Assorted publications

1. **Galgóczy, L**., Papp, T., Pócsi, I., Leiter, É., Marx, F., Papp, T. and Vágvölgyi, Cs. (2005) Sensitivity of different Zygomycetes to the *Penicillium chrysogenum* antifungal protein (PAF). J. Basic Microbiol. 45 (1), 136-141.

2. **Galgóczy L.**, Papp T., Lukács Gy., Leiter É., Pócsi I. and Vágvölgyi Cs. (2007) Interactions between statins and *Penicillium chrysogenum* antifungal protein (PAF) to inhibit the germination of sporangiospores of different sensitive *Zygomycetes*. FEMS Microbiol. Lett. 270, 109-115.

PhD THESIS

Examination of low molecular mass antimicrobial proteins and their encoding genes

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A novel, yet undescribed phenomenon is that hyphae can grow but they can not produce sporangiola in the inhibition zone in the case of *M. elongata*. F8AP had no effect on bacterial growth (examined: *Bacillus subtilis*, *Escherichia coli*, *Micrococcus luteus*, *Serratia marcescens*).

In silico examination revealed the physical and chemical properties of F8AP: it is small molecular mass (6357.16 Da), basic (pI= 9.26) protein consisting of 55 amino acids, which contains two domains and is stabilized by three disulfide bridges. The tertiary and quaternary structure of the protein (based on the presumed 3D model of F8AP) is very similar the defenzin-like molecules: 2 β-sheets connected by one α-helix.

Construction of a heterolog expression system for a novel antimicrobial protein

The *f8ap* gene was built into the pPICZαA vector and transformed into *Pichia pastoris*. F8AP expression was observed when methanol was added into the medium. Purified F8AP from the supernatant maintained its biological activity.

membrane perturbation and intracellular oxidative stress. The effect of PAF on fungal species has been investigated on a very limited number of isolates until now, and its ability to cooperate with any antifungal drugs has not been revealed yet (Marx, 2004). The *Rhizopus microsporus* var. *oligosporus* antibiotic peptide encoded by two genes (*abp1* and *abp2*) has an antibacterial effect against *Bacillus* spp. only. ABP1 of the both is the biologically active form (Kobayashi *et al*., 1992; Yamada *et al*., 2005).

The aims of this study

The aims of this study were:

1. To investigate the effect of PAF on some practically important fungal isolates.
2. To investigate the possible interactions between statins and PAF against various PAF/statin sensitive Zygomycetes.
3. To investigate the possible antifungal effect of PAF-fluconazole combination against dermatophyte species.
4. To determine the presence of possible novel antimicrobial proteins and their encoding genes in the case of F-, R- and A-isolates.
5. To optimize the expression of a novel antimicrobial protein.
6. To develop a procedure for the isolation and purification of the novel antimicrobial peptides, and for the determination of its antimicrobial spectrum.
7. To create a heterolog expression system for the novel antimicrobial protein.

Determination of the presence of possible novel antimicrobial proteins and their encoding genes

Fifteen F-isolates representing 10 species have been screened via PCR experiments. Sequences corresponding to hypothetical defensin-like proteins have been found in all isolates. These revealed high similarity to the nucleic acid sequence of the *gama* gene. Taking into account their nucleic acid and hypothetical protein sequences, 6 types of antifungal proteins could be differentiated. Aside from the possible signal sequences, only 3 novel antifungal protein groups were detectable: F7, F2 and F5/a-d, F1.

The presence of *abp1-2* gene was observed in 12 R-isolates representing 6 species. The percentage of homology between the DNA sequences was 100% to *abp1* and *abp2*.

We also examined A-isolates representing 7 species. One, non functional *afp/anafp* homologue mutant gene was detected in the case of the A6-isolate. An AFP-similar protein encoding gene was established in the case of A2-isolate, which carries one amino acid mutation in its hypothetical signal sequence compared to AFP.

The presence of defensin-like antifungal proteins is more frequent among filamentous fungi than presumed before.

Optimization of the expression of a novel antimicrobial protein

The production of antifungal poteins in the case of F-isolates has been optimized with using of antifungal protein induction medium. Their biological activities on hyphal growth of *Trichoderma longibrachiatum* and *Mortierella elongata* was investigated with an agar diffusion technique. The extracts of eight of ten species showed similar

Syncephalastrum racemosum and *Saksenaea vasiformis* proved to be insensitive to PAF. Similar results were observed in PAF-treated *Hypocrea orientalis*, *Trichoderma atroviride*, *T. citrinoviride*, *T. harzianum*, *T. inhamatum* and *T. longibrachiatum*. Only the *T. viride* isolate was insensitive to influence of PAF, and the rate of inhibition in case of *T. harzianum* and *T. inhamatum* depended on the applied medium. Sprawling hyphae were observed in the region of earlier inhibition zones after 48 or 72 hours of incubation depending on the applied medium. All pathogenic yeast isolates (*Candida albicans*, *C. glabrata*, *C. guilliermondii*, *C. inconspicua*, *C. krusei*, *C. lipolytica*, *C. lusitaniae*, *C. norvegica*, *C. tropicalis*, *C. parapsilosis*, *C. pulcherrima*, *C. zeylanoides* and *Malassezia pachydermatis*) proved to be insensitive species to PAF treatment. Higher concentrations of PAF (≥50 μg/ml) were effective in inhibiting the growth of dermatophytes (*Trichophyton mentagrophytes*, *T. rubrum*, *T. tonsurans*, *Microsporum canis*, *M. gypseum*) and fungicidal effect was observed in every cases at 200 μg/ml of PAF.

