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ORIGINAL PAPER

In vitro activity of *Penicillium chrysogenum* antifungal protein (PAF) and its combination with fluconazole against different dermatophytes

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Abstract Strains of five dermatophyte species (*Microsporum canis*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Trichophyton tonsurans*) were selected for testing against *Penicillium chrysogenum* antifungal protein (PAF) and its combination with fluconazole (FCZ). Inhibition of microconidia germination and growth was detected with MICs of PAF ranging from 1.56 to 200 μg ml^{-1} when it was used alone, or at constant concentration (100 μg ml^{-1}) in combination with FCZ at from 0.25 to 32 μg ml^{-1}. The MICs for FCZ were found to be between 0.25 and 128 μg ml^{-1}. PAF caused a fungicidal effect at 200 μg ml^{-1} and reduced growth at between 50 and 200 μg ml^{-1}. Total growth inhibition with fungistatic activity was detected at 64 μg ml^{-1} of FCZ for *M. gypseum*, *T. mentagrophytes*, and *T. tonsurans*, and at 32 μg ml^{-1} FCZ for *M. canis* and *T. rubrum*. PAF and FCZ acted synergistically and/or additively on all of the tested fungi except *M. gypseum*, where no interactions were detected.

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Abbreviations

ADD	Additive effect
FCZ	Fluconazole
FI	Full inhibition
IR	Interaction ratio
NI	No interaction
PAF	*Penicillium chrysogenum* antifungal protein
SY	Synergism

Introduction

The dermatophytes are a group of morphologically and physiologically related molds which affect the keratinous tissue of humans and other vertebrates (Fernández-Torres et al. 2002; Santos and Hamdan 2005). Most of them are widely distributed throughout the world, but some display a restricted geographic distribution. The dermatophytes infect the hair, skin, and nails, because they can utilize keratin for growth. An atypical manifestation with more severe and more extensive lesions can evolve in immunocompromised patients. Direct contact or exposure to infected desquamated cells can trigger transmission (Santos et al. 2006). A number of enzymes, such as acid proteinase, elastase, keratinases and proteinases, which can permit their invasion into keratinized tissues, are the major virulence factors of these fungi

(Weitzman and Summerbell 1995). The incidence of dermatophytoses has been increasing continuously during recent years as a consequence of the rise in the number of immunocompromised patients (Fernández-Torres et al. 2002). The treatment with antifungal agents is based on the site and extent of the infection, the species involved, and the efficacy, safety profile and kinetics of the available drugs. Topical therapy with clotrimazole is generally used for localized, relatively inextensive lesions. Systemic antifungal treatment with oral drugs such as itraconazole and terbinafine is necessary in cases of tinea unguium, scalp ringworm, extensive infections, or skin lesions with folliculitis (Fernández-Torres et al. 2002). In recent years, a number of safe and highly effective antifungal compounds [terbinafine, itraconazole, fluconazole (FCZ), voriconazole, posaconazole, ravuconazole and a new triazole, UR-9825] have been introduced into clinical practice.

The low molecular mass, cysteine-rich antifungal proteins synthesized by filamentous fungi may improve the range of promising candidates available for the treatment of dermatophytosis. One of these is the *Penicillium chrysogenum* antifungal protein (PAF). PAF is secreted as a 6.3-kDa, highly basic, cysteine-rich extracellular protein containing three disulfide bonds (Marx 2004). It is an effective inhibitor of hyphal extension and spore germination. PAF-treated mycelia are swollen and short, with multiple branches. A fragmented cytoplasm and the accumulation of nuclei at broken hyphal tips have been described in sensitive fungi (Kaiserer et al. 2003). PAF has been shown to be localized intracellularly and to exert multiple detrimental effects: the induction of morphological changes, membrane perturbation, intracellular oxidative stress and a programmed cell death mechanism (Marx et al. 2007). PAF is an effective inhibitor of some Ascomycetes and Zygomycetes (Galgóczy et al. 2005; Kaiserer et al. 2003; Marx 2004). The influence of PAF on isolates of dermatophytes has not been investigated to date.

