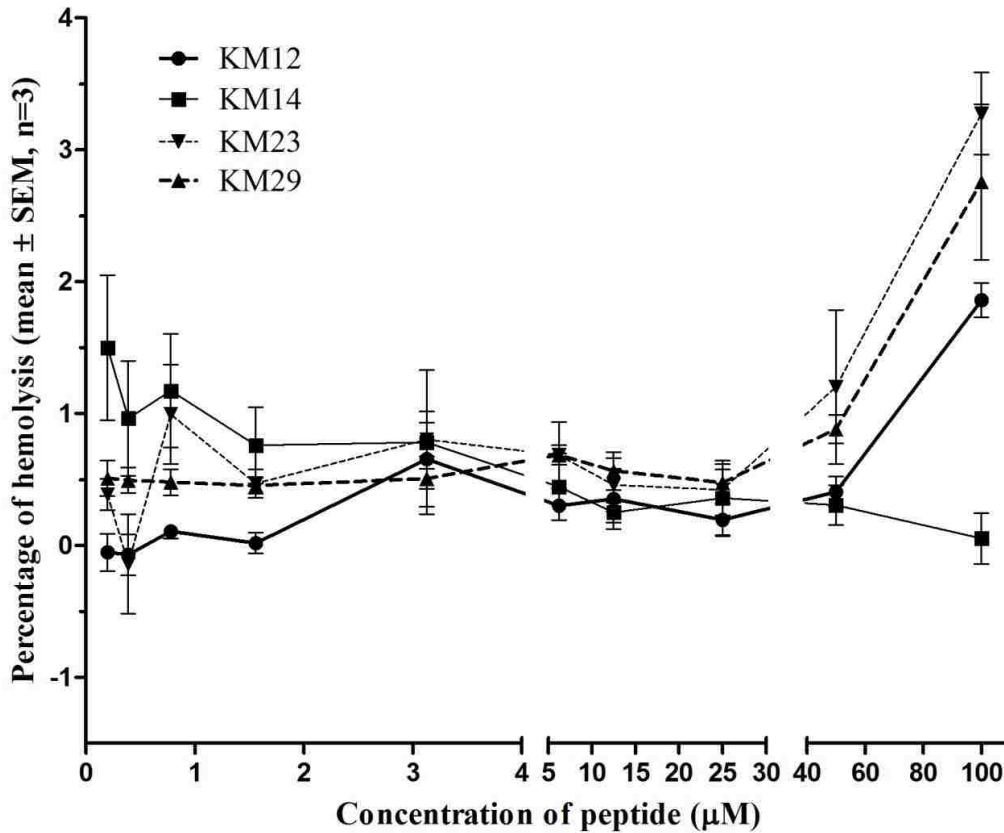**Figure 3.9: Hemolysis of sheep red blood cells treated with KM12, KM14, KM23 and KM29 peptides**. Sheep red blood cells in phosphate-buffered saline were incubated with KM12, KM14, KM23 and KM29 peptides at concentrations ranging from 0.2-100 μM. The supernatants were obtained after 1 h at 37°C and absorbance was measured at 405 nm. The percentages of hemolysis were expressed as mean ± SEM, n = 3.

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CHAPTER 4

**EVALUATION OF ACUTE TOXICITY AND POTENTIAL IMMUNOGENICITY OF
ANTIFUNGAL PEPTIDES IN MICE**

A. Introduction

Acute toxicity and immunogenicity are among the major concerns that arise when peptide-based drugs are to be administered to a human subject. Antimicrobial peptides with potential effects on phospholipid membranes may inadvertently target and disrupt the phospholipid membranes of host cells via nonspecific interactions. Many antimicrobial peptides have in their structures positive or negative charges which allow them to interact with charged phospholipid molecules on the target membranes. Those interactions could lead to the disruption of the target cell membranes via barrel-stave, toroidal or carpet models. Antimicrobial peptides with strong activities against bacteria such as melittin (Sessa *et al.*, 1969, Hermetter & Lakowicz, 1986, Laine *et al.*, 1988) and alamethacin (Dathe *et al.*, 1998) are known to cause hemolysis of mammalian red blood cells. That unwanted effects obviously poses a great harm to host subjects if the peptides are to be used in human therapeutics.

As many antimicrobial peptides are non-self molecules, possible immune responses of the human body to those peptides must be taken into consideration. The body is equipped with innate and acquired immune systems that constantly scan for the presence of foreign material and respond accordingly. The innate immune system is a line of defense that uses neutrophils, eosinophils, mononuclear phagocytes and natural killer cells to identify and remove foreign pathogens that enter the body (Janeway & Medzhitov, 2002). Pattern recognition receptors present on the plasma membranes of those immune cells such as toll-like receptor-2 (TLR2), toll-like receptor-1: toll-like receptor-2 (TLR1:TLR2) could recognize foreign proteins or peptides that are structures of bacteria and viruses (Xu *et al.*, 2000, Takeuchi *et al.*, 2002, Aliprantis *et al.*, 1999). The activation of those pattern recognition receptors results in the

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activation of intracellular signaling pathways and the production of cytokines known to be involved in cellular signaling and inflammation (Dunne & O'Neill, 2003).

