A study on the activity of plant symbiotic peptides with therapeutic potential against pathogenic fungi and investigation of their cytotoxicity

Ph.D thesis

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INTRODUCTION AND OBJECTIVES

The agents of fungal infections, so-called mycosis concern every field of life all over the world. Nosocomial infections originating from hospitals or health-care institutes, or other diseases caused by fungi threaten not just human population, but also animals and plants.

The increasing number of drug resistant microbes became a global public health problem, that urges the identification of novel antimicrobial agents. Antimicrobial resistance concerns not just bacteria but fungi too. More and more study report about such fungal isolates which are resistant against conventional antifungal agents.

As new generational antibiotics, antimicrobial peptides (AMPs) might provide effective solutions for these problems. AMPs are present in all organisms from bacteria across the evolutionary spectrum. In plants and animals, these peptides are the effector molecules of the host innate immune system. They possess antiviral, antibacterial, antiprotozoal and antifungal activities, furthermore, some of them have both antibacterial and antifungal activity. Thanks to their broad-spectrum and rapid killing, or inhibiting activities, these natural molecules can be promising candidates in the battle against pathogens.

Plant AMPs have been isolated from each part of the plant, such as from the roots, seeds, flowers, stems, and leaves. The recently identified nodule-specific cysteine rich (NCR) peptides are produced by certain legume plants in the infected cells of the symbiotic root organ called nodule. There, their primary function is to control the terminal differentiation of the endosymbiotic bacteria (rhizobia). NCR peptides contain a relatively conserved secretory signal peptide and a highly diverse mature peptide composed of 30-50 amino acids with conserved positions of four or six cysteines that make them similar to a group of

AMPs, the defensins. So far, more than 500 NCR peptide coding genes have been identified in the genome of *Medicago truncatula*. Mature peptides show high sequence diversity, hence, their isoelectric point (pI) ranges from 3.2 to 11.2 and there are high differences in their expression pattern and activity, too.

Our group has shown earlier, that some cationic NCR peptides have bactericid effect on various Gram-negative and Gram-positive bacteria.

1. In the light of that, first, we wanted to check the possible activity of NCR peptides against fungi, too. For this purpose, the effect of 19 NCR peptides with different isoelectric point were investigated against ten pathogenic fungal species.

2. Furthermore, our aim was to reveal the mode of action of those NCR peptides that had antifungal activity on yeast-like growing fungi. In addition, we aimed to identify the potential targets including cell organelles and proteins.

    a. For this purpose, we wanted to investigate the possible membrane damaging effect of NCR peptides.

    b. We wanted to determine the localization of NCR peptides in the target cell.

    c. We tried to identify the target proteins that interact with the NCR peptides.

3. Our final aim was to study whether NCR peptides are applicable for therapeutic use. This requires, that peptides must be non-toxic for human/mammalian cells and be able to arrest fungal infection *in vitro* and *in vivo*.

We chose *Candida albicans,* one of the most common opportunistic human pathogen, to achive these two latter aims (2., 3.).

METHODS

Microorganisms and their growing conditions

Candida species (*Candida albicans, Candida krusei, Candida parapsilosis, Canida glabrata*) were maintained on YPD medium. *Malassezia furfur* was grown on Pityrosporum medium. *Trichophyton mentagrophytes* was maintained on Sabouraud agar, whereas *Aspergillus niger*, *Aspergillus flavus, Fusarium graminearum* and *Rhizopus stolonifer var. stolonifer* grew on malt medium. Hyphal growth of *C. albicans* was induced in Complete Keratinocyte Medium (CKM; Life Technologies) without serum.

The effect of the NCR peptides on free-living fungi was investigated either in a phosphate buffer completed with 5% glucose (PBgluc) or in Low-Salt fungal Medium (LSM) which had been shown to be appropriate to study the antifungal effect of defensins.