In the present study, the effects of PAF and its combination with FCZ against five dermatophytes representing eight isolates (*Microsporum canis*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Trichophyton tonsurans*) were tested in vitro. Though, FCZ is not the most effective azole antifungal agent used against dermatophytes, it could be the drug of choice in some cases one other drugs are contraindicated.

Materials and methods

Strains and media

The following strains were used in the present study: *M. canis* (American Type Culture Collection, USA; ATCC 36299; Szeged Microbial Collection, University of Szeged, Szeged, Hungary SzMC 90253), *M. gypseum* (ATCC 24102), *T. mentagrophytes* (ATCC 9533, SzMC 3633, SzMC 30522), *T. rubrum* (ATCC 28188) and *T. tonsurans* (ATCC 28942). All these isolates were maintained on potato dextrose agar (PDA) slants (Sigma-Aldrich) at 4°C. Tests were performed in RPMI 1640 medium (Sigma-Aldrich), with L-glutamine and without sodium bicarbonate buffered, with 0.165 M morpholinepropanesulfonic acid at pH = 7.0.

Antifungal agents

PAF was purified in a modification of the method described by Marx et al. (1995). Briefly, *P. chrysogenum* Q176 was grown in a sucrose (20 g l^{-1})—$NaNO_3$ (3 g l^{-1}) minimal medium for 96 h at 25°C with shaking (220 rpm) (Marx et al. 1995). After harvesting of the mycelia by centrifugation and separation of the low molecular weight protein fraction in Amicon Stirred Cells (V = 50 ml, Biomix PBTK ultrafiltration discs, size exclusion limit M_r = 30,000; Millipore), PAF was purified by ion-exchange chromatography on a CM Sephadex Fast Flow column (2 × 18 cm, equilibrated with 50 mM sodium phosphate buffer, pH = 6.6, flow rate 1 ml min^{-1}, t = 4°C; Amersham-Pharmacia). PAF was eluted with a NaCl gradient (0.05–1.0 M) prepared in the equilibrating buffer. The quality of the PAF preparation was always checked with SDS-PAGE on pre-cast Novex 16% Tris/Glycine gels (Invitrogen). Protein bands were visualized with Coomassie Brilliant Blue R staining. The purified protein was prepared in 25 mM Tris/HCl, pH = 7.5 and diluted in RPMI 1640 medium. FCZ (Sigma-Aldrich) was provided by the manufacturer as standard powder. The drug was dissolved in dimethyl formamide to obtain the stock solution. Drug

dilutions were performed in RPMI 1640 medium to yield twice the final strength required for the tests.

Inoculum preparation

Inocula were prepared in a modification of the method described by Santos et al. (2006). The fungal colonies were grown on PDA slants except for *T. rubrum*, which was subcultured onto oatmeal agar to induce conidium production (Jessup et al. 2000; Ghannoum et al. 2006b) and incubated at 30°C for 7 days. The fungal colonies were covered with 5 ml RPMI 1640 medium and suspensions were made by gently probing the surface with the tip of a pipette, generating a mixture of conidia and hyphal fragments. The heavy particles were allowed to settle for 10 min at room temperature, and the upper fraction was then filtered through a membrane (pore size 8 μm, Sartorius) which retains hyphal fragments and permits the passage only of dermatophyte microconidia. Inoculum quantification was performed by plating 0.01 ml of each kind of inoculum suspension in Sabouraud dextrose agar (Sigma-Aldrich). The plates were incubated at 30°C and examined daily for the presence of colonies. The inoculum suspensions were diluted (1:50) in RPMI 1640 medium. Colonies were counted as CFU ml^{-1} when they had become visible.

