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Understanding a Possible Wonder Drug: A Radial Diffusion Assay for the Rapid Evaluation of Antimicrobial Peptides

Dustin Walter
Ouachita Baptist University

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Dr. Ruth Plymale, thesis director

Dr. Sara Hubbard, second reader

Dr. Matt Douglass, third reader

Dr. Barbara Pemberton, Honors Program director

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Dustin Walter

Ouachita Baptist University, Arkadelphia, AR

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Abstract

Antimicrobial peptides (AMPs) have been a major research focus due to their potential to combat a variety of human pathogens. Our laboratory has identified several novel peptides that display significant antifungal activity. The effectiveness of these peptides *in vitro* has been promising; however, it has been shown that physiological concentrations of various salts along with other conditions are inhibitory to peptide activity. To further explore the inhibitory effects of these salts, a new assay was developed whereby we can observe the effects of various salts on the peptide killing activity. For our studies, we employed several clinical isolates of *Candida* species to evaluate the killing activity of peptides in the presence of physiologically relevant salts at varying concentrations. By adding the salts individually, we are able to examine the inhibitory effect of each. When compared to other assays, the new assay requires less time and resources by allowing us to test the AMPs under numerous conditions simultaneously. After testing the AMPs, we determined that $CaCl_2$, $MgSO_4$, NaCl, and KCl are all inhibitory to peptide killing activity at varying degrees. In addition, we discovered that circularization or hexanoic acid modification of the peptide bypasses the inhibition of salts. Our long term goal is to modify the peptides in a way that will allow for their use *in vivo*.

Introduction

Due to the increase in the rate of resistance to current infection therapeutics, a search for alternative treatment methods has been ongoing. One area that is being investigated is the use of antimicrobial peptides. Antimicrobial peptides have been found in numerous organisms such as plants, animals, and bacteria. In each of these, AMPs act as the first line of defense against infectious microorganisms including bacteria (both Gram positive and Gram negative), fungi, and some viruses [1].

The great potential for these peptides is attributed to the mechanism by which they directly confront the microbes. AMPs kill their targets by permeabilizing the plasma membrane or by disrupting the membrane. Due to the anionic character of the microbial membrane, the positively charged peptide is attracted. These electrostatic forces and the hydrophobic attractions between the amino acids and the phospholipid bilayer's core, which is composed of fatty acid tails, allow the peptide and the membrane to bind to each other. The addition of the peptides causes stress to build in the membrane. After a threshold value of stress is reached, the membrane cannot effectively act as a barrier. An aqueous pore forms, and ions and peptides travel through this pore [2]. From this point, the exact mechanism of action is unknown [3], as it is possible that the permeabilization of the membrane may kill the cells via loss of membrane integrity; alternatively, the peptide may enter the cell and target an essential pathway for growth.

The fact that peptides attack the membranes is significant for numerous reasons. Animal and plant cells have overall neutrally charged membranes, as compared to bacteria or fungi that have negatively charged membranes. This difference promotes the ability of the peptides for distinguishing between the pathogen and the host's cells. This selective toxicity is essential for AMPs to be considered for therapeutic use [4]. In addition, by attacking such an essential aspect

of the cell morphology in a pathogen, the AMPs make it difficult for the pathogen to develop resistance; for resistance to form, the pathogen's membrane composition would have to be changed, likely resulting in negative alterations to other natural processes carried by the cell membrane [5].

In previous studies, AMP efficacy has been shown *in vitro*. Because of this, AMP-based medications have been synthesized and are in varying stages of FDA approval. AMPs have been utilized in clinical trials to treat a wide variety of infections such as diabetic foot ulcers (Pexiganan), ulcerative oral mucositis (Iseganan), catheter-related bloodstream infections (Omiganan), acne (MBI 594AN and XMP.629), and oral candidiasis (Histatin variants). While each drug produces varying results, utilizing AMPs as alternative treatment methods for infectious diseases seems promising and is rapidly moving forward to the commercial market [6].

Candida is a genus of fungus found as a part of the natural flora in humans, with *C. albicans* being the most common. While normally it causes no problems, changes in physiological conditions of the host can lead to candidiasis (*Candida* infection). Healthy individuals who are infected with *Candida* species tend to have mild infections, but immuno-compromised patients exhibit more severe infections. There are two commonly observed types of *Candida* infections: mucocutaneous superficial infections such as oral thrush, vaginitis, and systematic *Candida* infections. Out of these *Candida* infections, systemic *Candida* infections are the most severe. Out of the 72.8 cases of systemic *Candida* infections per million per year, the mortality rate ranges from 33% to 40%. In addition, systemic *Candida* infections account for $1.8 billion in yearly healthcare costs in the United States alone. These staggering statistics relay the need for new, effective antifungal treatment [7].

To pursue AMPs as possible therapeutics for fungal treatment, the KM (Kumar-McNabb) peptides were developed. These peptides were designed based on preliminary studies that sought to define the minimally active region of histatin 5, a naturally occurring AMP found in the human oral cavity (personal discussions with Dr. McNabb). The KM peptide series has been shown to have effective fungicidal activity against *Candida* species. Of the KM peptides, KM-12 is being used as a prototype for future development due to its high fungicidal activity. In addition to its efficacy, KM-12 is a small peptide that would be economically feasible to produce in mass. In order to test the limitations of KM-12, a new assay was needed. The current method used in the McNabb/Kumar labs for evaluating KM-12 is relatively inefficient, expensive, and time consuming. Moreover, these approaches are not readily adaptable to a high-throughput strategy for evaluation. Because of this, a new experimental method was created that utilizes a 24-well plate and the radial diffusion assay. By using this new process, multiple conditions could be evaluated simultaneously while using minimal resources. This assay will be used in the future to study additional KM-12 derivatives that resolve some of the physiological barriers that prevent the use of AMPs for *in vivo* therapy. In this study, the affects of salinity on AMPs was observed.

One of the barriers to using AMPs for therapeutic purposes *in vivo* has been the sensitivity of most AMPs to the presence of cationic salts. At physiological salt concentrations, AMPs typically have reduced antimicrobial activity [8]. As the McNabb/Kumar labs continue to develop AMPs, one goal is to find a solution to the "salt problem". Thus, the radial diffusion assay offers a relatively simple approach to determining the salt-sensitivity of a given peptide derivative. Through use of this assay, the researchers were able to evaluate the affects of cationic salts on KM-12 peptide derivatives and identify two candidates that resist salt inhibition.

