Every single conidium in *Aspergillus fumigatus* caspofungin tolerant strains are intrinsically caspofungin tolerant

Clara Valero¹, Ana Cristina Colabardini¹, Patrícia Alves de Castro¹, Jorge Amich²,³, Michael J. Bromley³,⁴ and Gustavo H. Goldman¹

¹Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brazil

²Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III (ISCIII), Majadahonda, Madrid, Spain.

³Manchester Fungal Infection Group, Division of Evolution, Infection, and Genomics, Faculty of Biology, Medicine and Health, University of Manchester, , Manchester, United Kingdom.

⁴Antimicrobial Resistance Network, University of Manchester, Manchester, United Kingdom.

*Corresponding author:

Dr. Gustavo Henrique Goldman

Faculdade de Ciências Farmacêuticas de Ribeirão Preto

Universidade de São Paulo

Av. do Café S/N  CEP 14040-903

Ribeirão Preto, São Paulo, Brazil

e-mail: ggoldman@usp.br

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Abstract

*Aspergillus fumigatus* is a human fungal pathogen that causes a disease named aspergillosis. Echinocandins, such as the fungistatic drug caspofungin (CAS) are used as second-line therapy. Some *A. fumigatus* clinical isolates can survive and grow in higher CAS concentrations, a phenomenon known as “caspofungin paradoxical effect” (CPE). Here we investigate if CPE is due to a subpopulation of conidia produced by a CAS tolerant strain, indicative of a persistence phenotype or is caused by all the conidia which would be consistent with a tolerance phenotype. We evaluated 67 *A. fumigatus* clinical isolates for CPE growth and used a novel CPE Index (CPEI) classified them as CPE⁺ (CPEI ≥ 0.40) or CPE⁻ (CPEI ≤ 0.20). Conidia produced by three CPE⁺ clinical isolates, CEA17 (CPEI=0.52), Af293 (CPEI=0.64), CM7555 (CPEI=0.58) all showed the ability to grow in high levels of CAS while all conidia produced by the CPE⁻ isolate IFM61407 (CPEI=0.12) strain showed no evidence of tolerance. Given the importance of calcium/calcineurin/transcription factor CrzA pathway in CPE regulation, we also evaluated Δ*crzA*Af293 (CPE⁻) and Δ*crzA*CEA17 (CPE⁺) conidia tolerance to CAS. All Δ*crzA*CEA17 conidia showed CPE⁺ while 100 % of Δ*crzA*Af293 spores are CPE⁻. As all spores derived from an individual strain are phenotypically indistinct with respect to CPE it is likely that CPE is a genetically encoded adaptive trait that should be considered an antifungal tolerant phenotype. As the CPEI shows that the strength of the CPE is not uniform between strains we propose that the mechanisms that govern this phenomenon are multi-factorial.
Importance

*Aspergillus fumigatus* is the most important human fungal pathogen causing pulmonary infections. Caspofungin (CAS) is a fungistatic drug used as a second-line therapy against aspergillosis, the group of diseases caused by *A. fumigatus*. CAS inhibits the function of Fks1 a β-1,3-glucan synthase that has a critical role in the synthesis of the cell wall. Resistance to CAS is commonly associated with mutations in *fks1*, however, some *A. fumigatus* clinical isolates are able to grow in the presence of higher CAS concentrations, a drug tolerance phenomenon known as the “caspofungin paradoxical effect” (CPE). Here, based on the characterization of CPE presence in a series of *A. fumigatus* clinical isolates, we demonstrate that *A. fumigatus* CAS tolerant strains do not exhibit a CAS heterogeneous response. Our results indicate that every single conidium in *A. fumigatus* CAS tolerant strains are intrinsically CAS tolerant.
Observation

Fungal diseases are a significant health problem affecting more than 1 billion people around the world and causing more than 1.5 million deaths (1). *Aspergillus fumigatus* is the most important agent of fungal pulmonary infection causing a group of heterogeneous clinical diseases named aspergillosis (2). Few antifungal agents, such as the fungicidal azoles (first-line therapy, itraconazole, posaconazole, voriconazole, and isavuconazole), amphotericin B, and the fungistatic echinocandins (caspofungin, CAS, second-line therapy) are available to treat aspergillosis while worryingly clinical azole resistance has been increasingly reported (3-5). While azoles inhibit the ergosterol biosynthesis pathway by directly targeting the eburicol 14-demethylase Cyp51A/ERG11 (6), CAS acts by noncompetitively inhibiting the fungal β-1,3-glucan synthase (Fks1), which is essential for the biosynthesis of β-1,3-glucan in the fungal cell wall (7). In patients suffering from invasive aspergillosis, strains resistant to the azoles have often been shown to be acquired from the environment however in those suffering from chronic forms of aspergillosis resistance typically, and frequently occurs during the course of infection (8). CAS resistance has been increasingly observed in *Candida* spp. (9), and although infrequently described, there are reports of *A. fumigatus* CAS resistance from patients with chronic aspergillosis (10). Mutations in specific “hotspots” of the FKS1 gene are the main genetic mechanisms of CAS resistance described in both *Candida* spp. and *Aspergillus* spp. (10, 11).