Interaction between PAF and statins

The effects of combination of statins with PAF were investigated on four isolates of Zygomycetes (*Rhizopus stolonifer*, *Mortierella wolfii*, *Syncephalastrum racemosum* and *Mycotypha africana*) which represented combinations of lovastatin sensitive/insensitive and PAF sensitive/insensitive properties based on preliminary experiments. The efficiency to inhibit sporangiospore germination was studied with 4 statins (lovastatin, simvastatin, rosuvastatin, atorvastatin) at different concentrations (1-128 μg/ml) with a constant concentration of PAF (50 μg/ml) in *in vitro* experiments. Interactions were not found in *R. stolonifer*. This species was completely insensitive to both lovastatin and PAF.

Introduction

The number of microbial infections has increased continuously over the past years in consequence of increasing number of immunocompromised patients and inefficient using of of broad-spectrum antifungal drugs (Ribes *et al*., 2000). Infections caused by opportunistic filamentous fungi are especially problematic, because most of the antifungal treatments available have serious side effects and could not be applied without a damage of the host (Vicente *et al.*, 2003). Therefore, there is a substantial demand for new types of compounds with antifugal activity.

The pest controll has some challenge to defense against fungal infections. Its aim is the keeping or enhancing of yield with using of effective antifungal compounds. The requirement of these kinds of agents is that they should not do harm to the enviroment, plants, animals and human. Other aspect, is that they should be produced at a low of price.

The extracellular antimicrobial proteins secreted by some filamentous fungi are interesting from this respect, as they have effective inhibitory potential as concerns both the hyphal extension and the germination of the spores. The features of these proteins are a low molecular mass (5.8-6.6 kDa), a basic character, and the presence of 6-8 cysteine residues and several disulfide bonds. Proteins with such properties with antifungal activity have been isolated and investigated from 5 fungal species (*Penicillium chrysogenum*, *P. nalgiovense*, *Aspergillus clavatus*, *A. giganteus* and *A. niger*), furthermore, *in silico* investigation of genomic databases has been revealed a putative protein with high homology to *P. chrysogenum* antifungal protein (PAF) in *Gibberella zeae*. It has been shown that, in sensitive fungi, PAF is localized intracellularly and exerts multiple detrimental effects: induction of morphological changes,

Summary

Our results have revealed:

1. PAF-sensitivity of some practically important fungal isolates including human pathogenic clinical isolates.
2. Effective interaction between PAF and statins and antifungal activity of the observed statins.
3. Effective interaction between PAF and statins against dermatophyte isolates.
4. The presence of defensin-like antifungal proteins is more frequent among filamentous fungi than presumed before.
5. Some investigated novel antifungal proteins may be regarded as promising candidates in future antifungal drug research.
6. Presence of a small molecular mass antifungal protein in the case of F8-isolate (F8AP), which maintained its antifungal activity after the purification.
7. *Pichia pastoris* can synthetise the F8AP in a biologically active form.

Methods

- *In vitro* antifungal susceptibility tests
- Assays for i*n vitro* antifungal drug interactions
- DNA and RNA techniques
- Polymerase chain reaction (PCR)
- Inverse PCR
- DNA sequencing
- Techniques for the production and purification of novel antifungal proteins
- Protein gel electophoresis
- Isoelectric focusing protein gel electrophoresis
- *In silico* methods
- Heterolog expression (EasySelect^{TM} Pichia Expression Kit, Invitrogen)

Results

The effect of PAF on some practically important fungal isolates

PAF treatment (50 μg/ml) resulted in a strong inhibition of the germination of spores and the hyphal extension in the case of *Absidia corymbifera*, *Micromucor ramanniana*, *Mortierella elongata*, *M. nanthalensis*, *M. wolfii*, *Mycotypha africana*, *Rhizomucor miehei*, *R. pusillus*, *Rhizopus microsporus* var. *oligosprus*, *R. oryzae*, *Thamnostylum piriforme*, *Umbellopsis isabellina*, *Zygorhynchus macrocarpus*. *Actinomucor elegans*, *Cokeromyces recurvatus*, *Gilbertella persicaria*, *Mucor hiemalis* f. *luteus*, *M. racemosus*, *Rhizopus stolonifer*,

inhibitory effect like PAF, while the ferment broth of two isolates (F1 and F5/a) proved to be inactive in these tests. The supernatant of F8 was the most effective in the inhibition of hyphal extension.