Discussion of Antimicrobial Peptides

Benefits and drawbacks to using AMPs as therapeutic agents:

AMPs provide themselves as intriguing potential therapeutic agents against numerous pathogenic diseases due to their numerous benefits; however, before these peptides can be used, the drawbacks must be understood and overcome. The arguments for the use of AMPs as novel therapeutic options include their rapid killing activity, additional benefits not seen in antibiotic use, wide range of targets, and mode of action that limits the possibility of resistance formation. In spite of these benefits, as of 2012, no AMPs had been approved by the Food and Drug Administration as antimicrobial therapeutics [8, 9, 10].

One of the largest benefits for AMP therapy is the short time needed for the microbial killing to conclude. This killing often occurs in seconds to minutes after initial contact with the membrane of pathogens. To put this into perspective, the immune response usually takes minutes to hours to elicit microbial death [8, 11]. On top of the shorter killing time, AMPs limit resistance formation by pathogens by directly targeting the plasma membrane. In the small number of cases where resistance to AMPs develops, AMP efficacy is only lowered, not nullified [9]. By decreasing the time needed to eliminate pathogens and limiting the possibilities for resistance formation to occur, AMPs increase a patient's ability to fight off infection and decreases the time of sickness.

Another exciting aspect of AMP therapy is that many AMPs are able to perform more functions than antibiotics. Often, steroids are given to patients who are taking antibiotics to reduce the inflammatory response. With the use of some AMPs, this additional medication is unnecessary; the inflammatory response is lessened by AMPs during their release [8]. Another

drawback to antibiotics is seen in microbial resistance methods. Some strains of bacteria are able to produce biofilms and some microbes produce persister cells, variants of a microbe that are highly resistant to antibiotics and are found in most microbial populations; both of these forms of resistance decrease antibiotic efficacy. While antibiotics often prove ineffective in these cases, some AMPs are able to reduce bacterial numbers in spite of biofilms formation [9] and AMPs can target and kill persister cells [8]. As researchers look for alternatives to antibiotics, AMPs present themselves as great candidates by performing the job of antibiotics better.

Despite the aforementioned benefits, there are drawbacks to using AMPs in humans that prevent their immediate administration. This first drawback stems from a benefit. Because AMPs can target a wide range of cells, there is the potential for host toxicity. While this is true for some peptides, most show targeting preference for prokaryotic cell membranes and fungal cell walls, not eukaryotic cell membranes. With humans or other animals as the intended hosts, this drawback can be easily evaded by avoiding peptides that stray from the normal preference [8].

In order for AMPs to be effective, they must be able to survive and function in an environment that would mimic one seen *in vivo*. This aspect has presented some obstacles for AMP therapy. For instance, variance in pH or salinity can change the secondary structure of some AMPs. With the altered shapes come differences in function which may limit AMP efficacy. Because researchers cannot alter the pH or salt content of a host's body, they must produce AMPs that resist conformational changes in these conditions. Another environmental obstacle is the presence of proteases. Certain invading organisms may produce proteases that target and degrade AMPs before their effects can take place. In order to combat this, researchers can implement the "D" form of amino acids into peptides in place of the natural "L" form. After these changes are made, the stereospecific proteases will be unable to cleave the AMPs. Also,

degradation can be avoided by adding terminal end modifications to AMPs. These modifications stabilize AMP structure by preventing protease binding. Finally, proteases can be completely bypassed by delivering AMPs directly to the plasma membranes of pathogens. Through the use of manufactured vehicles, AMP exposure to the environment surrounding a pathogen would be limited until it successfully binds to the microbial membrane [8]. While all of these options for decreasing protease action would work theoretically, further experimentation needs to be done to make these theories realities.

As with most things in life, money is a major consideration in AMP synthesis. Financially, peptides can be a poor option if one is not careful, because peptide synthesis is expensive, increasing in cost with each additional amino acid residue. In order for AMPs to be viable options, limiting the length of the peptides will be necessary. This would require researchers to determine the smallest possible fragment of an AMP that continues to exhibit its antimicrobial actions [8]. Before the benefits of AMPs may be utilized, all of these drawbacks must be addressed. A deep understanding of peptide structure and how it affects activity and killing mechanism must be gained by researchers [10].

AMP interaction with the host immune system:

In general, the immune system is a complex system, providing multiple different pathways for combating pathogens. This fact is also seen in the various ways the immune system synthesizes, releases, and stores AMPs. Usually, AMPs are synthesized constitutively (continually being produced until a signal halts transcription) [12]. Under this type of control, AMPs may be released into the surrounding area as they are synthesized, resulting in a non-receptor-mediated response. In other words, no invading pathogen is needed to cause peptide

release; they are always present and work to stop an infection before it starts by killing any targets with which they come into contact [8]. Other peptides are synthesized under inducible transcriptional control where certain molecules that signify microbial presence, such as lipopolysaccharides and lipoteichoic acid, initiate AMP synthesis and release, resulting in microbial targeting and death [12]. In some circumstances, AMPs are stored as granules in neutrophils and other phagocytic cells; the AMPs are released after the immune cells detect pathogens. This allows a very concentrated, rapid response to the presence of a specific microbe. It has also been noted that motile cell bring some AMPs to the site of an infection, increasing their concentration [8, 11].

All of the methods of peptide release above play a role in innate immunity; however, AMPs can also act in the adaptive immune response. In this case, AMP release is dictated by the presence of specific microbial molecules, as with the inducible peptides. Once release is stimulated, however, the AMPs act as effectors, stimulating B and T cells and directing them to target the invading microbes; the peptides themselves do not attack the pathogens. Through the AMPs' role in the adaptive immune response, the host's immune system is trained to better target the pathogen that stimulated AMP release [12].

Surprisingly, there are even more ways that AMPs interact with the immune system. AMPs have been shown to affect the expression of molecules responsible for the host's defense system. By increasing the amount of defensins, proteins that are released to aid in host defense, AMPs cause more neutrophils to accumulate in the infected tissue. For example, the release of Dermaseptin, an AMP, increases the amounts of reactive oxygen species, molecules that are destructive to pathogens and signal the host's immune system to act. Additionally, chemokines

and their receptors, integrins, and transcriptional factors are examples of molecules whose production is altered based on the presence of specific AMPs [11].

AMP structure:

Natural AMPs are normally less than 50 amino acid residues in length, making them fairly small. Generally, they have an overall positive charge and are amphipathic, having both hydrophobic and hydrophilic regions. These qualities lead to their unique mode of action [10]. Cross-species examination reveals few occurrences of duplicate AMP amino acid residue sequences, even in closely related species, indicating the great diversity of AMPs. Secondary structures are often used to categorize AMPs into four groups for classification: alpha-helical, beta-sheet, extended, and loop AMPs [11].