Determination of antifungal activity

The in vitro antifungal activities of PAF, FCZ and their combinations were determined in 96-well microtiter plate bioassays by measuring the absorbance of fungal cultures at 620 nm, based on a modified CLSI M38-A method derived from Ghannoum et al. (2004). To assign the MIC values of each antifungal agent, 100 μl of the twofold drug concentration was mixed with 100 μl diluted microconidia suspension in each microdilution well. The final FCZ concentration ranged from 0.25 to 128 μg ml^{-1}. To determine MIC values, PAF was used in the range from 200 to 1.56 μg ml^{-1}. In the interaction experiments, 100 μl of the twofold drug concentration was used, but PAF was dissolved in the microconidia suspension. To reveal the interaction between the two antifungal compounds, FCZ at from 0.25 to 32 μg ml^{-1} was combined with a constant concentration of 100 μg ml^{-1} PAF. Each test plate contained a sterile control (200 μl medium alone), a growth control (100 μl medium plus 100 μl microconidia suspension), a drug-free control (200 μl microconidia suspension alone) and a sterile drug control (100 μl of twofold concentration of drug plus 100 μl medium). The plates were incubated at 37°C, and absorbances were measured with a microtiter plate reader (ASYS Jupiter HD-ASYS Hitech) after 4, 7 and 10 days. The sterile control was used as a background for the spectrometric calibration. In the case of *T. tonsurans*, a further checkerboard titration was used in the concentrations of the PAF, ranging from 50 to 3.125 μg ml^{-1}, were mixed with concentrations of FCZ ranging from 32 to 2 μg ml^{-1}. To decide whether the antifungal effect was fungistatic or fungicidal, 200 μl of each suspension in the microdilution plate was pelleted for 15 min at 10,000 g, washed twice with 500 μl potato dextrose broth (Sigma-Aldrich) and resuspended in a concentration of 10^3 conidia ml^{-1} in culture medium. 10 μl suspension was dropped and dried on the PDA plate. After incubation for 4, 7 and 10 days at 37°C, plates were checked visually. When a colony evolved from the drop, the antifungal effect was fungistatic; otherwise, it was fungicidal. Experiments were repeated three times.

Data analysis

For calculation of the inhibition rates, the absorbances of the untreated control cultures were in each case referred to 100% growth. The interaction ratio between the antifungal agents was calculated via the Abbott formula: $I_e = X + Y - (XY/100)$, where I_e is the expected percentage inhibition for a given interaction, and X and Y are the percentage growths inhibited by the compounds used alone. If I_0 is the observed percentage inhibition, the interaction ratio (IR) is given by $IR = I_0/I_e$, which corresponds to the nature of the interaction between the antifungal compounds. When IR is between 0.5 and 1.5, the interaction is additive, whereas $IR > 1.5$ denotes synergism, and $IR < 0.5$ denotes antagonism (Moreno et al. 2003).

Microscopy

Drops of PAF-treated fungal suspensions from the microtiter plate wells were examined and photographed by means of a light microscope (LR 66238C,

Carl Zeiss, Axilab) and a digital camera (Nikon, Coolpix 4500).

Results

Inoculum preparation

Inoculum count ranges are presented in Table 1. All were between 3.1×10^4 and 1.2×10^5 CFU ml^{-1}.

Sensitivity to FCZ and PAF

All isolates tested produced clearly detectable growth after 7 days of incubation. PAF at 200 μg ml^{-1} caused complete growth inhibition of all of the tested fungi. This effect was fungicidal in every case. The hyphae were swollen and microscopic observation revealed multiple branching at 100 μg ml^{-1} PAF after 4 days as compared with the untreated controls (Fig. 1). 100 μg ml^{-1} PAF decreased the growth rates of *M. canis* (two isolates), *M. gypseum*, *T. mentagrophytes* (three isolates), *T. rubrum* and *T. tonsurans* to 83 (±1.9%), 62 (±2,3%), 75 (±2.8%), 76 (±2.3%) and 42% (±6.2%), respectively. Inhibitory effects were not detected ≤50 μg ml^{-1} PAF. The growth of isolates of *M. gypseum*, *T. mentagrophytes* (three isolates) and *T. tonsurans* were affected fungistatically at 64 μg ml^{-1} FCZ, as were those of *M. canis* (two isolates) and *T. rubrum* at 32 μg ml^{-1}. Higher concentrations of FCZ were fungicidal (Table 1).