Foreign peptides entering the body are processed by phagocytic antigen-presenting cells and then presented to T lymphocytes in major histocompatibility complexes. Intracellular peptides such as those of viral origin are processed inside antigen-presenting cells by an endogenous pathway in which they are bound to class I MHC molecules for the presentation to CD8+ T lymphocytes. Extracellular protein or peptide antigens are taken into the intracellular space of antigen-presenting cells and processed by another pathway called the exogenous pathway. In this pathway, digested protein or peptide antigens are bound to class II MHC molecules in cytoplasmic vesicles before being delivered to the host cell membrane where they interact with CD4+ T lymphocytes. The presentation of extracellular peptides bound to class II MHC molecules are performed by professional antigen-presenting cell such as dendritic cells and macrophages (Vyas *et al.*, 2008). The presentation of small foreign peptides in combination with MHC molecules to T lymphocytes will result in the activation of the two important branches of the adaptive immunity.

Responses of the adaptive immune system to foreign protein or peptide antigens could include the activation cell-mediated and humoral-mediated immunity. Important effectors of the cell-mediated immunity branch are CD8+ cytotoxic T lymphocytes, macrophages and natural killer cells. These effector cells kill infected cells by toxic chemicals or phagocytosis. Beside the cell-mediated immunity branch, the humoral-mediated immunity branch can also react to the presence of foreign protein or peptide antigens by the formation of immunoglobulins. B lymphocytes of the humoral-mediated immunity can produce IgG antibodies which neutralize the activity of foreign protein or peptide antigens. Furthermore, B lymphocytes can form IgE

antibodies known to be involved in hypersensitivity reactions. Examples of formation of antibodies to peptide-based drugs have been described (Yagi *et al.*, 1963, Ferraiolo *et al.*, 1988).

The formation of memory cells and secondary immune responses should also be taken into account when a newly-developed peptide is to be administered to a human subject for an extended period of time. Foreign peptides introduced into the body could initiate a mild primary immune response but with the formation of memory cells, secondary immune responses could be strong and dangerous in subsequent administrations of the peptide. The formation of memory cells is one principle for the protective effects of vaccination. Unfortunately, this phenomenon could be dangerous if the body forms memory cells and strongly respond to the re-administration of therapeutic peptides.

For safety reasons, acute toxicity testing is a step to determine the dose that either cause no adverse effects or life-threatening toxicity (NIH, 1996). Acute toxicity testing is performed with the administration of a single dose or multiple doses of drug during a period that does not exceed 24 h (NIH, 1996). The NIH guidance for industry recommends that the testing should include two routes of drug administration in which one is the intended route to be used in human and the other is the intravenous route. Besides, NIH also recommends the test to be performed in at least two mammalian species that includes one non-rodent species. In this study, we evaluated potential acute toxicity of two newly developed antifungal peptides namely KM12 and KM14 in Hsd: ICR (CD1®) mice. This was a step that followed our previous study which showed that these peptides were nontoxic to NIH3T3 cells grown in culture media. We also investigated potential immunogenicity of these two peptides in mice in order to further determine their safety profile. The results of these testing were presented in the following sections.

B. Materials and methods

Chemicals and reagents. Chemicals and reagents were purchased and stored at conditions specified by the corresponding manufacturers. Glucose was purchased from EMD and phosphate buffered saline from Gibco-BRL. Acetic anhydride (J.T. Baker) methanol (EMD) and 10N NaOH) were purchased from VWR Scientific. Ammonium bicarbonate was purchased from Fluka. PVDF membrane 0.45 μm was purchased from Biotrace. Mouse anti-acetylated lysine (catalog number 9681S) was purchased from Cell Signaling and HRP conjugated goat anti-mouse IgG antibodies (catalog number SC-2005) from Santa Cruz Biotechnology.

Peptides. The peptides CFKRKY and CWKRKW were commercially synthesized by Peptide Internationals. The peptides were dimerized to form KM12 and KM14 peptides, respectively. Purified KM12 and KM14 were recovered by high-performance liquid chromatography and their sequence was confirmed by mass spectrometry. KM12 and KM14 were stored at $-20^{o}C$ and dissolved in either phosphate-buffered saline or 5% glucose. Stock concentrations of KM12 and KM14 were prepared in phosphate-buffered saline at concentrations of 35 mg/ml and 45.58 mg/ml, respectively. The peptides were diluted in sterile phosphate-buffered saline or 5% glucose to the appropriate concentration before injection into mice. The total volume per injection was less than 0.1 ml. Hsd:ICR (CD1[®]) mice were purchased from Harlan [TM] and kept in clean cages in accordance with current guidelines for animal care in research.

Evaluation of acute toxicity of KM12 and KM14 in mice. The protocol used for the testing of acute toxicity of KM12 and KM14 on mice was approved by the Institutional Animal Care & Use Committee (IACUC) of the University of Arkansas. Hsd: ICR (CD1[®]) mice were randomly allocated into three groups of control, KM12 and KM14 treatment. There were five

mice per treatment group. The peptides were prepared in sterile phosphate-buffered saline or 5 % glucose at the appropriate dose and the volume per injection was no greater than 0.1 ml. Injection was done via the tail veins of the mice and started with a dose of 8mg/kg of body weight using a standard up-down dosing protocol. Based on the responses of the mice to the starting dose, an adjustment in the dosage of the peptide would follow. If good tolerance to the starting dose was recorded, the peptide dosages would then be doubled until toxicity was detected or an upper limit dosage of 64 mg/kg of body weight per injection was reached. Alternatively, the peptide dosage would be reduced stepwise by a decrement of 2 mg/kg of body weight until no signs of toxicity were recorded. Tolerance to the injected peptides was evaluated in each mouse by the monitoring of clinical signs for at least 3 h post-injection. Those signs include reduced motor activity, piloerection, redness of the ear lobe, cyanosis, protruding eyeballs, slow or labored breathing, loss of response in the rear legs, convulsions and death. A score was given based on the criteria described in *Table 4.1*. All the injected mice were followed up for at least 14 days and sacrificed for gross evaluation of internal organs. The brain, heart, lungs, liver and spleen of those sacrificed mice were evaluated by gross morphology and the weight of each organ was recorded.