Alpha-helical AMPs do not fold until they enter plasma membranes and are thus unstructured in aqueous solutions. Once they insert themselves into a membrane, helices form [10]. Beta-sheet AMPs contain at least two beta-strands stabilized by disulfide bonds [8]. Extended AMPs have no regular secondary structure but usually have an overrepresentation of one or more amino acid. These extended peptides are not known for their membrane disruption. Instead, extended AMPs insert themselves through the membrane and exact an attack on metabolic machinery within the cells. Loop AMPs form a loop using one disulfide bridge. They have few amino acid residues, are easy to synthesize in a laboratory, and resist proteolytic degradation. Because of these qualities, many researchers hold high hopes for the use of loop AMPs as therapeutic agents [10, 11]. As expected in biological agents, there are AMPs that do not fit into one of the above categories and some that contain two different structural

components. It is also important to note that these structures may only be observed if the AMPs are interacting with the membrane [8].

AMP mode of action:

By evaluating the structure of AMPs (discussed above), their functions can be understood and explained. The most common mode of action for AMPs is disruption of the microbial membrane, leading to permeabilization, ion leakage (possibly leakage of larger molecules), and microbial death. It has been observed that, in some cases, AMPs enter through the pores they form and accumulate around intracellular targets. The exact mode of action past permeabilization and accumulation is unknown and a source of immense interest [11]. Cell death is caused by the consequences of permeabilizing the membrane. Because ions are now free to enter or exit the microbe as they please, the cell's primary method of creating energy through chemiosmosis is disabled. Due to the loss of osmotic control, water enters the cell leading to swelling and lysis [12].

The driving forces that allow membrane-peptide interaction are electrostatic attraction and hydrophobic interactions [8]. As stated before, AMPs are cationic, giving them an overall positive charge. Microbial membranes, conversely, are anionic. Because of the charge difference, AMPs are able to accumulate on the membrane. After a critical peptide-to-lipid ratio is met, hydrophobic segments of the peptides interact with the lipid tails within the membrane while the hydrophobic portions of the peptides interact with the lipid polar head groups. During this process, AMP-AMP interactions occur as well. All of these actions lead to the formation of a pore [13].

There are various pore structure models. Under the barrel-stave model, the AMPs form a transmembrane pore by bundling together. For this pore to form, the AMPs must assemble either on the plasma membrane once they have attached or within the membrane. The end result is multiple AMPs folded in such a way that the hydrophobic regions interact with the lipids within the membrane core and the hydrophilic residues of one AMP interact with the hydrophilic residues on the adjacent AMPs. The pore that is formed is oriented perpendicularly to the membrane. Ions and other molecules are able to freely enter the cell by utilizing the channel in the center of the pore, bypassing the plasma membrane [11]. In the case of alpha-helical AMPs, pore formation must occur on the plasma membrane surface because their hydrophilic portions are exposed, hindering single peptides from entering the hydrophobic core of the plasma membrane. The helices align parallel to the membrane and then are inserted perpendicularly [8].

Another proposed pore formation model is the carpet model. In this model, the peptides cause the membrane to form micelles, with the peptides surrounding the lipid spheres [8]. To form these micelles, highly and thoroughly positive AMPs (cannot be zwitterionic unless very slightly so) coat the cell surface until a proper lipid-to-peptide ratio is reached. The membrane will then begin to fold into itself, forming channels with the peptide-coated polar head groups facing the center of the pore (these pores are known as toroidal pores). As the membrane continues to invaginate in multiple locations, micelles form. No specific peptide structure is needed in this model. The only requirements are that the peptides are highly positive and sufficiently hydrophilic [11]. This model differs from the barrel-stave model in pore shape. In the barrel-stave model, the pores have the lipids all in the same orientation, parallel to the peptide channel. In this model, the lipids bend such that those closest to the pore range in orientation

from perpendicular to parallel to the pore. This toroidal pore resembles the shape of a doughnut [8].

While most AMPs cause cell death through sustained pore formation, there are some AMPs that induce cell death in ways that are very different. As mentioned earlier, extended AMPs do not form pores; these AMPs insert themselves completely through the membrane, allowing the cytoplasmic end of the peptide to interact with cellular machinery of the microbe. This interaction leads to decrease metabolic efficacy and microbial death [10].

It has been noted that some AMPs form pores, but then disassemble them. In the sinking-raft model, pore formation occurs but is not sustained and does not directly cause cell death. Instead, some AMPs translocate across the membrane through the pore and relieve the imposed membrane stress, resulting in pore closure. From there, the translocated AMPs complete their metabolic attack, killing the microbe [13].

Some AMPs do not even interact with the cytoplasmic side of the membrane. Instead, these AMPs may host their attack on the plasma membrane proteins. The bacterial membrane is associated with one third of the proteins in a bacterial cell. Many of these proteins provide functions essential to the bacterial cell such as producing ATP, moving nutrients across the membrane, creating a proton gradient, and communicating with surrounding cells. Some AMPs disrupt these activities, causing cell death [8].

AMP selectivity:

While many AMPs have a wide range of microbial targets, some affect only specific types of microbes; further, all broadly targeting AMPs do not have the same target range. As one may guess, this fact is largely due to AMP-membrane interactions. It was previously stated that

AMPs generally target prokaryotic cells preferentially over eukaryotic cells. This discrimination is made possible by the distinctive components of prokaryotic and eukaryotic cell membranes. Bacteria contain lipopolysaccharides or teichoic acids depending on the Gram determination, making the bacterial membrane negatively charged [11]. Animal cell membranes, on the other hand, lack these molecules, using phosphatidyl choline and sphingomyelin polar head groups on the outer leaflet and cholesterol as the imbedded support. These molecules cause the membrane to have a zwitterionic character and lead to a more positively-charged outer leaflet for the eukaryotic cells [12]. Membrane potential also affects the peptides' ability to bind. Eukaryotes have a less negative membrane potential than bacteria. As one may assume, a larger negative potential better attracts the cationic peptides [11]. When cationic AMPs interact with bacterial and animal cells, many more peptides are needed to induce eukaryotic cell death because there is less electrostatic attraction [12].