Interaction between FCZ and PAF

When PAF was applied at constant concentration (100 μg ml^{-1}), combined with eight different concentrations of FCZ (0.25-32 μg ml^{-1}), *T. rubrum* and *T. tonsurans* proved the most sensitive species to the PAF-FCZ combinations. Synergistic interactions were detected between PAF and FCZ at 8-16 and 32 μg ml^{-1}, respectively. PAF interacted additively with FCZ at 4–2 μg ml^{-1} for *T. rubrum*, and at 16–4 μg ml^{-1} for *T. tonsurans*. For *M. canis* and *T. mentagrophytes*, additive interactions between PAF and FCZ were detected at ≥8 μg ml^{-1} FCZ. Interactions were not found in the case of *M. gypseum*. This species was the one of the most sensitive to 100 μg ml^{-1} PAF. The inhibitions induced by the PAF-FCZ combinations were the same as those observed for PAF and FCZ alone. The complete blockade of *M. canis* and *T. rubrum* at 32 μg ml^{-1} FCZ in combination with PAF was fungicidal. These experimental data are to be found in Table 2.

Against *T. tonsurans*, which proved to be the most sensitive to PAF, checkerboard titration revealed further effective PAF + FCZ combinations where 50, 25 and 50 μg ml^{-1} of PAF combined with 16,

Table 1 MICs of PAF and FCZ against the tested dermatophytes, and their growth reduction rates

Species	Drug	MIC (μg ml^{-1})	Effect
M. canis	PAF	200	Fungicidal
(2 isolates)		100	83% (±1.9%) growth rate
3.9–4.8 × 10^4 CFU ml^{-1}	FCZ	32	Fungistatic
M. gypseum	PAF	200	Fungicidal
3.1 × 10^4 CFU ml^{-1}		100	62% (±2.3%) growth rate
	FCZ	64	Fungistatic
T. mentagrophytes	PAF	200	Fungicidal
(3 isolates)		100	75% (±2.8%) growth rate
4.2-6.8 × 10^4 CFU ml^{-1}	FCZ	64	Fungistatic
T. rubrum	PAF	200	Fungicidal
1.2 × 10^5 CFU ml^{-1}		100	76% (±2.3%) growth rate
	FCZ	32	Fungistatic
T. tonsurans	PAF	200	Fungicidal
4.6 × 10^4 CFU ml^{-1}		100	42% (±6.2%) growth rate
	FCZ	64	Fungistatic

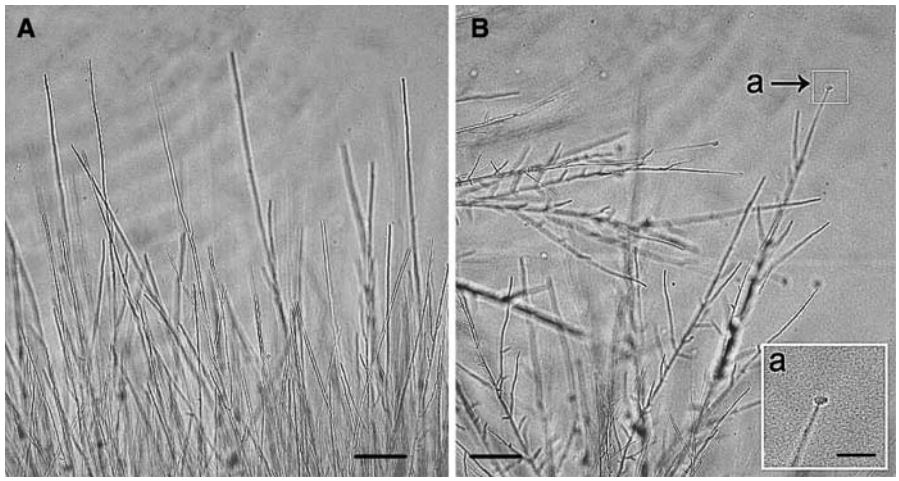

Fig. 1 Effect of PAF on hyphal growth in *M. gypseum*. Hyphal growth without PAF (**A**), and in the presence of 100 μg ml^{-1} PAF (**B**); swollen tips (a). Scale bars 50 μm (A, B), 20 μm (a)

16 and 8 μg ml^{-1} of FCZ, respectively, acted synergistically (IR = 2.03, 1.72, 1.76, respectively). Interactions between the two compounds were not observed below these values.