Human cell line

The immortalized human vaginal epithelial cell line PK E6/E7 was cultured in serum-free CKM supplemented with 0.005 μg/ml recombinant epidermal growth factor, 50 μg/ml bovine pituitary extract, L-glutamine and antibiotic/antimycotic solution in a CO_2 thermostat at 37 ℃. Cells at 60-70 % confluence were used.

Peptides

Mature (without the signal peptide) and N-terminally fluorescein isothiocyanate (FITC) labeled NCR peptides were chemically synthesized

(>95% purity, Proteogenix, France) and dissolved in MilliQ water and were diluted in LSM or PBgluc.

Antifungal assays

The antifungal activity of the NCR peptides was investigated *in vitro* by a microdilution assay using 96-well flat bottom microtiter plates. The effects of NCR peptides on the growth of fungi were determined by microscopic analysis and measuring absorbance at 600 nm with a microtiter plate reader (FLUOstar OPTIMA) after 24 and 48 hours.

Fluorescent and confocal microscopy

Morphological changes of *C. albicans* caused by NCR peptides were investigated with an Axio Observer Z.1 (Zeiss) fluorescent microscope. To study the localization of the peptides in the target cells, FITC-conjugated peptides were used. Images of the treated *C. albicans* cells were taken with an Olympus Fluoview FV1000 confocal laser scanning microscope.

Cell permeability assay

Cell permeability after peptide treatment was monitored by the release of the intracellularly accumulated dye calcein. Cell membranes are permeable for the non-fluorescent dye Calcein-acetoxymethyl (Calcein-AM) which is converted into green-fluorescent calcein through the hydrolysis of the acetoxymethyl ester by intracellular esterases in living cells. Membranes are inpermeable for the hydrophil Calcein which can be released from the cell only by a membrane permeabilizer agent.

Affinity chromatography assay on *C. albicans* protein extracts

Affinity chromatography was applied to look for intracellular target molecules of the cationic NCR247. At first, we used whole cell lysate, then pull-down experiments were repeated with plasma membrane isolates, too. To identify the pulled-down proteins, LC-MS/MS mass spectrometry was applied.

Yeast two-hybrid assays

Four putative interacting proteins identified by the pull-down experiments were chosen (Elongation factor 3, *CEF3*; translation elongation facrtor 2, *EFT2*; eukaryotic initiation factor 4A, *eifA4*; heat-shock protein, *hsp*) to verify their interaction with NCR247 which was used as a bait in yeast two-hybrid experiments.

Real-time quantitative PCR (qRT-PCR)

The expression changes of another possible NCR-interacting protein coding gene *PIL1* was investigated with qRT-PCR. cDNAs were synthetized from RNA samples purified from peptide treated *C. albicans.* As control, we used untreated and amphotericin B treated cells.

Cytotoxicity assays

To analyse the cytotoxic effect of NCR peptides on human epithelial cells we used two viability assays:

MTT assay: a semi-quantitative method, which informs about the metabolic state of the cells by measuring the activity of mitochondrial dehydrogenases via the conversion of the tetrazolium dye MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromid) to formazan.

Real-Time Cell Analysis (RTCA): provides real-time, quantitative information about the number of the living, attached cells by measuring electrode impedance.

The latter method was used to analyse the activity of NCR peptides *in vitro* in an infection model, where human vaginal epithelial cells were infected with *C. albicans.*

RESULTS

In the frame of the presented work, we found that some NCR peptides with high isoelectric point have intense activity against both yeast like (*Candida albicans, C. parapsilosis, C. crusei, C. glabrata, Malassezia furfur*) and filamentous fungi (*T. mentagrophytes, A. flavus, A. niger, R. stolonifer, F. graminearum*). NCR peptides inhibited the budding of yeast like growing fungi, the germination of spores and the growth of hyphae at a concentration range from 6.25 to 25μg/ml. The activity was not just static in this range, but also fungicid in the case of *C. albicans* which was investigated in more detail. As a general fact, cationic NCR peptides with isoelectric point above 9 had inhibitory effect.