There is one type of eukaryotic cells that is targeted by AMPs: tumor cells. In tumor cells, membrane symmetry is lost, leading to the homogenization of charges that were previously separated by leaflet location. With lipid translocation occurring in tumor cells, a more negative charge is seen on the outer membrane leaflet. Also, normal eukaryotic cells can be targeted by negatively charged or hydrophobic AMPs; these AMPs lose selectivity and thus target both eukaryotic and prokaryotic cells. This is favorable in the sense that it leads to a wider range of targets for the AMPs. However, eukaryotic cells are more at risk as well [11].

It may seem surprising that AMPs are able to target viruses, but many viruses are surrounded by a membrane envelope obtained from the host cell. AMPs attack the viral envelope in the same way they target the plasma membranes of prokaryotes. By integrating themselves into the membrane, AMPs destabilize it, leading to lysis of the viral particle [8]. Another more

surprising method of enveloped virus targeting is when the peptides prevent virus particles from binding with the membrane of the host. This inhibition can be accomplished by occupation of the host cell's receptors or by destabilization of the viral envelope [8, 11]. For the non-enveloped viruses, AMP attack occurs when the virus enters the host cell. The AMPs can alter the gene expression within the host, leading to either increased production of host proteins that combat the virus or to a decrease in viral gene expression. Transitioning to antifungal AMPs, one would assume these eukaryotic cells are safe from AMP targeting. However, some AMPs will target the chitin in the fungal cell wall, allowing them to attach [8].

Pathogen resistance to AMPs:

While microbial resistance to AMP attack is rare, it can occur. Usually resistance is caused by membrane surface modifications or proteases that degrade AMPs. Increased membrane fluidity allows AMPs to more effectively enter cells. To resist this entry, some cells will produce proteins that alter the membrane lipids, resulting in more lipid-lipid interaction and decreased membrane fluidity. Alpha-helical peptides are more inhibited than other AMPs by this microbial modification [11]. Another type of modification leads to change in the charge of the pathogen by either shielding the membrane charges or changing the membrane potential. In addition, some microbes have inducible genes that are activated to reduce AMP efficacy. The proteins formed by these genes lead to membrane molecule modification, substitution, or acylation in some cases. Some pathogens produce proteolytic enzymes that degrade AMPs once they enter the cytoplasm. Efflux pumps can be synthesized to remove AMPs from the cell's cytoplasm as well. Since some AMPs alter metabolic pathways, microbes can modify the intracellular targets of the AMPs to gain AMP resistance. The formation of biofilms also

provides pathogens with protection from AMPs. While these resistance methods are effective in delaying the actions of AMPs, it is uncommon for cell death to be completely avoided [8].

Synthesizing AMPs:

As stated previously, the end goal for researchers is the production of AMPs that can be used as antimicrobial therapeutic agents. Currently, AMPs are best suited for external application only because it is difficult for the peptides to pass through the gastrointestinal tract and the blood to arrive at the infected tissues. Some alternative dosing methods that are being considered are injections, delivery vehicles for peptide transport, or mixing the peptides with some substance, such as a muco-adhesive polymer or an acrylic bone cement, so the peptides are not rapidly cleared by the body [11]. While possible, these methods will require more research and pose problems when considering cost. Another alternative (and the method behind this research) is synthesizing peptides that can withstand and overcome biological obstacles.

In *de novo* synthesis, AMPs are looked upon favorably for many reasons. With many AMPs occurring naturally, researchers have numerous templates from which to build; they can alter these templates, creating peptides that have higher efficacy or better protease resistance than the originals. Being chains of amino acids, AMPs are easily modified. With this ability, researchers can change AMP structure and observe the outcomes of the changes without much trouble. Also, synthesis can occur either on the bench-top or in recombinant systems. Having these two modes of synthesis, researchers are afforded greater flexibility in AMP synthesis. Bench top chemical synthesis allows for the incorporation of unnatural amino acids. AMP-producing recombinant systems have already been established in plants and allow for savings in

time and money. These systems also allow for easy addition of post-translational modifications, such as methylation, amidation, phosphorylation, or glycosylation [8].

In synthesizing AMPs, multiple factors must be considered in order to produce peptides that are effective in killing pathogens and have reasonable costs. First of all, for peptides to even bind to the targeted cells, they must be water soluble. Without dissolving, pathogen killing is impossible. The second consideration is AMP length. In order to from structures with hydrophobic and hydrophilic faces, the smallest a peptide can be is 7 to 8 amino acid residues long. To form the alpha-helices for the barrel-stave model, 22 residues are needed while only 8 are needed if forming beta-sheets. While length is a major concern for cost, decreasing length too much can severely decrease AMP efficacy. Thirdly, overall charge is a concern that must be considered as well. Because these peptides are to be used as therapeutic agents for humans, an overall cationic character must be created; otherwise, selectivity between eukaryotic and prokaryotic cells is lost. As a fourth consideration, the hydrophobicity must be controlled in order to specify targets. Some pathogens are better targeted at specific ranges of hydrophobicity. Changing this quality of the peptide changes its target range. It is important to note that while hydrophobicity is important, amphipathicity has a larger affect on membrane binding and should be given preference when designing peptides. Lastly, adding disulfide bonds or cross linkages can change the antimicrobial effect [8].

Methods and Materials

Strains, growth medium, and culturing of organisms:

The *Candida* species and strains used in this study were *C. albicans* SC5314, *C. albicans* ATCC90028, *C. albicans* ATCC36082, *C. krusei* ATCC6258, *C. tropicalis* ATCC750, and *C. glabrata* ATCC90030. All strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The strains were grown and maintained on Sabouraud Dextrose agar plates at 30°C. For growth in liquid culture, the strains were grown in Yeast extract-Peptone-Dextrose (YPD) medium at 37°C overnight. After overnight growth in liquid culture, the concentration of cells in each culture was determined by hemocytometer counts to determine the number of cells/ml.

Radial diffusion assay:

Following growth of the appropriate *Candida* strain as outlined above, the cells were diluted to a concentration of 1 X 10^6 cells/ml in a 1% (wt/vol) agarose solution prepared in 10 mM phosphate buffer (pH 7.4). A volume of 300 μL of the cell/agarose solution was deposited in each well of a 24-well plate. Once the cell/agarose solution solidified, a 2 mm diameter hole was punched into the cell/agarose layer for each well. A 4 μL aliquot of a 50 μM peptide solution prepared in 10 mM sodium phosphate buffer at a pH of 7.4 was deposited into the hole. The plate was incubated for 2 hr at 37°C, and the peptide diffused into the agarose and killed the fungal cells. Following this incubation, each well was overlaid with 300 μL of a 1% agarose/6% Sabouraud Dextrose solution to provide nutrients to the surviving fungal cells to grow. The plate was subsequently incubated overnight at 37°C. For a control that exhibited no killing of fungal cells, 10mM sodium phosphate buffer at a pH of 7.4 was deposited into the hole

instead of the peptide. The cells killed by the peptide would leave a circular clearing in the middle of the surviving fungal cells that grew overnight. The diameters of the clear kill zones were compared to determine the relative killing ability of a specific peptide in the varying conditions.