Developing of a procedure for the isolation and purification of a novel antimicrobial peptides, and for the determination of its antimicrobial spectrum

Protein gel electrophoresis revealed the presence of a small protein (approximately 6.3 kDa) in the eight biologically active species. These proteins were purified further with ultrafiltration and ion exhcange chromatography on a CM-sepharose column. The purified protein of F8-isolate maintained its antimicrobial activity. It was determined with several tests against *Trichoderma longibrachiatum* and *Mortierella elongata*, that this ~6.3 kDa protein is responsible for the antifungal activity, and it was named F8-isolate antifungal protein (F8AP). The effect of F8AP on germination efficiency of sensitive conidiospores was examined in *T. longibrachiatum*. The conidiospores displayed abnormal and delayed germination when cultivated in an F8AP-containing medium. F8AP-treated conidiospores formed very short, swelled hyphae with multiple branches. F8AP treatment (50 μg/ml) resulted in the inhibition of growth in the cases of most of the 18 examined Zygomycetes and the 11 yeasts and 4 filamentous fungi from Ascomycetes. The species specifity of F8AP was the same as that of PAF in most of the cases, but differences were observed in the case of *Candida* spp.: *C. inconspicua*, *C. lipolytica*, *C. lusitaniae*, *C. norvegica* and *C. parapsilosis* were slightly sensitive. However, it has to be mentioned that there were differences in the applied test methods. Sprawling hyphae were not observed in the region of earlier inhibition zones in the case of *Trichoderma/Hypocrea* species like in case of PAF.

The inhibitions induced by the combinations of simvastatin-PAF, rosuvastatin-PAF and atorvastatin-PAF were the same as those observed for the statins alone. *M. wolfii* was practically insensitive to the applied statins. The approximately 40% decrease in the growth of *M. wolfii* in the presence of the statin-PAF combinations corresponds with the activity of PAF applied without a statin. For *S. racemosum*, no interaction between lovastatin and PAF was detected: there was no significant difference between sensitivity to lovastatin or lovastatin-PAF. Simvastatin at 4 μg/ml acted synergistically with PAF, decreasing the growth rate to 17% (±2%). Higher concentrations of simvastatin added together with PAF led to the total inhibition of growth. Rosuvastatin acted synergistically with PAF even at low statin concentrations (≥2 μg/ml); but it is worth to mention that rosuvastatin alone can evoke a complete growth inhibition at concentrations above 32 μg/ml. Atorvastatin acted synergistically with PAF on *S. racemosum* at 1-8 μg/ml concentrations, and additively at 16 μg/ml of atorvastatin; higher statin concentrations combined with PAF resulted in complete growth inhibition. *M. africana* was sensitive to all statins and PAF, and the inhibition was increasing when the two types of compounds were applied in combinations. Synergistic interactions were detected between lovastatin (at 1-8 μg/ml) and PAF. The addition of 8 μg/ml lovastatin together with PAF reduced the growth rate to 8% (±0.8%); higher concentrations resulted in further slight decreases in the growth, but complete inhibition was not achieved. Simvastatin interacted additively with PAF at 1-4 μg/ml. However, no effect of PAF was detected at higher simvastatin concentrations: the inhibition rates were the same as those with the same amounts of simvastatin without PAF. One μg/ml of rosuvastatin and PAF acted synergistically against *M. africana*, causing an inhibition of 92% (±0.2%). Additive interactions were observed at 2-4 μg/ml; higher rosuvastatin concentrations led to complete growth

inhibition. Atorvastatin at 1-4 μg/ml acted additively with PAF; higher concentrations in the presence of PAF inhibited germination completely. The inhibition rates obtained with the rosuvastatin-PAF and atorvastatin-PAF combinations were very similar. In conclusion, the activities of the statin-PAF combinations on the different strains varied and depended considerably on the activities of the components alone. When a strain was resistant to one of the components, *e.g. R. stolonifer* to PAF or *M. wolfii* to the statins, significant interactions could not be detected; only the effect of the active component was observed.

Interaction between PAF and fluconazole

A constant concentration of PAF (100 μg/ml) combined with eight different (0.25-32 μg/ml) concentrations of fluconazole (FCZ) was investigated against dermatophytes. The activities of PAF-fluconazole combinations on different strains varied and depended on the activities of the components alone. *Trichophyton rubrum* and *T. tonsurans* were the most sensitive species to PAF-fluconazole combinations. Synergistic interactions were detected betweeen PAF and fluconazole at 8-16 μg/ml and 32 μg/ml in several cases. PAF interacted additively with fluconazole at 4-2 μg/ml for *T. rubrum*, and 16-4 μg/ml of fluconazole in the case of *T. tonsurans*. For *T. mentagrophytes* and *Microsporum canis,* additive interactions were detected between the two compounds at ≥8 μg/ml of fluconazole. Interactions were not found in the case of *M. gypseum*, only the effect of PAF or fluconazole was detectable. Fluconazole was dominant ranging from 32 μg/ml to 8 μg/ml, than the influence of PAF was prevailed. The fungistatical effect of fluconazole at 32 μg/ml in the case of *T. rubrum* and *M. canis* turned into fugicidical when 100 μg/ml of PAF was added.