Drug tolerance has been extensively studied in bacterial pathogens, where it has been defined as the ability of all cells of an isogenic strain to survive and even grow at slow rates in the presence of drug concentrations that are greater than the minimal inhibitory concentration (MIC) (12). The term persistance is a type of tolerance that describes a phenomenon where only a sub-population of cells within an isogenic strain are tolerant. To date, the description of tolerance in fungi has focused almost exclusively on yeast-like fungi where tolerance is frequently observed as occurring in sub-populations within an isogenic strain (13-15). Very little attention has been given to defining
drug tolerance in filamentous fungi however one adaptive phenomenon exhibited by some A. fumigatus clinical isolates allows them to grow and tolerate high CAS concentrations and is known as the "caspofungin paradoxical effect" (CPE). Although there are several mechanisms already described for A. fumigatus CPE (for reviews, see 16, 17), there is a little understanding if CPE occurs as a result of phenotypic heterogeneity within an isogenic population. Here, based on the characterization of CPE presence in a series of A. fumigatus clinical isolates, we demonstrate that conidia from A. fumigatus CAS tolerant strains do not exhibit CAS heterogeneity and hence CPE should be considered as a tolerant but not persistant phenotype.

We have investigated the CPE in 67 A. fumigatus clinical isolates (Supplementary Tables S1 and S2 at 10.6084/m9.figshare.1917888; S. Zhao, A. Martin-Vicente, A. C. Colabardini, L. P. Silva, J. R. Fortwendel, G. H. Goldman, and J. G. Gibbons, submitted for publication) and defined a CPE index (CPEI) = average radial diameter in MM+8 µg/ml CAS/average radial diameter in MM, where CPEI ≥ 0.4 are CPE+, CPEI ≥ 0.25 and ≤ 0.4 have an intermediate (partial) phenotype (CPEP), and CPEI ≤ 0.2 are CPE-.

Figure 1A shows the heat map representing the CPEI of 67 A. fumigatus clinical isolates grown for 5 days at 37 °C on MM+0.125 to 8 µg/ml of CAS. Radial growth in the presence of CAS is exemplified for three clinical isolates CEA17/A1163 (CPEI=0.52), CM7555 (CPEI=0.58) and IFM61407 (CPEI=0.12) (Figure 1B). It should be noted that the CPEI cannot be assessed for strains that are resistant to CAS using the drug concentrations outlined here however by adjusting the CAS concentrations the CPEI can still be calculated.

A. fumigatus sexual and asexual spores are the single developmental “cell-like”-structures with a single nucleus in the fungus. Germlings or mycelia are syncytia with several nuclei present in a common cytoplasm. Is CAS tolerance present in a “fraction” of the conidial population or in every single conidium in a single CAS-tolerant CPE+ clinical isolate? In order to address this question, we have grown two A. fumigatus reference isolates CEA17 (CPEI=0.52) and Af293 (CPE=0.64) in MM in the presence or absence of CAS 8µg/ml for 5 days at 37 °C. Then, conidia were harvested, filtered, diluted to 10³ conidia/ml and 100 µl were plated them on MM (control) or MM+8 µg/ml (a CPE

5
concentration) (Figure 1D). After 48 hs (MM) or 72 hs (MM+CAS) of growth at 37 °C, the number of colonies ≥ 0.5 cm radial diameter were counted (Figure 1C), and the CAS growth index, CGI (%) = (number of colonies radial diameter ≥0.5 cm on MM+8 µg/ml CAS/number of colonies radial diameter ≥0.5 cm on MM) x 100, was determined (Figure 1C). When CEA17, Af293, and CM7555 clinical isolates were grown on either MM or MM+8.0 µg/ml, we observed a CGI of 100 % (Figure 2A, Supplementary Table S3 at 10.6084/m9.figshare.19178888). We did not observe radial diameter size heterogeneity in any of the colonies grown on MM or MM+8 µg/ml (all c. 0.5 cm radial diameter; Figure 2A). These results indicate that every single conidium in A. fumigatus CPE+ strains are intrinsically CAS tolerant. We then evaluated the CGI for the clinical isolate IFM61407 strain that is CPE− (Figures 1C). IFM61407 conidia derived from MM or MM+8.0 µg/ml CAS show a CGI of 0 %, respectively (Figure 2A, Supplementary Table S3 at 10.6084/m9.figshare.19178888).