Optimization of the radial diffusion assay:

The radial diffusion assay was optimized by examining several relevant parameters that would allow reproducible results. For these studies, *C. albicans* SC5314 was used to assess various parameters. First, the minimum amount of cell/agarose solution needed was evaluated using the KM-12 peptide with both 300 μL and 600 μL of the cell/agarose solution deposited into the wells of the 24-well plate. It was found that 300 μL of the cell/agarose solution was adequate for the assay. Second, the optimal width of the hole in the agarose was examined by using holes of 1 mm and 2 mm diameter in the cell/agarose layer, and the 2 mm hole was found to give optimal results. Third, the optimal concentration of the peptide was examined. Initially, the peptides KM-10, KM-12, and WD-16 were prepared at concentrations of 3 μM, 6 μM, 12 μM, 25 μM, 50 μM, and 100 μM, and a 3 μL aliquot of each peptide was deposited into the hole of the cell/agarose solution. For a second series of experiments, both the concentration of the peptides and the volume of each peptide were altered. The peptides KM-10, KM-12, and KM-33 were prepared at concentrations of 25 μM, 50 μM, and 75 μM; 3 μL of the 75 μM concentration, 4 μL of the 50 μM concentration, 4 μL of the 25 μM concentration, or 5 μL of the 25 μM concentration of each peptide was deposited into a hole of the cell/agarose layer.

Effect of cationic salts on peptide activity:

To test the effects of cationic salts on fungicidal activity of peptides, *C. albicans* SC5314 was used as the test strain along with the indicated peptide, either KM-12 or KM-33. The radial diffusion assay was performed as outlined above except the indicated salts were added to the agarose/cell solution at the indicated final concentration prior to addition to the 24-well plate. The following salts were evaluated: NaCl, $ZnCl_2$, $CaCl_2$, KCl, and $MgSO_4$.

Testing KM-12 derivatives in fetal bovine serum:

To test the effectiveness of KM-12 derivatives in fetal bovine serum (FBS), KM-12, KM-18, and KM-34 were tested against *C. albicans* in varying concentrations of FBS. The cell/agarose solution was mixed with FBS to make cell/FBS/agarose solutions containing 0%, 5%, 10%, 20%, and 40% FBS. A volume of 300 μL of each solution was deposited into wells, and 4 μL of 50 μM KM-12, KM-18, or KM-34 were placed into the appropriate wells and the radial diffusion assay was performed as outlined above.

Results and Discussion

Optimization of the radial diffusion assay:

For the radial diffusion assay to work reproducibly, a variety of parameters needed to be standardized. Thus, the initial goal in developing the assay was to evaluate several parameters of the assay and establish standardized conditions. It was determined that 300 μL of the cell/agarose solution added to each well of the 24-well plate provided a kill zone that was larger and more distinct than that observed with 600 μL of agarose/cell solution (data not shown). In wells that received 600 μL of the cell/agarose solution, small colonies of *C. albicans* SC5314 were seen

growing within the kill zone. This generated an undesirable haziness within the kill zone that made comparative analysis more difficult. It is presumed that increasing the volume of the cell/agarose solution from 300 μL to 600 μL increased the number of fungal cells present per well; however, the amount of peptide deposited was kept the same. Moreover, the volume of the hole created in the agarose was deeper for the 600 μL volume while the dispensed peptide remained constant. This resulted in an uneven diffusion of the peptide causing cell growth within the kill zone. Thus, the volume of cell/agarose solution and the peptide solution were adjusted such that the diffusion from the hole in the agarose was evenly distributed from the top to bottom.

By altering the width of the hole within the agarose, the volume capacity changed. Having a hole that was too narrow would allow only a small volume of peptide to diffuse into the cell/agarose layer resulting in smaller kill zones. In contrast, a hole with a diameter that is too wide would result in the uneven diffusion of the peptide throughout the agarose. When comparing the results of 1 mm versus 2 mm diameter holes, both produced the same diameter kill zones. However, the kill zones with a the 1 mm hole were hazy, indicating some cell growth. It was found that when the same volume of peptide solution was added to the 1 mm versus 2 mm hole, some of the peptide solution settled on top of the cell/agarose layer with the 1 mm hole. This produced an uneven lateral diffusion of the peptide, resulting in differential killing of the fungal cells through the agarose. To remedy this, the volume of the dispensed peptide solution could be reduced for the 1 mm hole; however, the size of the kill zone produced would be also smaller. Since the kill zone produced by 4 μL of the peptide solution is optimal, the 2 mm diameter hole was chosen since it balanced volume and kill zone area optimally.

Once the optimum volume of cell/agarose and the peptide volume were established, the next goal was to determine the optimum concentration of peptide that would give an appropriate kill zone. Initially, three peptides (KM-10, KM-12, and WD-16) with varying efficiencies of fungicidal activity were tested at different concentrations. WD-16 is the functional component of histatin, a human AMP, and thus functions as a wild type. As shown in Figure 1, KM-12 displayed the most efficient fungicidal activity as indicated by the diameter of the kill zone. KM-10 showed an intermediate level of killing and WD-16 showed very limited killing activity. By testing these peptides at different concentrations, the concentration that produced an optimum kill zone was established. For all of the peptides, the diameters of the kill zones decreased as the concentration of the peptide solution decreased. The fungicidal activity of KM-12, KM-10, and WD-16 were not observed at or below concentrations of 3μM, 12μM, and 50μM respectively. The wild type WD-16 was ineffective when compared to the KM peptides. When comparing the kill zones of KM-12 and KM-10, a concentration of 25 to 50 μM could be used to obtain an obvious kill zone for these peptides.