Evaluation of KM12 immunogenicity in mice. The immunogenicity of KM12 peptide in Hsd:ICR mice were investigated in a group of Hsd:ICR (CD1®) mice injected with KM12 once a month for four months at a safe dosage determined by the acute toxicity testing. Control mice were injected with 5% glucose only. Mice were injected once per month with KM12 prepared in sterile 5% glucose. Injections were intravenous, intramuscular or intra-peritoneal routes with five mice used per route of injection. At the fifth month, the injected mice were subjected to general anesthesia for the collection of blood via cardiac puncture and then euthanized by cervical dislocation. Blood samples were allowed to stand at room temperature for

30 min and serum was recovered by centrifugation at 1500 x g for 10 minutes. Serum samples were stored at -20°C until being used.

Detection of antibodies against KM12 in mouse serum. The presence of antibodies, if any, in the mouse serum samples was investigated by Dot Blot. In brief, 0.45 μm PVDF membrane was soaked in 100% methanol for 5 min and then in TBS (30ml of 5M NaCl, 100ml of 1M Tris-HCl pH 8, and water up to 1000 ml) for at least 5 minutes before being used use. 10μg of KM12 prepared in 30 μl of phosphate-buffer saline was applied onto the PVDF membrane with the use of Hybridot manifold BRL 1050mm (Bethesda Research Laboratories). 10μg of acetylated KM12 in buffer was used as control and applied onto PVDF membrane at a remote position. The membrane was allowed to dry completely for several hours at room temperature before being used. The control acetylated KM12 was prepared by a method described by Hunt (Hunt, 2001). In brief, lysine residues of the KM12 peptide were acetylated by a reagent composed of acetic anhydride and methanol mixed at a volume ratio of 1:3, respectively. Thus, 10μg of KM12 per well (approximately 6 nmol) in 120 μl of 50 mM ammonium bicarbonate was added to 300 μl of acetylating reagent and the whole mixture was allowed to stand at room temperature for one hour. The mixture was neutralized with 175μl of 10N NaOH and dotted onto one well of PVDF membrane with the use of a vacuum Hybridot manifold. Membranes dotted with peptides were allowed to dry at room temperature and standard protocol for Western Blot was performed. Nonspecific binding sites on PVDF membranes were blocked with TBS-0.05% Tween 20 -5% milk (30ml of 5M NaCl, 100ml of 1M Tris-HCl pH=8, 5 ml of 0.5% Tween 20, 50g low fat dry milk, and water up to 1000ml) for at least 2 hours at 37°C. The membranes were incubated with anti-acetylated lysine mouse IgG$_2$ antibodies (dilution ration 1:1000) or diluted mouse serum (dilution ratio 1:100) in TBS- 0.05% Tween 20 -2% milk at 4°C overnight on a roller and then

gently washed three times with TBS-0.05% Tween 20. The membranes were then incubated with

goat anti-mouse IgG for 45 minutes at room temperature and then subjected to three washes of

10 minutes each with TBS-0.05% Tween 20 to remove unbound antibodies. The blotted

membranes were developed with Immobilon Western Chemiluminescent HRP substrate and

analyzed with the use of FluorChem [TM] 8900 and images were acquired for further analysis by

Quantity One software (Basic version, BioRad). In the analysis step, a circle was drawn around

each recognizable dot present at corresponding sites of KM12 or acetylated KM12 on the

membranes and the average intensity of those circles were determined by the Quantity One

software. An increase of at least 20% in average chemiluminescent intensity of a circle compared

to that of adjacent local background was considered positive for an immune response.

C. Results

Acute toxicity evaluation of KM12 and KM14. To determine the toxicity of the antifungal peptides KM12 and KM14 *in vivo*, mice were chosen. An up-down protocol was employed in which a dose of the peptide is selected (8 mg/kg) and based on the response of the animal the dose is either doubled (16 mg/kg) or reduced by half (4 mg/kg) and the study proceeds until a tolerable dose is identified. For the KM12 peptide, a dosage of 8mg/kg in phosphate-buffered saline was well tolerated by the mice. However, when the dose was increased to 16 mg/kg some mice were noted to develop signs of acute toxicity such as reduced motor activities (n=4), protruding eyeballs accompanied by convulsion (n=3) or death (n=1) (*Table 4.2*). A reduction of the injected dose to 12 mg/kg did not improve significantly the signs of acute toxicity; however, a dosage of 10 mg/kg was tolerated by the mice. When the solvent for the KM12 peptide was switched from phosphate-buffered saline to 5% glucose the KM12 peptide seemed better tolerated. Two mice were injected with KM12 prepared in 5% glucose at a dose of 16 mg/kg and they developed convulsions but survived the injection. Given the poor tolerance of 16 mg/kg, the dose determined to be safe for the immunogenicity analysis was 10mg/kg of body weight.

The KM14 peptide was poorly tolerated by the mice even at a starting dose of 8 mg/kg. Two mice injected with KM14 peptide prepared in phosphate-buffered saline died with signs of general muscle stiffness. Post-mortem examination of the brain, heart, lung, liver and kidneys of those mice did not show any abnormality by gross morphology. When KM14 was prepared in 5% glucose, there seemed to be an improvement in the tolerance of the injected mice. One mouse injected with KM14 prepared in 5% glucose developed temporary loss of motor activity but survived the injection. Nevertheless, given the generally poor tolerance of KM14 by mice, this

peptide was no longer included in the evaluation since the antifungal activity *in vitro* was not improved as compared to KM12. Based on the results of this acute toxicity testing, KM12 in 5% glucose at the dose of 10mg/kg was selected for the next step of immunogenicity testing. The evaluation of KM14 was discontinued due to poor *in vivo* tolerance.

