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Agents that activate the High Osmolarity Glycerol pathway as a means to combat pathogenic molds

Annegret Wiedemann[a,1], Anja Spadinger[a,1], Axel Löwe[b], Allison Seeger[b], Frank Ebel[a,b,c,*]

[a] Institute for Infectious Diseases and Zoonoses, Ludwig-Maximilians-University, Munich, Germany
[b] Max-von-Pettenkofer-Institute, Ludwig-Maximilians-University, Munich, Germany
[c] German Center for Infection Research (DZIF), Munich, Germany

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ABSTRACT

Treatment of invasive fungal infections often fails due to the limited number of therapeutic options. In this study, we have analyzed the impact of agents activating the High Osmolarity Glycerol (HOG) pathway on molds that cause infections in humans and livestock. We found that agents like fludioxonil and iprodione, have a clear anti-fungal activity against pathogenic Aspergillus, Lichtheimia, Rhizopus and Scedosporium species. Only *A. terreus* turned out to be resistant to fludioxonil, even though it is sensitive to iprodione and able to adapt to hyperosmotic conditions. Moreover, the *A. terreus tcs*C gene can fully complement an *A. fumigatus* Δ*tcs*C mutant, thereby also restoring its sensitivity to fludioxonil. The particular phenotype of *A. terreus* is therefore likely to be independent of its TcsC kinase. In a second part of this study, we further explored the impact of fludioxonil using *A. fumigatus* as a model organism. When applied in concentrations of 1–2 μg/ml, fludioxonil causes an immediate growth arrest and, after longer exposure, a quantitative killing. Hyphae respond to fludioxonil by the formation of new septa and closure of nearly all septal pores. Mitosis occurs in all compartments and is accompanied by a re-localization of the NimA kinase to the cytoplasm. In the swollen compartments, the massive extension of the cell wall triggers a substantial reorganization resulting in an enhanced incorporation of chitin and, most strikingly, a massive loss of galactomannan. Hence, HOG-activating agents have dramatic cell biological consequences and may represent a valuable, future element in the armory that can be used to combat mold infections.

1. Introduction

Invasive fungal infections represent a major threat for severely immunocompromised patients and are often associated with high mortality rates. Currently, *Aspergillus fumigatus* is the most common and important pathogenic mold, but infections by non-Aspergillus species are also increasing (Douglas et al., 2016) and some of these pathogens are even more difficult to treat than Aspergillus infections (Cortez et al., 2008). The spectrum of antimycotic agents that are in clinical use for invasive mycoses is small and consists essentially of polyenes, azoles and echinocandines. Azoles are most widely used, but the development of resistance to some of these drugs has been reported for *A. fumigatus* (Azevedo et al., 2015), which underlines the need to develop new approaches in anti-fungal therapy.

Anti-fungal agents are not only used in human and veterinary medicine, but to a much larger extent in agriculture. Some of the agents used in the field attack fungi by harnessing the so-called High Osmolarity Glycerol (HOG) pathway (Kojima et al., 2004). This signalling cascade normally enables fungi to thrive under hyperosmotic conditions (Bahn, 2008). However, a pharmacological activation of the HOG pathway, e.g. by fludioxonil, causes a dramatic osmotic imbalance and consequently a massive influx of water (Okada et al., 2005). The use of HOG-activating agents therefore requires a humid environment, which limits their use in agriculture.

In bacteria, sensing and processing of stress signals largely relies on two-component systems that consist of a sensor histidine kinase and a response regulator. In filamentous fungi, hybrid histidine kinases (HHK) integrate both functions in a single polypeptide and

* Corresponding author at: Institute for Infectious Diseases and Zoonoses, Ludwig-Maximilians-University, Munich, Germany.
E-mail address: frank.ebel@lmu.de (F. Ebel).
[1] Both authors contributed equally.

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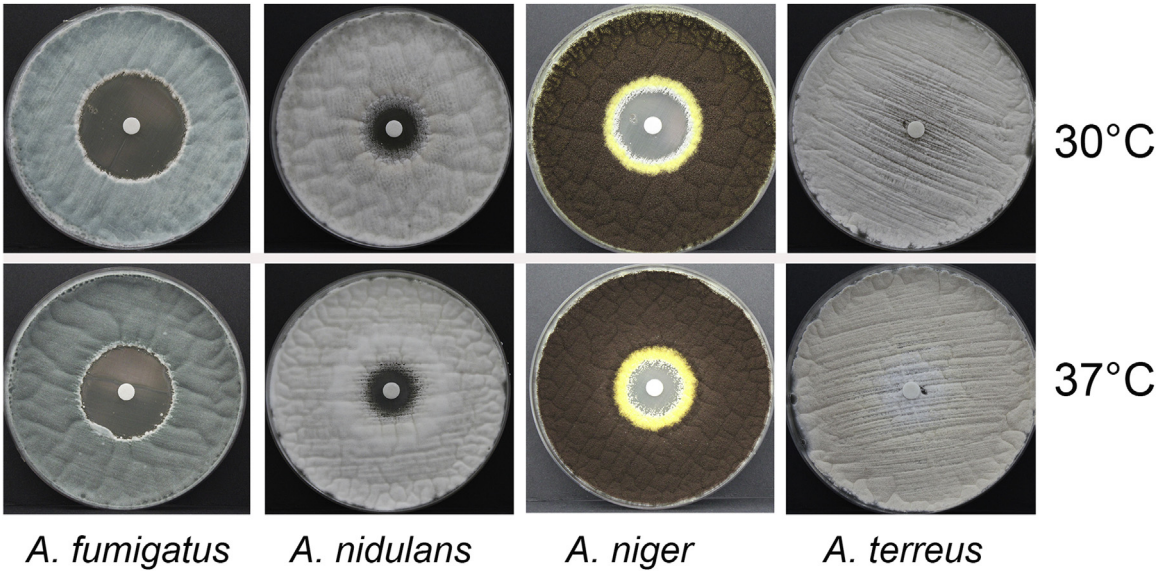

