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In vivo application of a small molecular weight antifungal protein of *Penicillium chrysogenum* (PAF)

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ABSTRACT

The antifungal protein of *Penicillium chrysogenum* (PAF) inhibits the growth of important pathogenic filamentous fungi, including members of the *Aspergillus* family and some dermatophytes. Furthermore, PAF was proven to have no toxic effects on mammalian cells *in vitro*. To prove that PAF could be safely used in therapy, experiments were carried out to investigate its *in vivo* effects.

Adult mice were inoculated with PAF intranasally in different concentrations, up to 2700 $\mu g \cdot kg^{-1}$ daily, for 2 weeks. Even at the highest concentration – a concentration highly toxic *in vitro* for all affected molds – used, animals neither died due to the treatment nor were any side effects observed. Histological examinations did not find pathological reactions in the liver, in the kidney, and in the lungs. Mass spectrometry confirmed that a measurable amount of PAF was accumulated in the lungs after the treatment. Lung tissue extracts from PAF treated mice exerted significant antifungal activity. Small-animal positron emission tomography revealed that neither the application of physiological saline nor that of PAF induced any inflammation while the positive control lipopolysaccharide did. The effect of the drug on the skin was examined in an irritative dermatitis model where the change in the thickness of the ears following PAF application was found to be the same as in control and significantly less than when treated with phorbol-12-myristate-13-acetate used as positive control.

Since no toxic effects of PAF were found in intranasal application, our result is the first step for introducing PAF as potential antifungal drug in therapy.

Introduction

Pulmonary aspergillosis is one of the most severe human diseases caused by fungi threatening especially immunodeficient patients. This expanding population is composed of patients with advanced HIV infection, prolonged neutropenia, and inherited immunodeficiency and patients who have undergone lung transplantation and allogeneic hematopoietic stem cell transplantation. It can be classified into three types: allergic bronchopulmonary aspergillosis, fungus ball type pulmonary aspergillosis and invasive pulmonary aspergillosis. The allergic bronchopulmonary aspergillosis is typical in patients with chronic obstructive pulmonary disease. In this case, the colonization of the fungi causes an allergic response. The fungus ball type pulmonary aspergillosis called aspergilloma means the development of a fungal ball in preexisting cavity usually caused by old tuberculosis or cystic diseases of the lung. In invasive pulmonary aspergillosis the hyphae invade the tissue of the lung. In histopathology discrete nodules can be seen consisting of round shaped coagulation necrosis with numerous hyphae in it, or fused lobular consolidation which means the filling of acute inflammatory exudates with a fungal proliferation in alveoli (Shibuya et al., 2004). The most important species in this respect is *Aspergillus fumigatus*. If chronic pulmonary aspergillosis is untreated, destruction can eventually encompass an entire lobe or lung; even with treatment, the morbidity and mortality remains high. Patients require long-term antifungal therapy to prevent relapse (Smith and Denning, 2011). In different types of pulmonary aspergillosis the treatment is diversified, but all include the application of one or more antifungal drug. For example in invasive aspergillosis, which is often rapidly progressive and has a high mortality rate; posaconazole,

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voriconazole, caspofungin, amphotericin B, or amphotericin B lipid formulations may be considered as empiric therapy (Maertens et al., 2004). Initial combination therapy is unusual and generally reserved for single drug treatment failures.

Fungal infections of the skin and nails of the foot caused by dermatophytes are common, reflecting the virulent nature of the organisms. They are a group of physiologically and morphologically similar molds which affect the keratinous tissue of humans and other vertebrates because they can utilize keratin for growth (Galgoczy et al., 2008). Many fungal infections of humans and animals affect only the outer layers of skin (the hair, skin, and nails) and they are sometimes difficult to cure. Although the symptoms produced by infection with different types of fungi diversified, they usually cause itching, reddened skin, and inflammation. While some superficial skin infections are mild and produce only few or no symptoms, others are more irritating (Crawford and Hollis, 2007). Albeit these fungal infections are rarely fatal, they may cause considerable embarrassment or discomfort. Most superficial infections respond well to topical antifungal creams, other skin infections, however, require systemic antifungal treatment. Infections with dermatophytes can be treated with allylamines like naftifine or nerbinafine, or azoles like bifonazole, oxiconazole miconazole or clotrimazole (Crawford and Hollis, 2007).

Although a number of antifungal drugs are in use, there is a rising tendency of resistant strains leading to a high mortality rate (Howard et al., 2009; Limper et al., 2011), thus rendering the finding of new antifungal treatments of high interest. It is widely accepted that one of the most important mechanisms of resistance is basically due to the deregulation of antifungal resistance effector genes. This is a consequence of point mutations in transcriptional regulators of these effector genes or in the genes coding antifungal targets (Vandeputte et al., 2012). Identification of new antifungals is essentially achieved by the screening of natural or synthetic chemical compound collections. One of the most practicable ways to develop new classes of antifungal drugs relies on the small antifungal proteins produced commonly by plants and fungi.