Immunogenicity of KM12 peptide. To determine whether mice would develop an immune response to KM12 an experiment was set up in which the peptide was injected monthly via tail vein (5 mice), intramuscular (5 mice) or intraperitoneal (5 mice), along with the controls for each site of injection (5 mice per site X 3 sites). Thus, a total of 30 mice were used in the study with 29 mice completing the study, one mouse died at the third month following convulsions after the IV injection of KM12. Some other mice in the intravenous injection group also developed unwanted signs during or right after the injection of KM12 (*Table 4.3*), however they survived after fourth months of monthly KM12 intravenous injection. Two mice in the intraperitoneal treatment group showed skin lesions related to in-cage fighting and those lesions improved after they had been separated from other mice in the treatment group. None of the mice in the intramuscular injection group or the three control groups showed any signs related to the injection during the 4-month period. Autopsy at the end of 4-month period of injection did not show any gross abnormalities.

Thirty serum samples obtained from 15 mice injected with the KM12 peptide via subcutaneous, intramuscular or intraperitoneal routes, as well as from 15 control mice were used in dot blot experiments. The purpose of dot blot was to detect the presence of anti-KM12 antibodies, if any, in those serum samples. Due to the small size of KM12, a positive control peptide was developed by acetylating lysine residues on KM12 *in vitro*. This peptide could then

be detected using anti-acetylated lysine antibodies to confirm that the peptide was present on the surface of PVDF membranes and to control for the Western blotting technique.

With respect to Dot Blot, one serum sample from the KM12 intravenous treatment group was positive for anti-KM12 IgG antibodies (*Figure 4.1A*). That serum sample was obtained from a mouse that died at the third month of intravenous injection of KM12. The four other mice in the intravenous injection group did not develop any detectable levels of anti-KM12 antibodies in their serum although some of them also developed adverse reactions during the course of intravenous KM12 injection such as rapid breathing, reduced motility, jerking movements and convulsion (*Table 4.2*). In addition to the mouse in the intravenous treatment group, one mouse in the intramuscular treatment group also developed antibodies against KM12 (*Figure 4.1*B). However, none of the mice in the intramuscular treatment groups developed any observable adverse reactions to KM12 injection during the course of 4-month KM12 administration, and none of the mice in the intraperitoneal route of injection showed antibody production.

D. Discussion

The doses of 8 mg/kg and 10 mg/kg of body weight of KM12 peptide were well-tolerated by the mice. The starting dose of 8 mg/kg was chosen based on a search in the literature. The concentration of KM12 in the extracellular fluid of the injected mice, in theory, could reach a level of 7.0 - 9.3 µM if with assumptions that total body water accounted for 60-80% of total body weight in mice (Sheng & Huggins, 1979) and that the KM12 peptide distributed within that water compartment. In reality, the volume of distribution of a peptide drug could be much less or greater than the volume of total body water depending on the biochemical properties of the peptide such as its molecular weight, charges, lipid solubility and its binding capacity to serum proteins (Wilkinson, 2001). For that reason, actual blood concentrations of KM12 achieved after an intravenous injection must be determined by appropriate methods before any concrete conclusions on those concentrations are to be reached. The use of 5% glucose as solvent seemed to improve the tolerance of the mice to KM12 peptide compared to phosphate-buffer saline solvent. As 5% glucose is a typical solvent for the delivery of drugs in human while sodium phosphate buffer is not, this is a positive finding.

The presence of one disulfide bond in the structure of KM12 peptide could be suboptimal in terms of drug stability because the bond is subjected to reduction reaction after the peptide is introduced into the body. *In vitro* experiments at our lab suggested that KM12 peptide was reduced into two monomers of CFKRKY peptide when the former was added into 10% new born calf serum (Yazan Akkam, data not shown). Although KM12 in the presence of 10% new born calf serum did not show any toxicity to murine fibroblasts in WST-1 and propidium iodide assays, it cannot be ruled out that the monomer CFKRKY peptide played a role in the development of acute unwanted effects in the mice that developed symptoms. In addition to

possible cleavage at the disulfide bond, KM12 peptide is also subjected to cleavage at the aromatic peptide bonds by proteases present in the extracellular fluid of mice and human such as carboxypeptidase A (Rawlings et al., 2012, Stewart & Gilvarg, 1999, Wei et al., 2002, Fontenele-Neto et al., 2005, Lyons & Fricker). Small amino acid fragments of the KM12 peptide, in theory, could be responsible for the unwanted signs recorded in the KM12 injected mice.

The role of cysteine residues in the development of unwanted effects toward KM12 in injected mice needs further investigations. Although cysteine is a natural amino acid and does not contain sulfa moiety (SO_2-NH_2) well known for their potential risk of allergic reactions in human, its presence in the sequence of KM12 peptide could be suboptimal. The oxidation reaction between sulfhydryl groups to form unusual cross-links between body proteins has been implicated in the pathophysiology of glucose 6-phosphate dehydrogenase deficiency (Johnson *et al.*, 1979). We incorporated cysteine residue into the sequence of our prototype peptide for the purpose of dimerization of two monomer peptide of CFKRKY into a more potent antifungal peptide of KM12 peptide. However, this approach needs to be modified if the antifungal peptides being developed are to be delivered by parenteral routes due to issues with stability and immunogenicity.