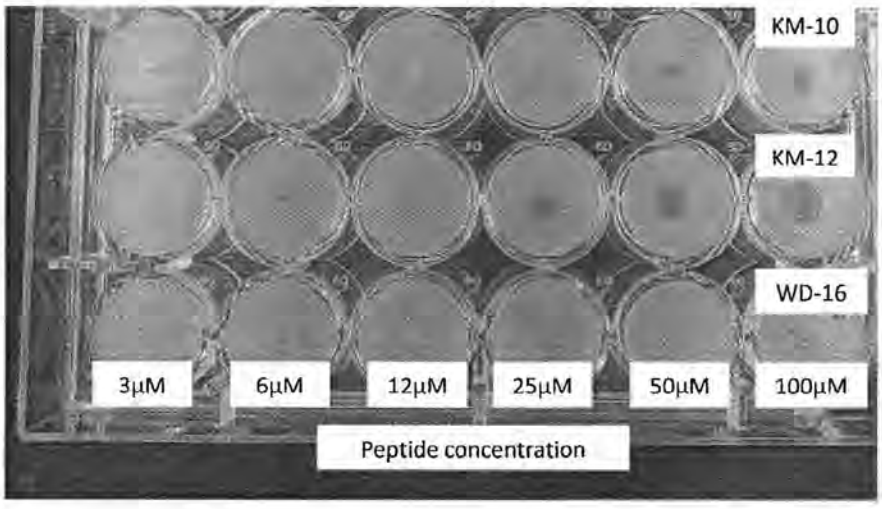

Figure 1: Optimum concentration of peptide. The indicated peptides were evaluated using the radial diffusion assay with increasing peptide concentrations as shown. *C. albicans* SC5314 was used as the test strain for this assay.

In a second round of optimizing the peptide, KM-33 was added to the peptides tested. To increase the size and thus improve the visibility of the kill zone at lower concentrations of peptide, we increased the volume of peptide solution deposited. As expected, the kill zone increased in size as the concentration and volume of the peptide solution increased (Fig. 2). In considering the amount of peptide solution dispensed, it was concluded that a volume of 4 μL of peptide solution provided an optimum kill zone. When comparing the 25 μM versus 50 μM concentration, the 50 μM gave a slightly larger kill zone. Thus, it was determined that the 50 μM concentration would be preferable since the larger kill zone would offer a broader effective range important in later experiments. Thus, the future experiments were conducted using 4 μL of a 50 μM peptide.

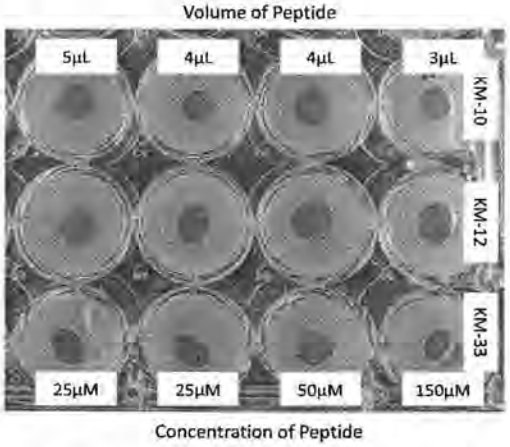

Figure 2: Optimum volume/concentration of peptide. The indicated peptides were evaluated using the radial diffusion assay with increasing peptide concentrations as shown. The volume of peptide added to each well was also optimized as shown. *C. albicans* SC5314 was used as the test strain for this assay.

Effects of cationic salts on peptide activity:

To examine the salt-sensitivity of AMPs, the radial diffusion assay was used with several individual physiological salts to determine the contribution of each to inhibitory activity. Five different salts were tested for this series of experiments: NaCl, $CaCl_2$, $MgSO_4$, KCl, and $ZnCl_2$. NaCl, $CaCl_2$, $MgSO_4$, and KCl were chosen as relevant physiological salts that are present in significant concentrations in the human body. $ZnCl_2$ was chosen because it has been speculated that $ZnCl_2$ increases AMPs' killing activity via an unknown mechanism [14]. In the initial

experiment, the effect of NaCl of on the fungicidal activity of KM-12 and KM-33 was evaluated. As shown in Figure 3, both KM-12 and KM-33 were completely inhibited at 60 mM NaCl or higher.

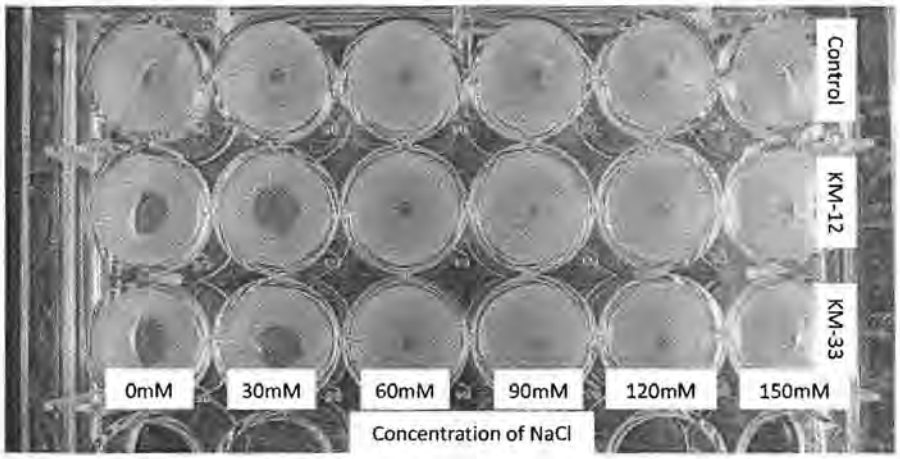

Figure 3: Effect of NaCl on AMP activity. Increasing concentrations of NaCl were added to the radial diffusion assay as indicated. The wells contained 10 mM phosphate buffer pH 7.4 (control) or 50 μM of the indicated peptide in the same buffer.

To evaluate the effect of salts, different concentrations of NaCl, $CaCl_2$, $MgSO_4$, and KCl were evaluated using the radial diffusion assay. Since RPMI-1640 is used for the growth of multiple human cell lines, the concentration of salts in this medium represents a good estimate of physiologic salt concentrations. Thus, the concentrations evaluated were selected based on their respective concentrations in RPMI-1640 medium. Because $ZnCl_2$ is not found in the RPMI-1640, the $ZnCl_2$ concentration was arbitrarily chosen.