Table 2 Interactions between PAF at constant concentration (100 μg ml^{-1}) and FCZ at various concentrations against the tested strains

FCZ (μg ml^{-1})	IR	Type of interaction	Growth (%)
M. canis (2 isolates)			
32	1	Additive	0[a]
16	0.67 ± 0.08	Additive	56 ± 2.9
8	0.53 ± 0.03	Additive	70 ± 1.2
T. mentagrophytes (3 isolates)			
32	0.87 ± 0.03	Additive	25 ± 1.8
16	0.67 ± 0.04	Additive	37 ± 2.3
8	0.57 ± 0.02	Additive	46 ± 1.7
T. rubrum			
32	1	Additive	0[a]
16	3.37 ± 0.09	Synergistic	8 ± 0.7
8	1.72 ± 0.06	Synergistic	11 ± 1.7
4	1.17 ± 0.05	Additive	21 ± 2.1
2	0.52 ± 0.01	Additive	49 ± 0.4
T. tonsurans			
32	2.59 ± 0.17	Synergistic	15 ± 1.8
16	0.86 ± 0.02	Additive	31 ± 1.1
8	0.65 ± 0.03	Additive	36 ± 2.3
4	0.62 ± 0.02	Additive	38 ± 1.8

The untreated control is taken as 100% of growth

[a] Fungicidal effect

Discussion

The aim of this study was to investigate the possible antifungal effects of PAF, and its interaction with FCZ against dermatophytes. PAF exerted fungicidal action on the five investigated species, and the treated hyphae displayed revealing morphological changes as described previously at lower concentrations (Kaiserer et al. 2003). This action is connected with the detrimental effects of PAF on sensitive fungi. The main symptoms are the inhibition of germination and hyphal growth, retardation of the lengthening of the hyphae, membrane perturbation, the induction of intracellular oxidative stress and an apoptosis-like phenotype (Kaiserer et al. 2003; Marx 2004). The influence on heterotrimeric G-protein signaling provides the antifungal effect of PAF. A sensitive mold, *Aspergillus nidulans* endures hyperpolarization of the plasma membrane, because PAF most probably interacts directly or indirectly with the plasma membrane H^+ pump and triggers ion effluxes (Kaiserer et al. 2003; Leiter et al. 2005). The PAF concentration of 100 μg ml^{-1} observed to inhibit the germination of microconidia and to cause growth retardation for these dermatophytes is higher than that earlier reported for different species of Ascomycetes and Zygomycetes; ~50 μg ml^{-1} (Kaiserer et al. 2003; Galgóczy et al. 2005). PAF would be an appropriate compound for the treatment of topical dermatophytosis, because it does not have a toxic effect on mammalian cells in vitro (Szappanos et al. 2005). This was recently confirmed by Jacobi et al. (2007), who investigated the penetration of

fluorescein isothiocyanate (FITC)-labeled protein mixtures (10–100 kDa in size) in vitro through porcine skin and in vivo through human skin. In vitro, the FITC-labeled proteins were observed within the complete stratum corneum and inside the hair follicles 15 min after application. They were also distributed inside the dermis around the hair follicles. They observed a similar pattern of distribution within the stratum corneum and the hair follicles in vivo (Jacobi et al. 2007). These results suggest the possibility for PAF to penetrate human skin in vivo. On the other hand, a protein similar to PAF, *Aspergillus giganteus* antifungal protein, is stable and effective in the surface even 14 days after its application and can prevent *Botrytis cinerea* infection at an active dose in *Pelargonium* species (Moreno et al. 2003).