Potential cleavage of KM12 at the disulfide bond and other peptide bonds raised an important question in terms of potential fast clearance of the peptide. Small peptides of less than 14,600 Da such as lysozyme were shown to have high glomerular sieving coefficients and high clearances by the kidney (Maack et al., 1979). If KM12 with a molecular weight of 1768 Da is broken down into two smaller peptides of 885 Da, those smaller peptides could be subjected to even faster clearance by the kidney. However, *in vitro* studies at our lab suggested that KM12 is

bound to serum proteins. In *in vitro* studies at our lab, trichloroacetic acid was able to precipitate KM12 in the presence of 10 % newborn calf serum while it failed to precipitate KM12 dissolved in sodium phosphate buffer. That finding suggested that the peptide bound to serum proteins, probably albumin, which was precipitated by trichloroacetic acid (Yazan Akkam, data not shown). The binding of KM12 or KM12 cleavage products to serum proteins could help solve the problem with fast renal clearance, however, it raises another important issue with the immunogenicity of KM12 peptide.

It is known that a hapten is non-immunogenic given its low molecular weight, however, it could induce an immunological response if bound to a carrier protein (Landsteiner, 1947). The monomer cleavage product of KM12 has one cysteine residue that could form disulfide bond with serum proteins under the influence of free radicals and the monomer-carrier protein complex may function like a hapten. The development of side effects at the second month of intravenous injection and the detection of IgG antibody against KM12 in intravenously and intramuscularly injected mice suggested that KM12 peptide was recognized by the mouse immune system and defensive immune reactions were developed against the peptide. The fact that all the first injections were uneventful regardless which route was used further supported the argument that immune responses were involved in the development of the side effects observed in the symptomatic mice. As the monomer peptide of CFKRKY had a molecular weight of less than 1000 Da, it was very likely that a hapten-protein complex was involved in the development of antibodies against KM12.

Our next question was the potential antigenic determinant, if any, of the CFKRKY monomer or KM12. Previous studies have provided valuable tools that could help with this question. A study on solvent accessibility of the side chains of peptides provided a tool to

calculate surface probability of each amino acid residue in the sequence of one peptide of interest (Janin & Wodak, 1978). As the region(s) of antigenic determinant on a peptide of interest should be exposed for recognition by immune cells, hydrophilicity of the amino acids in the sequence of a peptide was used as one parameter to help define antigenic determinant of that peptide (Hopp & Woods, 1981). A tool to plot backbone flexibility and help identify highly mobile regions in the structures of a peptide of interest was also developed (Karplus, 1985). Mobile regions of a peptide were shown to be indicator of antigenic determinant of peptides (Westhof et al., 1984). A group of researchers introduced a valuable algorithm that combined several parameters such as surface probability, hydrophilicity, backbone flexibility and secondary structure prediction to determine antigenic determinants of a protein or peptide (Jameson & Wolf, 1988). This algorithm is usually used by commercial companies in the development of antibodies against a peptide of interest.

With respect to the antigenic determinant of the monomer CFKRKY or KM12 peptides, it is very likely that the antigenic determinant, if any, resides within the region that has positive charges of the peptides. As lysine and arginine are amino acids that have net positive charge, they are more likely to be exposed to hydrophilic environment of the extracellular fluid than the aromatic amino acid of phenylalanine. Tyrosine residue with one hydroxyl group on its side chain is also likely to be exposed to hydrophilic environment of the extracellular fluid. An attempt to identify antigenic determinant of the monomer CFKRKY with the use of criteria set out by Hopp and Wood and the software program Antigenicity Plot provided by Bioinformatics Organization (Luca Toldo, 1997) did not show any results for the CFKRKY monomer. However, the program suggested that the antigenic determinant of KM23 and KM29 could reside within

the region of KRKY sequence and closer to the C- terminus than to the N- terminus of the

peptides (*Figure 4.2* and *Figure 4.3*).

In summary, this *in vivo* study with mice helped clarify the potential acute toxicity and

immunogenicity of two novel antifungal peptides developed at our lab. The dose of 10 mg/kg of

KM12 was tolerated by the mice; however, the same dose was toxic if KM14 was used. 1 out of

5 mice developed IgG antibodies against KM12 peptide after fourth months of monthly KM12

injection via either intravenous or intramuscular routes. The presence of cysteine residue and

disulfide bond in the structure of KM12 peptide affected its stability in extracellular fluid and

increased its ability to bind serum proteins. KM12 peptide or its CFKRKY monomer may

function as a hapten and induced immunological response in the mice after binding to serum

proteins. The data obtained from this study are useful for the development of novel histatin-5

based antifungal peptides that contains the functional motif of YKRKF.

Table 4.1: Clinical signs during the first three hours of intravenous injection of KM12 and KM 14 peptides in Hsd:ICR mice.

Score	Signs observed during a period of 3 h post-injection
5	No signs observed
4	Light redness of ear skin
3	Reduced motor activity and redness of ear skin
2	Reduced motor activity, piloerection and bright redness of ear skin
1	Protruding eyeballs, temporary loss of motor activity, piloerection, redness of earskin/legs and labored breathing
0.5	Protruding eyeballs, temporary loss of motor activity, piloerection, redness of ear skin/ legs, convulsions with subsequent loss of response in rear legs and labored breathing
0	Death

Table 4.2 Acute toxicity of KM12 and KM14 peptides on Hsd:ICR (CD1®) mice with different dosage regimens.

