PH.D. THESIS

INVESTIGATION OF THE DEVELOPMENT AND CONSEQUENCES OF ANTIFUNGAL RESISTANCE IN THE HUMAN PATHOGEN *CANDIDA AURIS*

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Introduction

Opportunistic infections caused by pathogenic fungi pose an increasing health risk globally. Although the majority of fungal infections affect the body surface, the colonization of these areas significantly increases the risk of developing invasive infections. Microorganisms can enter the bloodstream, leading to the formation of systemic infections. These deep seeded diseases are hard to eliminate since in the clinical setting only a limited number of antifungal agents are available for treatment.

A significant number of fatal infections associated with fungi is attributed to pathogens belonging to the *Candida* genus. While *Candida albicans* remains the most prominent member of the genus, recent epidemiological studies indicate an increase in the presence of non-*albicans* species. One of the most notable emerging species is *Candida auris* was first isolated in 2009. Apart from spreading predominantly in hospital environments and endangering already weakened patient populations, *C. auris* efficiently develops resistance to commonly used antifungal agents. Due to these characteristics, infections caused by this pathogen have a mortality rate ranging from 30% to 70%.

Approximately 90% of identified *C. auris* isolates show resistance to azoles, while around 30% are resistant to

amphotericin-B and 5% to echinocandins. Within the *Candida* genus, the proportion of isolates resistant to multiple antifungal drug classes (multi-drug resistant) is higher than the average. Some strains exhibit resistance to all antifungal drug class, rendering them pan-drug resistant. The high number of isolates resistant to therapy suggests that this pathogen possesses characteristics that facilitate the development of acquired antifungal resistance, independent of the mechanism of action of antifungal agents, without significantly reducing the viability of fungal cells.

In our study, our aim was to investigate the mechanisms how *C. auris* is able to develop resistance to antifungal therapy and to determine the correlation between acquired resistance and changes in phenotype, genotype, and virulence.

Methods

Cultivation of Cells Used in Experiments

Maintenance and cultivation of yeast cells, cultivation of J774.2 cell line, *in vitro* microevolution procedure

Methods Applied in the Characterization of Created Strains

Determination of antifungal susceptibility using with microdilution method, growth tests in liquid medium, investigation of abiotic stress tolerance on solid media, determination of cell wall composition through fluorescence microscopy and flow cytometry, examination of efflux activity with fluorescent labeling, XTT reduction assay for studying biofilm formation, *in silico* analysis of whole-genome sequencing, *in vitro* phagocytosis assay

Molecular Techniques

Isolation of DNA and RNA from yeast cells, cDNA synthesis, PCR, gel electrophoresis, RT-qPCR

Methods Used in In vivo Studies

Intravenous infection in a mouse model of systemic *candidiasis*, determination of organ colonization

Analytical Methods (in collaboration)

Determination of sterol composition using LC-MS method, metabolomic studies (GC-MS), lipidomic analysis (UHPLC-HRMS)

Results

Generation of Resistant *C. auris* Strains

Resistant strains against all three antifungal agent groups (polyenes, azoles, echinocandins) were generated through *in vitro* microevolution method. For this, two clinically sensitive *C. auris* isolates (0381, 0387) were selected. These strains were cultured in media containing increasing concentrations of each antifungal agent, creating the necessary selection pressure for the appearance of resistant cells. Using this method, we generated strains resistant to amphotericin B from the polyene group, to fluconazole, posaconazole, and voriconazole from the triazole group, and to micafungin from the echinocandin group. After reaching the predetermined final concentration of the antifungal agents, the generated strains were grown without selective pressure, ensuring the stabilization of the resistant phenotype.

Characterization of the Amphotericin B Resistant Strains

In the amphotericin B evolved strains, no stable resistance was developed. The 0381 AMB^{evo} strain exhibited antifungal susceptibility similar to the initial sensitive isolate, while the 0387 AMB^{evo} strain showed cross-resistance to fluconazole and voriconazole. Compared to the initial isolate

the latter strain demonstrated decreased growth capability in complex medium, and increased sensitivity to cell wall stress-inducing agent, caffeine and membrane detergent SDS. In *in vivo* experiments, the created strains showed virulence similar to the initial clinical isolate, as the 0381 AMB^{evo} strain displayed no significant changes in pathogenic potential, and the 0387 AMB^{evo} strain colonized the kidneys and heart of the animals slightly less effectively.

Characterization of Triazole Resistant Strains

Strains selected in the presence of triazoles became resistant to the respective antifungal agents. Additionally, the 0381 FLU^{evo} strain showed cross-resistance to voriconazole, while the 0381 VOR^{evo} strain exhibited resistance to fluconazole. The 0381 POS^{evo} strain lost sensitivity to all three triazoles. Similar results were observed for the 0387 FLU^{evo}, POS^{evo}, and VOR^{evo} strains, with the added cross resistance to caspofungin, indicating multi-drug resistance. The triazole-resistant strains derived from the 0387 clinical isolate displayed increased division rates, although they achieved the maximum cell concentrations at lower cell counts.