Filamentous fungi produce a wide spectrum of defensive proteins some of which, including the antifungal protein secreted by *Penicillium chrysogenum* (PAF), are designed to hinder the growth of other fungi. PAF is a low molecular weight (6.5 kD), basic, and cysteine-rich protein whose N-terminal amino acid sequence (GenBank accession number AAA92718) has a homology with other antifungal proteins produced by other fungi (Marx et al., 1995). PAF inhibits the growth of various pathogen fungi including *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus niger*, *Botrytis cinerea* (Kaiserer et al., 2003) and some dermatophyte species (Galgoczy et al., 2008). It causes an increased production of reactive oxygen radicals, reduces the metabolism of the fungal cells, and initiates an increased potassium efflux (Kaiserer et al., 2003). PAF enters the cells *via* an active transport mechanism (Oberparleiter et al., 2003) and acts intracellularly, probably through hetero-trimeric G-protein signaling (Leiter et al., 2005; Marx et al., 2008). It causes the hyperpolarization of the membranes of the fungal cells, which leads to the disintegration of the membranes and, consequently, an apoptosis-like phenotype appears. Furthermore, reactive oxygen species oxidize intracellular proteins, lipids, and nucleic acids, causing not only the disintegration of the cellular membranes but also that of the mitochondria (Leiter et al., 2005). These toxic, when they reach a critical limit, also contribute to the programmed cell death of the affected fungi (Hegedűs et al., 2011).

On the other hand, PAF had no toxic effects in a variety of mammalian cells (Szappanos et al., 2005). The ionic currents of neurons and astrocytes, and the potassium and L-type calcium currents of skeletal muscle fibers were not altered. No toxic effects were found on endothelial cells and only a minor pro-inflammatory effect was observed on human blood incubated with PAF when the level of cytokines was measured.

These *in vitro* measurements however did not give a direct assessment of how PAF would alter physiological parameters *in vivo*. The first step to answer this question is to characterize any morphological changes that might occur in the most likely affected organs including possible therapeutic targets. In addition, since the administration of PAF might cause an inflammatory or immune response, these should also be specifically addressed.

Notwithstanding that generic inflammatory mediators are usually assessed on bronchoalveolar lavage specimens or blood, it becomes more and more important to characterize and resolve cellular mechanisms *in vivo*, and to study physiological reactions, the development of diseases, and their treatment directly at the level of organs. For that, *in vivo* imaging would allow real time mechanistic investigation of inflammatory mediators. Nuclear imaging methods such as magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT), and positron emission tomography (PET) have been applied for visualizing mechanical and physiological responses. Imaging techniques used in human medicine have been modified and adapted to small animal experiments, to serve basic research and provide a preclinical, translational step towards clinical practice. PET is a functional and molecular imaging method, permitting repeated noninvasive determination of several biological and pharmacological processes (Harris et al., 2011). With this technique different organs, including the whole lung, can be visualized *in vivo*. ^{18}FDG PET imaging has been used to determine the metabolic activity of neutrophils in the lung and to clarify the relationship between activation and migration of neutrophils in obstructive lung diseases like bronchiectasis and in inflammatory lung diseases like pneumonia (Chen and Schuster, 2004).

Employing these techniques, this is the first study to indicate that the low molecular weight antifungal protein PAF has no toxic effects *in vivo*. Although further investigations should be carried out *in vivo*, including safety and efficacy studies, our results support the possible therapeutic application of PAF against aspergillosis, and fungal infections of the skin.

Part of this work has been presented to the Hungarian Physiological Society (Palicz et al., 2010).

Materials and methods

Purification of PAF. Purification of PAF was carried out as described previously (Szappanos et al., 2005). Briefly, *P. chrysogenum* NCAIM 00237 was grown in a sucrose (20 $g \cdot l^{-1}$)–$NaNO_3$ (3 $g \cdot l^{-1}$) minimal medium for 72 h at 25 °C with shaking (Marx et al., 1995). Mycelia were removed with centrifugation and the low molecular weight protein fraction of the supernatant was separated in Amicon Stirred Cells (Millipore, Billerica, MA, USA). PAF was purified with ion exchange chromatography on a CM Sephadex Fast Flow column (Amersham-Pharmacia, Uppsala, Sweden). The purity of the protein was tested on SDS-PAGE and by Western blotting (see Supplementary Fig. 1). PAF was dissolved in physiological saline for the *in vivo* experiments.

Animal care. Animal experiments conformed with the guidelines of the European Community (86/609/EEC). The experimental protocol was approved by the institutional Animal Care Committee of the University of Debrecen. The mice were housed in plastic cages with mesh covers, and fed with pelleted mouse chow and water *ad libitum*. Room illumination was an automated cycle of 12 h light and 12 h dark, and room temperature was maintained within the range of 22–25 °C.

Administration of PAF. 12 week old C57/Bl6 mice of both sexes were used in all *in vivo* experiments. First the mice were randomized into six groups (a control group and mice treated with 5, 25, 125, 250 and 2700 $\mu g \cdot kg^{-1}$ PAF dissolved in sterile physiological (0.9%) saline; 10 animals per group) for the long time experiment and into two groups (control group and mice treated with 2700 $\mu g \cdot kg^{-1}$ PAF; 5 animals per group) for the short time experiment. In the latter case the animals were treated daily for 2 weeks. To investigate the long