PEPTIDE USED	DOSAGE	SIGNS OBSERVED ON TESTED ANIMALS
Control with PBS (n=3)	PBS, IV	None
KM12 IN PBS (n=6)	8mg/kg, IV	None
KM12 IN PBS (n=6)	16 mg/kg, IV	Reduced motor activity (n=4), protruding eyeballs accompanied by convulsion (n=3), death (n=1)
KM12 IN PBS (n=5)	12 mg/kg, IV	Labored breathing (n=4), reduced motor activity (n=4), protruding eyeball (n=1), redness in ear skin (n=1)
KM12 IN PBS (n=5)	10 mg/kg, IV	None
KM12 in 5 % glucose (n=2)	16mg/kg, IV	Convulsion (n=2), no death
KM14 in PBS (n=2)	8mg/kg, IV	Death (n=2)
KM14 in 5% glucose (n=1)	8mg/kg, IV	Temporary loss of motor activity, no death

Table 4.3 Signs observed in Hsd:ICR mice during a four- month course of injection of KM12 and KM14.

| MONTH	ROUTE USED N=5 per group	DOSAGE	SIGNS OBSERVED ON TESTED ANIMALS
First month	IV, IM, IP	10μg/g	None
Second month	IV	10μg/g	Rapid breathing and temporary reduced of motility (n=3)
	IM, IP	10μg/g	None
Third month	IV	10μg/g	Convulsion (n=4), rapid breathing (n=1), bulging eyes (n=2), reduced motility (n=2), death (n=1)
	IM, IP	10μg/g	None
Fourth month	IV	10μg/g	Reduced motility (n=4), convulsion (n=1), Jerking movements (n=1)
	IM, IP	10μg/g	None
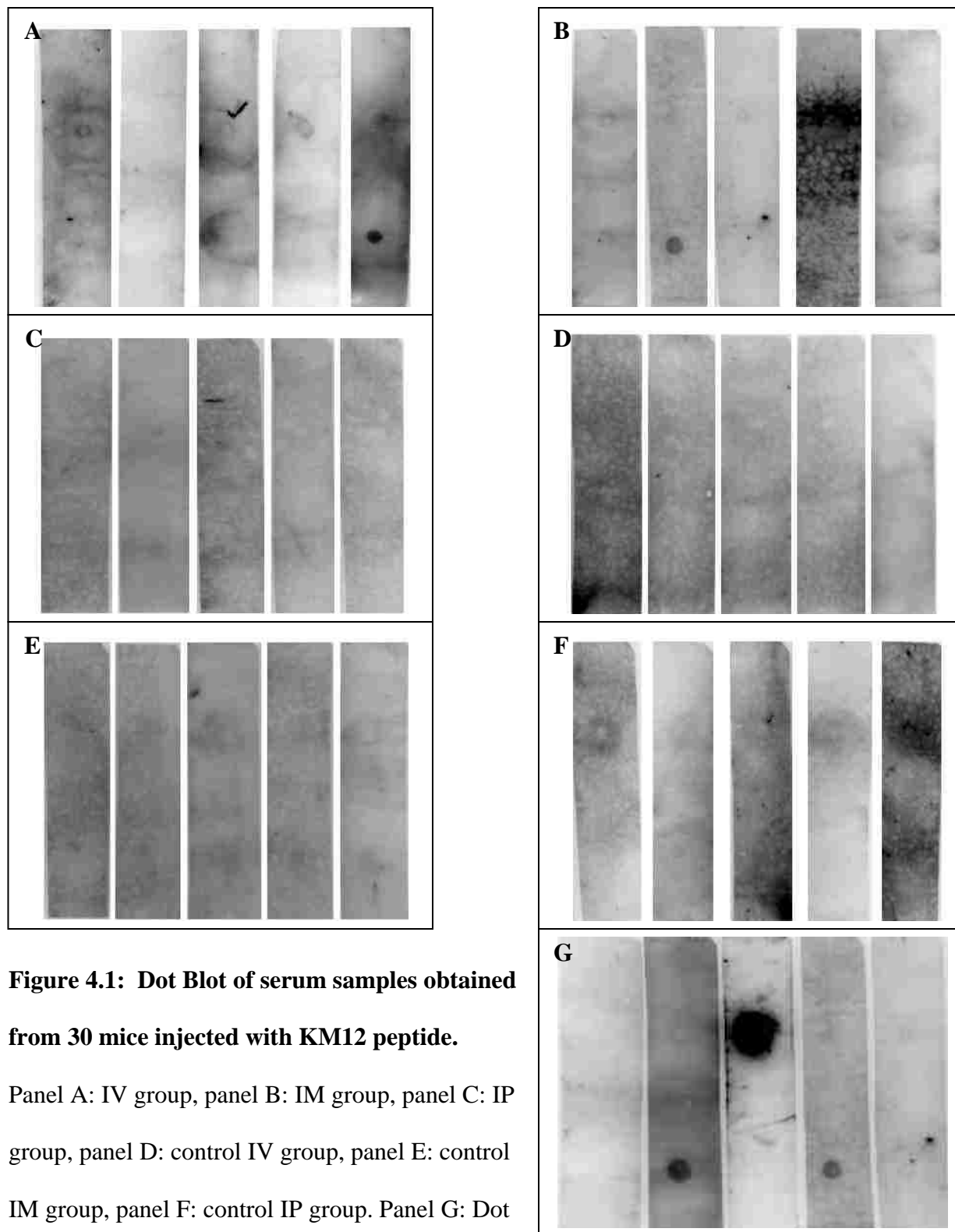
Figure 4.1: Dot Blot of serum samples obtained from 30 mice injected with KM12 peptide.