The 0381 POS^{evo} strain showed sensitivity to cell wall-damaging agents and the membrane detergent SDS. The 0387 POS^{evo} and VOR^{evo} strains were also sensitive to the membrane-

damaging agent, and all three strains derived from the 0387 clinical isolate consistently showed tolerance to caffeine. Strains sensitive to membrane stress exhibited reduced ergosterol concentrations, and in these strains, we detected a sterol derivative, portensterol. A sterol product previously unkonown in *Candida* species. Following to triazole treatment decrease in ergosterol content occurred in each strain. In the evolved strains derived from the 0381 isolate and in 0387 FLU^{evo}, an alternative branch of the ergosterol biosynthesis pathway activated, resulting in the appearance of the toxic byproduct 14-Me-ergostadien-diol. Furthermore, these strains contained a yet unclassified sterol derivative, stigmasterol.

Examination of the surface exposition of individual cell wall components indicated increased levels of mannan and β-glucan in the 0387 POS^{evo} and VOR^{evo} strains. Analysis of efflux processes in evolved strains suggested that in 0381 FLU^{evo} and VOR^{evo} strains the ABC transporter Cdr1 is involved in the antifungal resistance, while in 0387 derived triazole-evolved strains MFS transporters such as Mdr1 and Tpo3 are participating in the detoxification process. The altered efflux processes in the 0381 strains are likely due to point mutations in the *TAC1b* gene.

Whole-genome sequencing revealed that the 0387 triazole-evolved strains carried a point mutation in the *BCY1*

gene, that encodes the regulator protein of the cAMP-dependent protein kinase A (PKA) signaling pathway. Compared to the initial susceptible isolate these strains exhibited differences in biofilm-forming ability and rapamycin sensitivity, suggesting that *BCY1* may play an indirect role in antifungal resistance. Additionally, the 0387 POS^{evo} strain showed a point mutation in the *ERG3* gene that encodes a key enzyme of the sterol biosynthesis pathway.

In vivo studies with triazole-evolved strains indicated that the 0381 derived resistant strains were more efficient in colonizing the liver and brain of the experimental animals, while uniform virulence attenuation was observed in the 0387 triazole-evolved strains.

Characterization of Micafungin Resistant Strains

The 0381 MICA^{evo} strain exhibited resistance to all three tested echinocandins (anidulafungin, caspofungin, micafungin). In addition to general echinocandin resistance the 0387 MICA^{evo} strain, showed cross-resistance to triazoles and increased MIC values of amphotericin B. Despite extensive antifungal resistance, the 0387 MICA^{evo} strain did not show significant sensitivity to tested abiotic stressors, while the 0381 MICA^{evo} strain was more susceptible to the cell wall-damaging

agents calcofluor white and congo red compared to the initial isolate.

Flow cytometric analysis indicated increased surface presence of mannans in both micafungin-selected strains, while the ratio of β-glucans in the two evolved strains changed in the opposite manner.

Genome analysis of both micafungin evolved strains, showed point mutations in the *FKS1* gene. This gene encodes the target enzyme of echinocandins, 1,3-β-D-glucan synthase, therefore it directly contributes to antifungal resistance. Besides this classic echinocandin resistance mechanism, numerous additional genomic modifications were also identified. In the 0387 MICA^{evo} strain, a mutation in the *ERG3* gene was found, that possibly contributed to the described triazole resistance. Additionally, amino acid change was found in the *BCY1* gene, which encodes the regulator of the cAMP-dependent protein kinase A (PKA) pathway, and also in the Tpk2-encoding gene responsible for the catalytic functions of the same pathway.

Despite extensive resistance profile, neither micafungin-selected strain showed attenuated virulence in intravenous mouse model. The 0381 MICA^{evo} strain was significantly more efficient in colonizing the kidney, brain, and heart of the animals, while the 0387 MICA^{evo} strain exhibited significantly higher CFU values in the brain and heart. Due to

the high level of brain tissue colonization, we examined the pathogenic potential of the evolved strains over a long-term infection (7 days, 15 days, and 30 days). The detected CFU values indicated a gradual clearance of the examined organs. However, in mice infected with the 0387 MICA^{evo} strain, we identified a stable persisting population of fungal cells in the heart of the animals.

Summary

1. From two antifungal susceptible *C. auris* isolates we generated evolved strains resistant to amphotericin B, fluconazole, posaconazole, voriconazole, and micafungin using *in vitro* microevolution method.
2. We characterized the overall growth capabilities, stress tolerance, and cell wall composition of the generated strains. Therefore, we identified correlations between acquired antifungal resistance and the viability of the fungal cells in several conditions.
3. We analyzed the sterol composition of the triazole-evolved strains and identified the presence of sterol derivatives previously unplaced on the *Candida* ergosterol biosynthetic pathway.
4. We examined the genomic, metabolomic, and lipidomic features of certain generated strains. Numerous genomic changes were found that play a direct (*ERG3*, *TAC1b*, *FKS1*) or indirect (*BCY1*, *TPK2*) role in antifungal resistance.
5. *In vivo* experiments suggested that in the case of *C. auris*, the development of resistance does not always result in a decreased virulence despite fitness-loss.

List of publications

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Bohner, F., Papp, C., Takacs, T., Varga, M., Szekeres, A., Nosanchuk, J. D., Vágvölgyi, C., Tóth, R., & Gacser, A. (2023). Acquired Triazole Resistance Alters Pathogenicity-Associated Features in *Candida auris* in an Isolate-Dependent Manner. *Journal of fungi* **IF (2023): 4,7**

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