Fig. 1. Impact of fludioxonil on the growth of different Aspergillus species. The following strains have been analyzed in this agar diffusion assay: *A. fumigatus* D141, *A. nidulans* A26, *A. niger* DSM737 and *A. terreus* NIH 2624. 10^6 conidia of each strain were homogenously spread on the surface of two Sabouraud plates. Paper disks containing 10 μg fludioxonil were placed in the centre of the plates. Pictures show plates after incubation for 2 days at either 30 °C or 37 °C.

transfer the phosphoryl group intramolecularly from a histidine to an aspartate residue (Shor and Chauhan, 2015). While their primary function is adaptation to high osmotic stress, group III HHK have also been shown to contribute to the virulence of several fungi (Bahn, 2008; Defosse et al., 2015). The sensing module of group III HHK consists of several HAMP domains (present in Histidine kinases, Adenyl cyclases, Methyl-accepting proteins and Phosphatases), which control the activity of the C-terminal kinase module (Defosse et al., 2015). Remarkably, group III HHK are not found in vertebrates (Shor and Chauhan, 2015), which makes them an attractive therapeutic target.

We have recently shown that the dramatic impact of HOG-activating agents, like fludioxonil, on *A. fumigatus* is mediated by the type III HHK TcsC (McCormick et al., 2012). In the current study we have investigated the impact of fludioxonil on *A. fumigatus* in more detail and furthermore analyzed the impact of the HOG-activating agents fludioxonil and iprodione on other major pathogenic molds.

2. Result

Fludioxonil inhibits growth of *A. nidulans* and *A. fumigatus* (Hagiwara et al., 2007; McCormick et al., 2012). Using a disk-diffusion assay we confirmed this and observed similar effects for *A. niger* and *A. flavus* (Fig. 1 and data not shown). *A. fumigatus* showed the highest sensitivity, whereas *A. terreus* strain NIH2624 was not inhibited at all (Fig. 1). Further testing of NIH2624 and two additional *A. terreus* strains on plates with paper disks containing 1, 10 and 100 μg fludioxonil revealed that *A. terreus* has a natural resistance to this agent (Suppl. Fig. S1A–C in the online version at DOI: http://dx.doi.org/10.1016/j.ijmm.2016.09.005). Nevertheless, we observed a partial growth inhibition around fludioxonil-containing disks, but only at early time points, when the mycelial growth was still sparse (Suppl. Fig. S1D in the online version at DOI: http://dx.doi.org/10.1016/j.ijmm.2016.09.005).

Fludioxonil damages susceptible fungi by activation of a type III HHK. In *A. fumigatus* this kinase is called TcsC (Afu2g03560) and contains 6 HAMP domains. The TcsC homolog of *A. terreus* in the data base (Q0C9V0/ATEG_09534) contains only four HAMP domains and lacks the C-terminal receiver domain, which represents an essential element of type III HHKs (Suppl. Fig. S2 in the online version at DOI: http://dx.doi.org/10.1016/j.ijmm.2016.09.005). This truncation could explain the striking fludioxonil resistance of *A. terreus*, but should also result in an inability to cope with hyperosmotic stress. However, *A. terreus* grows normally on plates containing 1.2 M sorbitol (Suppl. Fig. S3 in the online version at DOI: http://dx.doi.org/10.1016/j.ijmm.2016.09.005) and in liquid culture it was able to tolerate even higher NaCl concentrations than *A. fumigatus* (2.5 M versus 2 M, data not shown). Even more striking, *A. terreus* is clearly sensitive to iprodione (Fig. 2A) and when treated with iprodione shows the characteristic phenotypes induced by HOG-activating agents, such as cellular swelling, elevated numbers of nuclei and tip lysis (Fig. 2B and D). This suggested that *A. terreus* possesses a functional TcsC protein. A closer inspection of the *A. terreus tcs*C gene in the data base (supercontig:CADRE:1.15:139557:141148:1) revealed that it is incomplete and contains a stretch of nucleotides that has not been defined yet. This prompted us to re-sequence the *tcs*C gene of strain NIH 2624 (accession number: HE995413). It turned out that *A. terreus* TcsC is highly homologous to *A. fumigatus* TcsC (91.2% identity) and contains all domains typically found in a type III HHK (Suppl. Fig. S2 in the online version at DOI: http://dx.doi.org/10.1016/j.ijmm.2016.09.005). When expressed in an *A. fumigatus* Δ*tcs*C mutant, *A. terreus tcs*C allowed growth under hyperosmotic conditions and conferred sensitivity for fludioxonil and iprodione (Fig. 3), i.e. it fully complemented the mutant phenotype.

The sensitivity of different *Aspergillus* species to fludioxonil and iprodione prompted us to extend our analysis to other pathogenic molds. Disk diffusion assays revealed that growth of the *Mucorales* species *Lichtheimia corymbifera* and *Rhizopus oryzae* is inhibited by both agents (Fig. 4). *Scedosporium minutisporum* is also sensitive to both agents, but the inhibition zone observed for fludioxonil was strikingly larger than for any other mold tested (Fig. 4). This high sensitivity to fludioxonil was also observed for *S. prolificans, S. aurantiacum* and *Pseudallescheria boydii* (data not shown).

We next searched the genomes of all species tested in Figs. 1 and 4 for TcsC homologs using BlastP. In case that a genome sequence was not available, we analyzed a closely related species. All these genomes contained one protein with high homology to TcsC (Table 1). The most striking differences were observed for the