Panel A: IV group, panel B: IM group, panel C: IP group, panel D: control IV group, panel E: control IM group, panel F: control IP group. Panel G: Dot Blot of serum samples used in IV lane 2 (lane1), IV lane 5 (lane2), IM lane 2 (lane 4), IM lane 3 (lane 5) and acetylated KM12 (lane3).
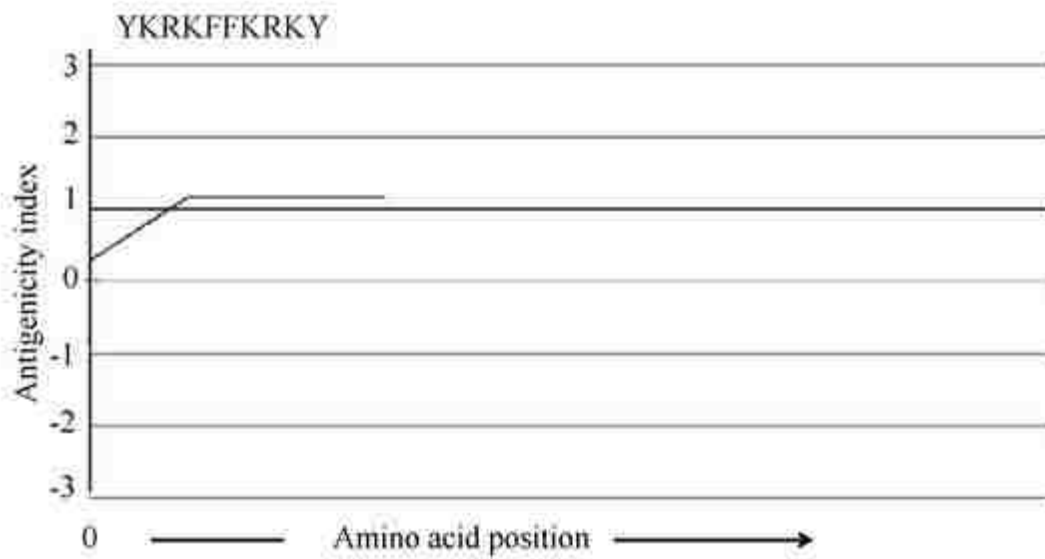
Figure 4.2: Antigenicity plot of KM23. The antigenicity plot of KM23 was obtained with the use of software provided by Bioinformatic Organization. Higher index means higher antigenicity property.

Figure 4.3: Antigenicity plot of KM29. The antigenicity plot of KM29 was obtained with the

use of software provided by Bioinformatic Organization. Higher index means higher antigenicity
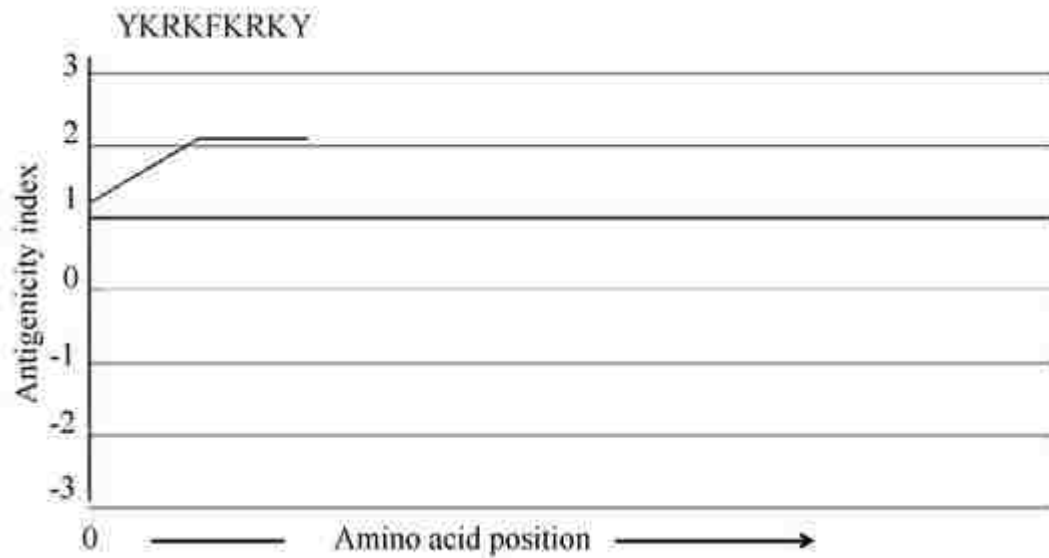
property.

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CHAPTER 5

CONCLUSIONS

The emergence of multidrug-resistant *Candida* strains demands the introduction of new agents into the current arsenal of antifungal drugs. *Candida* species are common pathogens found in cases of opportunistic fungal infections in humans. Furthermore, *Candida* species were ranked as the fourth leading cause of hospital acquired blood stream infections (Wisplinghoff *et al.*, 2004). Current antifungal drugs used for the treatment of candidiasis are known to work efficiently via their specific cellular targets in the affected fungal cells; however, the drugs could be toxic to the host and their cellular targets in fungi cells could be altered in drug-resistant strains. The development of new antifungal agents with new modes of action will be part of a solution to the current problem of antifungal drug resistance.

Histatin-5 peptide with known killing activity against C*andida* species is used in this study as a model for the development of new peptide-based antifungal agents. The peptide is present naturally in human saliva and active against several strains of fungi such as *Candida albicans* and *Candida glabrata*. The histatin-5 peptide was previously shown to kill *C. albicans* yeast cells by disrupting the cell membrane (Edgerton *et al.*, 1998) and mitochondrial functions of the affected yeast cells (Helmerhorst *et al.*, 1999, Ruissen *et al.*, 2001). Although histatin-5 has been discovered for more than 20 years, its mode of action is not fully understood. More studies are needed to help better understand how the peptide works on affected fungal cells. In this study, the mechanism of killing by histatin-5 was investigated with the use of four 16mer derivatives of histatin-5. Furthermore, the disruption of the *SSA2* gene that was proposed as the putative receptor for histatin-5 and the use of artificial membranes were also employed in order to better understand the interaction between its derivatives and their targets. We hoped the findings obtained from this study would help design new antifungal drugs based on the natural model of histatin-5 peptide.