FCZ has been described as a less effective antifungal compound as compared with other azoles (itraconazole, clotrimazole, ketoconazole, miconazole, voriconazole, posaconazole and isoconazole). The MIC of FCZ in previously studies was between 1 and >64 μg ml^{-1} depending on the test method applied and the organisms involved (Jessup et al. 2000; Fernández-Torres et al. 2001; Ghannoum et al. 2004, 2006a, b; Santos and Hamdan 2005; Santos et al. 2006). The MIC_{90} values of *M. gypseum*, *T. rubrum* and *T. mentagrophytes* were generally reported to be >64 μg ml^{-1} (Fernández-Torres et al. 2001; Santos and Hamdan 2005; Santos et al. 2006), that of *M. canis* 16 μg ml^{-1} and that of *T. tonsurans* 8 μg ml^{-1} FCZ (Jessup et al. 2000; Fernández-Torres et al. 2001). Our present data are in accord with this, except for at *T. rubrum*, where the MIC with a fungistatic effect was less (32 μg ml^{-1}), and for *M. canis* and *T. tonsurans*, where it was higher (64 and 32 μg ml^{-1}, respectively). Of other drugs, such as terbinafine, griseofulvin and ciclopirox, also used in the treatment of dermatophytosis, terbinafine is the most effective and commonly used clinically against dermatophytes (Jessup et al. 2000; Fernández-Torres et al. 2002; Ghannoum et al. 2006b). However, it is worrying that terbinafine-resistant *T. rubrum* has been appearing in recent years (Ghannoum et al. 2004). Otherwise topical and oral administration of terbinafine can induce several serious side effects. Side-effects occurred in 2% of patients using topical terbinafine cream (Villars and Jones 1989). Adverse effects believed to be terbinafine-related occurred in 4–5% among topically treated patients in another study. The events were primarily local skin reactions of mild to moderate intensity (Schopf et al. 1999). Gupta et al. (1998) reported a series of different cutaneous manifestations of the adverse effects of terbinafine and there are so many ocassion of side effect connected with terbinafine in the literature (Gupta and Porges 1998; Bennett et al. 1999; Hall and Tate 2000; Rogalski et al. 2001; van Puijenbroek et al. 2001; Aksakal et al. 2003; Abecassis et al. 2004; McKellar et al. 2004; Kim et al. 2007). Combined application of the less effective azoles with novel topical or oral antifungal compounds may solve this problem in case of terbinafine sensitive patients.

The activities of the PAF–FCZ combinations on the different strains in the present work varied and depended on the activities of the components. The fungistatic effect of FCZ at 32 μg ml^{-1} for *M. canis* and *T. rubrum* became fugicidical when 100 μg ml^{-1} PAF was added. The synergistic effect of 16 μg ml^{-1} FCZ on *T. tonsurans* became additive at ≤4 μg ml^{-1} FCZ, and additive interactions were observed at ≤8 μg ml^{-1} FCZ for *T. mentagrophytes*. The synergistic and additive effects detected suggest that PAF and FCZ can function together and generate a significant antifungal effect. The case of *M. gypseum* is problematic: no interaction between the two compounds was observed, even though this species was one of the most sensitive to the applied 100 μg ml^{-1} PAF [inhibition rate: 38% (± 2.3%)]. FCZ predominated between 32 and 8 μg ml^{-1}, after which the influence of PAF was the stronger. Clinical administration of 200 mg FCZ for 5 days resulted in a concentration of this agent of 127 μg g^{-1} in the stratum corneum (Wildfeuer et al. 1994). This observation is compatible with our present findings. Analogous interaction effects were described between PAF and non-antifungal agents in a recent study: it was demonstrated that PAF acts synergistically with some statins, which alone display antifungal activity against some Zygomycetes (Galgóczy et al. 2007).

The azole antifungal agents inhibit the synthesis of ergosterol by blocking the action of cytochrome P450 14a-demethylase (P450 14DM) (Odds et al. 2003). This action is not selective, and binding to human P450 14DM can also occur. The membrane-destroying effects of FCZ and of PAF are possibly triggered by each other when they are applied in combination. It is important to mention that only one concentration of PAF (100 μg ml^{-1}) was tested; it is possible that

inhibition can be induced with lower FCZ concentrations at higher PAF concentrations.

The observed activities of PAF and the PAF–FCZ combinations make them promising candidates for the treatment of topical and systemic dermatophytosis. Further studies, investigating the interaction of PAF with other clinically used azole compounds against dermatophytes would also be interesting.

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