Calcium homeostasis has been reported to play a central role in the CPE cellular response in A. fumigatus (18, 19, Figure 2B). CAS increases the intracellular calcium (Ca²⁺) concentration activating the calcineurin-CrzA pathway (20). CrzA regulates the activation of several stress responses and cell wall modifications (21, 19). Interestingly, crzA deletion in the clinical strain Af293 results in CPE loss (22, Figure 2C), while crzA deletion in the CEA17 background is CPE+ (23, 19, Figure 2C), demonstrating intra-species differences or CPE heterogeneity. Differently from ∆crzA<sup>C</sup>CEA17 mutant, the ∆crzA<sup>A</sup>Af293 mutant cannot activate cell wall remodeling genes upon CAS exposure, affecting its CPE (23). The CGIs for ∆crzA<sup>C</sup>CEA17 and ∆crzA<sup>A</sup>Af293 mutant strains are 100 and 0 %, respectively, when grown on MM+8 µg/ml independently if the conidia are derived from MM or MM+8 µg/ml (Figure 2D, Supplementary Table S3 at 10.6084/m9.figshare.19178888). Taken together these results indicate that the transcription factor CrzA, whose deletion results in heterogeneity in the response of the CEA17 and Af293 strains to CAS does not show CPE heterogeneity since all the conidia from the CPE− ∆crzA<sup>A</sup>Af293 strain do not exhibit CPE, while all the conidia from the CPE+ ∆crzA<sup>C</sup>CEA17 strain are CPE+ (Figure 2D).
Our results emphasize the view that every single conidium in an *A. fumigatus* CPE strain is able to grow and be CAS tolerant. In contrast, conidia from *A. fumigatus* strains lacking CPE have reduced growth in CPE concentrations, strongly indicating that there are no *A. fumigatus* CAS tolerant subpopulations. As a conclusion, *A. fumigatus* CPE is a homogeneous trait within an isogenic population while CPE heterogeneity exists between strains.

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**References**


Figure legends

**Figure 1** – Distribution of *A. fumigatus* CAS tolerance in 67 clinical isolates and definition of CPE index (CPEI) and CAS Growth Index (CGI). (A) Heat map depicting the CPE Index (CPEI) = radial diameter in MM+8 µg/ml CAS/radial diameter in MM, where CPEI ≥ 0.4 are CPE⁺, CPEI ≥ 0.25 and ≤ 0.4 are partially CPE⁺ (CPEP), and CPEI ≤ 0.2 are CPE⁻. Heat map scale and gene identities are indicated. Hierarchical clustering was performed in MeV (http://mev.tm4.org/), using Pearson correlation with complete linkage clustering. (B) Growth of *A. fumigatus* CEA17, CM7555, and IFM61407 clinical isolates on MM and MM+CAS (increasing concentrations). The strains were grown for 5 days at 37 °C. (C) Scheme showing how the CAS Growth Index (CGI) was calculated. *A. fumigatus* isolates were grown on MM or MM+8 µg/ml for 5 days at 37 °C. Conidia were harvested, filtered, diluted to 10³ sp/ml and 100 µl were plated in MM or MM+8 µg/ml and incubated for 2 or 3 days at 37 °C. The number of colonies were counted in both treatments and determined the CGI as follows: CGI (%) = (number of colonies radial diameter ≥0.5 cm on MM+8 µg/ml CAS/number of colonies radial diameter ≥0.5 cm MM) x 100.

**Figure 2** – CAS growth index (CGI) for *A. fumigatus* clinical isolates. (A) CEA17, Af293, CM7555, and IFM61407 clinical isolates were grown on MM and MM+8 µg/ml of CAS for 5 days at 37 °C. Conidia were harvested, filtered, diluted to 10³ sp/ml and 100 µl were plated in MM or MM+8 µg/ml and incubated for 2 or 3 days at 37 °C. The number of colonies were counted in both treatments and the CGI was determined. (B) Scheme showing the calcium/calcineurin/CrzA pathway. Upon CAS cell wall damage, calcium concentrations increase in the cytoplasm by calcium transport or mobilization of endogenous calcium deposits. Calcium binds to calmodulin, which activates calcineurin that directly dephosphorylates CrzA, resulting in its translocation to the nucleus. CrzA binds to calcineurin-dependent response elements (CDRE) promoters, activating the transcriptional programs that promote stress tolerance. (C) CEA17, Af293, Δ*crzA*<sup>CEA17</sup>, and Δ*crzA*<sup>Af293</sup> strains were grown on
MM and MM+CAS for 5 days at 37 °C. (D) \( \Delta \text{crzA}^{\text{CEA17}} \) and \( \Delta \text{crzA}^{\text{Af293}} \) strains were grown on MM and MM+CAS for 5 days at 37 °C. Conidia were harvested, filtered, diluted to \( 10^3 \) sp/ml and 100 µl were plated in MM or MM+8 µg/ml and incubated for 2 or 3 days at 37 °C. The number of colonies were counted in both treatments and the CGI was determined.
**Figure A.** Heatmap showing the expression levels of genes with varying concentrations of CAS (Caspofungin) in µg/ml.

**Figure B.** Comparison of growth inhibition (CPEI) of different fungal strains with varying concentrations of Caspofungin. CPEI values for:
- CEA17: 0.52
- CM7555: 0.58
- IFM61407: 0.12

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**Figure C.** Diagram illustrating the process of counting colony forming units (CFUs) and calculating the Caspofungin Growth Index (CGI) ranging from 0 to 100%.