As shown in Figure 4, significant inhibition of the KM-12 fungicidal activity was observed at 50 mM NaCl, consistent with the data shown in Figure 3. For $CaCl_2$, activity was greatly diminished at 0.8 mM; however substantial activity was retained in the presence of 0.4 mM $CaCl_2$. For $MgSO_4$, KM-12 activity was completely inhibited at 16 mM; however, the activity remained noteworthy at 0.8 mM. Not surprisingly, KCl and NaCl inhibition were very similar. By comparing inhibition of fungicidal activity caused by these four salts, one can

evaluate the relative detriment the salts have on KM-12 activity. In order of increasing detriment, there is KCl < NaCl < $MgS0_4$ < $CaCl_2$. Thus, divalent cations had a stronger inhibitory effect than monovalent cations on fungicidal activity.

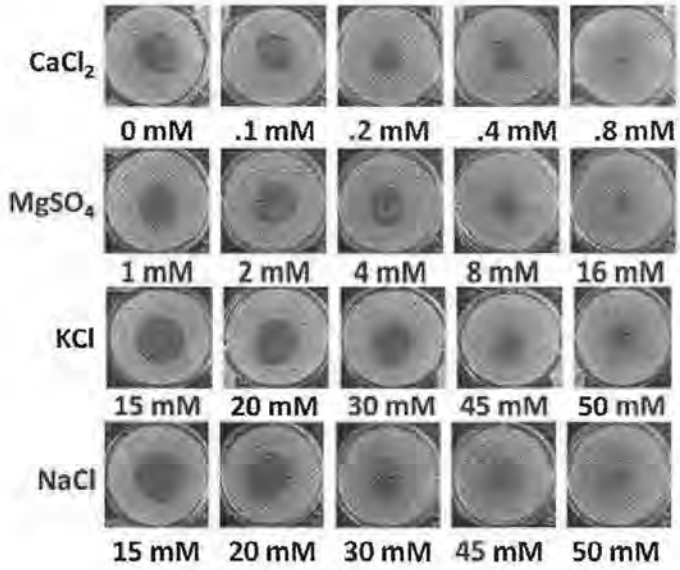

Figure 4: Effect of various cationic salts on the activity of KM-12. *Candida albicans* SC5314 was exposed to KM-12 in the presence of the indicated salts at the concentrations shown using the radial diffusion assay as described in the Materials and Methods. The peptide was used at 50 μM concentration.

Previous studies have suggested that zinc enhances the fungicidal activity of some AMPs. To determine how zinc influences the activity of KM -12, the radial diffusion assay was conducted with KM-12 in the presence of varying concentrations of $ZnCl_2$. It was found that 1 mM $ZnCl_2$ inhibited the activity of KM-12; however, at the 4 mM concentration, the zinc was fungicidal in the absence of KM-12 (Fig. 5).

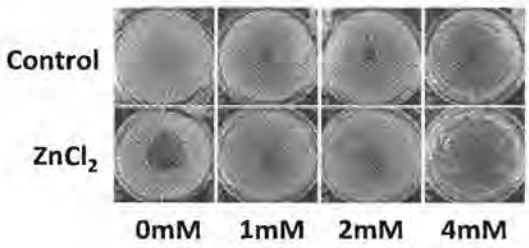

Figure 5: Effect of $ZnCl_2$ on the activity of KM-12. *Candida albicans* SC5314 was exposed to KM-12 in the presence of the indicated concentrations of $ZnCl_2$ shown using the radial diffusion assay as described in the Materials and Methods. The peptide was used at 50 μM concentration. Control contains only 10 mM phosphate buffer pH 7.4.

Specificity of the KM peptides:

One of the problems with the current antifungal therapeutics is the lack of broad range specificity (having numerous pathogens a therapeutic specifically targets). For example, some non-*albicans* species of *Candida* are not sensitive to fluconazole, a common antifungal therapeutic. For this reason, the specificity of KM-12 and its derivative, KM-34, were evaluated with non-*albicans* species known to be resistant to fluconazole. As shown in Figure 6, *C. tropicalis* and *C. krusei* are resistant to fluconazole, but are sensitive to killing by the KM-12 and KM-34 peptides. These results suggest that KM-12 and KM-34 have a broader specificity of fungicidal activity than fluconazole.

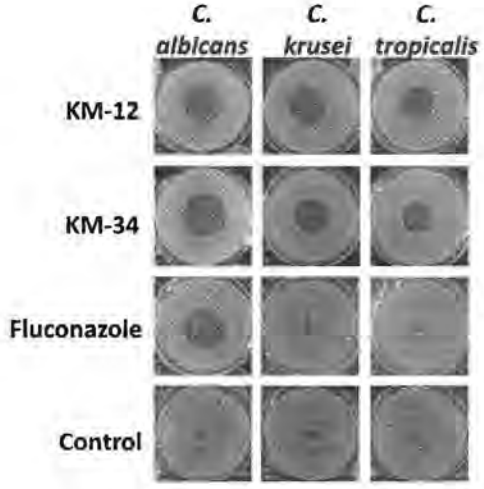

Figure 6: Specificity of the KM peptides with fluconazole-resistant strains of *Candida*. The peptides KM-12 and KM-34 were evaluated for fungicidal activity against *C. albicans* SC5314, *C. krusei* ATCC6258, and *C. tropicalis* ATCC750. The peptides were used at 50 μM concentration. For comparison, 50 μM fluconazole was evaluated in the same assay as indicated. Control contains only 10 mM phosphate buffer pH 7.4.

To further examine the range of fungicidal activity, the killing activity of KM-12 and KM-5 was evaluated against *C. albicans* clinical isolates and selected non-*albicans* species of *Candida*. KM-12 was found to effectively kill all species evaluated, while KM-5 killed the majority, but it was less effective against *C. albicans* ATCC90028 and *C. glabrata* ATCC90030 (Fig. 7). Based on these results, KM-12, the prototype KM peptide, can effectively kill multiple strains of *Candida*. This fact makes KM-12 or a derivative an attractive candidate for further

development. *C. albicans* ATCC36082 was slightly more resistant to KM-12 than the other isolates, producing a smaller, hazier kill zone.

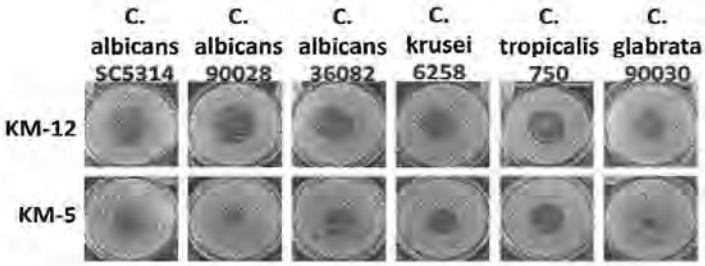

Figure 7: Specificity of the KM peptides with various strains of *Candida*. The peptides KM-12 and KM-5 were evaluated for fungicidal activity against *C. albicans* SC5314, *C. albicans* ATCC90028, *C. albicans* ATCC36082, *C. krusei* ATCC6258, *C. tropicalis* ATCC750, and *C. glabrata* ATCC90030 as indicated. The peptides were used at 50 μM concentration. Control contains only 10 mM phosphate buffer pH 7.4.