We chosed one of the most common opportunistic human pathogenic fungi, *Candida albican*s to investigate the mechanism of the activity of cationic NCR peptides on yeast-like growing fungi and to study which cell organelles or proteins could be their targets. The disturbance of cell membrane integrity is a frequent mode of action in the case of antimicrobial peptides. We determined with the help of fluorescently labeled peptides that NCR peptides predominantly localized to the fungal plasma membrane. Furthermore, calcein release indicating membrane permeabilization proved that they caused changes in membrane integrity. We found membrane damaging effect on pseudohyphae, as well. In addition, intracellular targets cannot be excluded either.

Using a cationic peptide in affinity chromatography experiments we identified numerous putative interacting partners from *C. albicans*. Most of them were ribosomal proteins and other polypeptides that are also implicated in translation. We tried to validate some of the results with yeast-two hybrid system or qRT-PCR, however, the interactions between the NCR peptides and the fungal proteins could not be confirmed yet.

As a therapeutic agent should not be toxic to the patients, the cytotoxicity of selected NCR peptides on an immortalized human cell line was investigated with viability assays. According to MTT assay, which provides information about the metabolic state of living cells, cationic peptides in the antifungal concentration range (<25 μg/ml) affected only slightly the viability of the human cells. On the other hand, when the cell proliferation ability was measured with the real-time cell analyser (RTCA), the surviving cells showed a highly reduced cell proliferation at 12.5 μg/ml concentration and at higher concentrations they could not maintain their proliferation ability.

The active cationic NCR peptides could eliminate not only the yeast form of the fungi but they were able to prevent the growth and the subsequent human cell killing activity of the hyphal form in an *in vitro* co-culture model. Based on our experiences salts and components of the serum inhibit the activity of NCR peptides similarly to other AMPs. These results indicate that in proper formulation that helps to maintain the activity of the peptides on the human epithelial surface, the cationic NCR peptides might be effective drugs for the treatment of candidiasis.

THE THESIS IS BASED ON THE FOLLOWING PUBLICATION

- **Ördögh L.**, Vörös A., Nagy I., Kondorosi É., Kereszt A. (2014) Symbiotic plant peptides eliminate *Candida albicans* both *in vitro* and in an epithelial infection model and inhibit the proliferation of immortalized human cells. BioMed Research International. Volume 2014, Article ID 320796
  (IF_{2014}=2,706)

ADDITIONAL PUBLICATIONS

- **Ördögh L.**, Hunyadkürti J., Vörös A., Horváth B., Szűcs A., Urbán E., Kereszt A., Kondorosi É., Nagy I. (2013) Complete genome sequence of *Propionibacterium avidum* Strain 44067, isolated from a human skin abscess. Genome Announcements. 1(3):e00337-13.

- **Ördögh L.**, Galgóczy L., Krisch J., Papp T. and Vágvölgyi Cs. (2010) Antioxidant and antimicrobial activities of fruit juices and pomace extracts against acne-inducing bacteria. Acta Biologica Szegediensis 54(1):45-49
  (IF_{2011}=0,55)

- Galgóczy L., **Ördögh L.,** Virágh M., Papp T., and Vágvölgyi Cs. (2009) In vitro susceptibility of clinically important Zygomycetes to combinations of amphotericin B and suramin. J. Mycol. Med. 19(4), 241-247.
- Galgóczy L., Papp T., Kovács L., **Ördögh L.** and Vágvölgyi Cs. (2008) In vitro activity of phenothiazines and their combinations with amphotericin B against zygomycetes causing rhinocerebral zygomycosis. Med. Mycol. 47, 331-335.

  (IF_{2012}=2,13)

- Krisch J., **Ördögh L.,** Galgóczy L., Papp T., and Vágvölgyi Cs. (2008) Anticandidal effect of berry juices and extracts from *Ribes* species. Cent. Eur. J. Biol. 4, 86-89.

  (IF_{2008}=0,250)