Previous studies on the mechanism of action of histatin-5 on *C. albicans* suggested that the peptide required Ssa2p of the yeast cells as a receptor to exert its killing action (Li *et al.*, 2003, Li *et al.*, 2006); however, the data of this study did not support that concept. In this study, the *ssa2* null mutant of *C. albicans* showed comparable susceptibilies to the four 16mer derivatives as the wild-type strain. The finding that both the 16mer wild-type peptide and its D-isomer (16mer WD) had comparable killing activities on *C. albicans* yeast cells suggested that the two peptides did not need a chiral-receptor-type interaction with its targets to exert their killing activity. As the 16mer wild-type peptide is known to function as well as the 24mer histatin-5 in antifungal killing activity (Raj *et al.*, 1990), it is very likely that the mode of action of histatin-5 does not require Ssa2p as reported.

Further *in vitro* experiments with artificial membranes and electron transport chain inhibitors provided more information on how the 16mer derivatives acted on the targeted *C. albicans* yeast cells. The release of fluorescence from liposomes as a result of treatment with the 16mer peptides suggested that the peptides could disrupt the membranes in a protein-independent manner to gain access into the intracellular space of the *C. albicans* yeast cells. The finding that the killing effects of the four 16mer peptides were attenuated with the use of electron transport chain inhibitors such as sodium azide or antimycin A suggested that the electron transport chain or cellular respiration was involved in the modes of action of the four derivatives as in that of histatin-5. It is logical for us to think that mitochondria were one potential intracellular target of the four 16mer peptides or that ATP was needed for the entry of the four derivatives into the intracellular space of *C. albicans* yeast cells.

The data obtained from the experiments with the four 16mer derivatives in this study helped confirm the existence of a short 5mer functional domain of histatin-5. The 5mer domain

was identified as YKRKF and located to the middle portion of histatin-5. Although the antifungal activity against *C. albicans* of this 5mer was no stronger than the 16mer WL, the dimerization of this functional domain generated new short peptides with very strong antifungal activities such as the KM12, KM14, KM 23 and KM 29. These new peptides were shown to have stronger antifungal activities against *C. albicans* than the 16mer WL wild-type peptide in killing assays. It is hoped that peptides synthesized by the dimerization or multimerization of the 5mer domain would have some pharmaceutical value.

The next step in this study after the discovery of the 5mer domain and the development of new peptides with strong antifungal activities was the testing of potential toxicity of these peptides in mammalian cells. Several toxicity assays such as toxicity assays with WST-1, dye exclusion assays and hemolysis assays were used to evaluate potential toxicity of these new peptides on mammalian cells *in vitro*. The results obtained from these *in vitro* toxicity assays were promising when the new antifungal peptides at concentrations up to 100 μM did not show signs of significant toxicity to the mammalian cells as compared to controls. The concentration used in toxicity testing was much higher than the concentration of 5μM needed to kill more than 95% of the yeast cells. The data on the safety profile of KM12 and KM14 on mammalian cells *in vitro* allowed us to move forward with the testing of acute toxicity and immunogenicity of KM12 and KM14 in mice.

The step of testing potential toxicity of KM12 and KM14 on mice provided useful information that could help us better design histatin-5-based antifungal peptides with potential pharmaceutical values. In this step, KM14 peptide was showed to be toxic to the injected Hsd:ICR (CD1$^{®}$) mice at an intravenous dose of 10 mg/kg while the same dose of KM12 peptide was well tolerated by the mice. In terms of immunogenicity, monthly intravenous and

intramuscular injection of KM12 at a dose of 10mg/kg for 4 months resulted in the formation of anti-KM12 antibodies in 20% of the mice that received either intravenous or intramuscular injection of the peptide. The formation of IgG antibodies against the KM12 peptide in the injected mice might be due to the cleavage at the disulfide bond of KM12 by reduction reaction in the extracellular fluid to form its constituent monomer of CFKRKY that had the potential to function as a hapten. A lesson that we have learned from this study is that the dimerization of CFKRKY monomer by disulfide bond is suboptimal. At least, the stability and immunogenicity of the peptides created from the dimerization of the 5mer monomers via disulfide bonds must be taken into account if this mode of peptide synthesis is to be used.

Although the multimerization of the 5mer domain has been shown in this study to generate new peptides with strong antifungal activities, the road toward the introduction of a new peptide-based antifungal drug into clinical practice is viewed as lengthy and will be full of hurdles. In addition to the issues of stability and immunogenicity that could be overcome by the use of D-amino acid, careful design and testing the final peptide products in animals, antimicrobial peptides usually possess charges that could interact with ions normally present in the body fluids. As a consequence of these ionic interactions, the activities of antimicrobial peptides could be significantly affected by the presence of those ions at physiological concentrations. The antifungal activities against *C. albicans* of the peptides used in this study were tested in the presence of 10mM sodium phosphate buffer which contains a sodium concentration much lower than that of the extracellular fluids of 135 mM. It is predicted that the antifungal activities of these peptides will be much weaker in serum which contains proteins and higher concentrations of ions such as sodium, calcium and chloride ions, etc. If the issue of reduction in antifungal activity due to the presence of high salt concentrations could be

overcome, peptide-based antifungal drugs will definitely play a role in the future fights against fungal infections in human.

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