Evaluation of KM peptide derivatives for resistance to cationic salts:

The studies shown in Figure 3 indicate that the KM peptides are sensitive to an ionic environment. To examine whether other derivatives of the KM peptides may have resistance to these salts, an experiment was conducted in which KM-18 and KM-34 were incubated with *C. albicans* SC5314 in the presence of physiological NaCl concentrations (150 mM). As a control peptide, the wild-type WD-16 was used since it was known to be highly salt sensitive. As shown in Figure 8, KM-18 and KM-34 were uninhibited by the presence of 150 mM NaCl. KM-18 and KM-34 are both derivatives of KM-12 that were synthesized to maintain fungicidal activity at physiological concentrations of salts. KM-18 is a circularized peptide containing the active sequence YKRKF which is found in KM-12. KM-34 contains the identical five amino acid sequence with the N-terminal covalent addition of a 6-carbon hexanoic acid. This suggests

that circularization and/or the addition of a hydrophobic tail to the peptide may improve fungicidal activity in the presence of cationic salts. This observation will be further explored by the McNabb/Kumar lab in the future as well as the effect that other N-terminal peptide modifications may have on fungicidal activity.

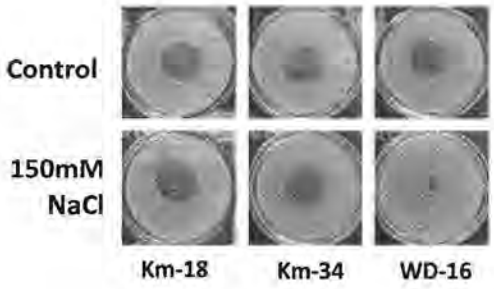

Figure 8: KM peptide derivatives that display resistance to cationic salts. The peptides KM-18 and KM-34 were evaluated for fungicidal activity against *C. albicans* SC5314 at 150 mM NaCl as indicated. The peptides were used at 50 μM concentration. Control contains the indicated peptide and no salt added.

KM peptide activity in blood serum:

To evaluate the effect of blood serum on the activity of the KM peptides, fetal bovine serum (FBS) was used to mimic conditions in the human blood stream. For the three peptides evaluated, KM-12, KM-34, and KM-18, as the concentration of FBS increased, the fungicidal activity decreased (Fig. 9). There are three possible reasons for the decrease in activity: 1) the cationic salts in the serum inhibited peptide activity; 2) the peptides were strongly bound to serum proteins such as albumin; or 3) proteases in the serum destroyed the peptides. It was already shown that KM-34 and KM-18 were not significantly affected by physiological salt concentrations (Fig. 8), suggesting that one or both of the other two reasons are likely to be involved in the loss of activity. The KM peptides will have to be further modified to combat these inhibitory effects; however, the radial diffusion assay offers an excellent platform for evaluating new KM peptide derivatives as the development of these novel fungicidal peptides progresses.

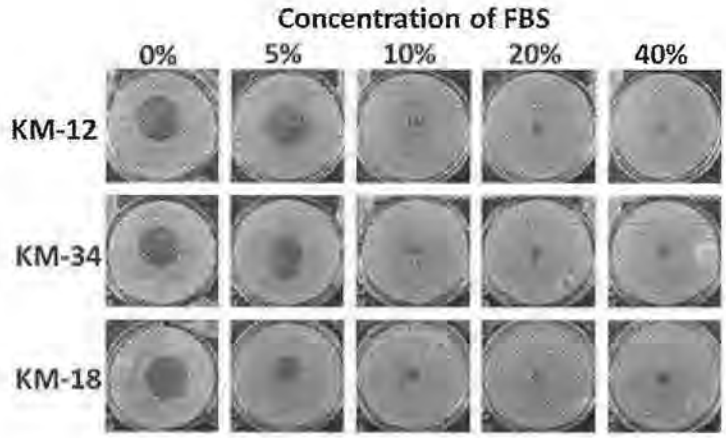

Figure 9: KM peptide activity in serum. The peptides KM-12, KM-34, and KM-18 were evaluated for fungicidal activity against *C. albicans* SC5314 in fetal bovine serum (FBS) at the concentrations indicated. The peptides were used at 50 μM concentration.

Conclusions

In summary, development of the radial diffusion assay has allowed us to develop a new AMP screening assay that is adaptable to high-throughput screening and is cost-effective for the initial evaluation of new peptides. The utility of this assay was demonstrated by evaluating the sensitivity of various *Candida* species to the KM peptides developed in a collaborative project between the laboratories of Dr. David McNabb and Dr. Suresh Kumar (personal interview with Dr. McNabb). It was found that the fungicidal response observed with the radial diffusion assay was the same as observed with the standard minimum inhibitory concentration (MIC) assays (Akkam and McNabb, unpublished results). The four common cationic salts found in the RPMI-1640 (NaCl, $CaCl_2$, $MgSO_4$, and KCl) inhibited the fungicidal activity of most KM peptides. Moreover, in spite of previous findings, $ZnCl_2$ seemed to inhibit the killing activity of the KM peptides at low concentrations while killing fungal cells alone at higher concentrations. In response to these inhibitory factors, we identified two KM peptide modifications that may alleviate some of the inhibitory effects of cationic salts, namely circularization (KM-18) or

hexanoic acid modification (KM-34) of the peptide. Further modification may be required before the KM peptides are stable and functional *in vivo*.

It is worth noting that the mechanism by which the KM peptides kill fungal cells is unknown. The radial diffusion assay offers an approach to identifying the genetic pathways involved in fungicidal activity of the peptides. The McNabb lab has a library of *Saccharomyces cerevisiae* mutants containing null mutations in every nonessential gene in the genome (>5000 different mutants). The radial diffusion assay presents a viable approach to screening through the mutants to identify pathways that affect KM peptide sensitivity. Once the pathways are identified in *S. cerevisiae*, the orthologous genes in *C. albicans* can be knocked out to determine whether the same pathways confer sensitivity or resistance in that organism. Using this genetic approach, the mechanism by which the KM peptides kill fungi could be elucidated.

Acknowledgements

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