SHORT COMMUNICATION

Environmental azole fungicide, prochloraz, can induce cross-resistance to medical triazoles in *Candida glabrata*

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Abstract

Acquisition of azole resistance by clinically relevant yeasts in nature may result in a significant, yet undetermined, impact in human health. The main goal of this study was to assess the development of cross-resistance between agricultural and clinical azoles by *Candida* spp. An *in vitro* induction assay was performed, for a period of 90 days, with prochloraz (PCZ) – an agricultural antifungal. Afterward, the induced molecular resistance mechanisms were unveiled. MIC value of PCZ increased significantly in all *Candida* spp. isolates. However, only *C. glabrata* developed cross-resistance to fluconazole and posaconazole. The increased MIC values were stable. *Candida glabrata* azole resistance acquisition triggered by PCZ exposure involved the upregulation of the ATP binding cassette multidrug transporter genes and the transcription factor, *PDR1*. Single mutation previously implicated in azole resistance was found in *PDR1* while *ERG11* showed several synonymous single nucleotide polymorphisms. These results might explain why *C. glabrata* is so commonly less susceptible to clinical azoles, suggesting that its exposure to agricultural azole antifungals may be associated to the emergence of cross-resistance. Such studies forward potential explanations for the worldwide increasing clinical prevalence of *C. glabrata* and the associated worse prognosis of an infection by this species.

Although *Candida albicans* is still the most frequently isolated *Candida* species, *C. glabrata* and *C. parapsilosis* have emerged as the second or third most common agent of invasive candidosis, depending on the region (Pfaller & Diekema, 2007; Costa-de-Oliveira *et al.*, 2008; Tortorano *et al.*, 2013).

In an attempt to understand the growing clinical relevance of *Candida* species, several facts were considered. *Candida* species are human commensals, but they are also ubiquitous in the environment (Odds, 1988); antifungal agents used for crop protection of the azole class, such as prochloraz (PCZ), very similar to those used in human therapy and are extensively used in agriculture within the EU (Hof, 2001). Such long antimicrobial pressure is recognized to lead to drug resistance. Fungal diseases are problematic for both human health and agriculture, and azole drugs represent the core therapy for both; such circumstance may represent an initial step in the emergence of clinically resistant fungal isolates. Therefore, the purpose of this study was to evaluate the potential development of cross-resistance by the extensive use of azole fungicides in agriculture, similar to azoles used in humans.

A preliminary broth microdilution susceptibility assay was performed in order to evaluate the initial MIC of the isolates to PCZ and to confirm results for all the clinical azoles, according to the Clinical and Laboratory Standards Institute M27-S4 protocol (Clinical & Laboratory

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Standards Institute, 2012). Within the fungal collection of the Microbiology Department of Faculty of Medicine – University of Porto, we found that isolates, irrespectively from their species, resistant to clinical azoles showed also high MIC value to PCZ. Therefore, three clinical isolates each of *C. albicans*, *C. parapsilosis,* and *C. glabrata* were selected based upon their susceptibility profile: susceptible to clinical azoles and low MIC value to PCZ. Strains were kept in a YPD medium broth supplemented with 10% glycerol, stored at −80 °C. Prochloraz (PCZ) was used as a representative of agriculture azoles because its initial minimal inhibitory concentration (MIC) value was the lowest encountered. Among clinical antifungals, we used fluconazole (FLC), voriconazole (VRC), and posaconazole (POS) – clinical azoles – and anidulafungin (AND) as representative of echinocandins. Prochloraz was resuspended in 80% acetone solution at a final concentration of 5 mg L^{-1}. Clinical azoles were dissolved in dimethyl sulphoxide to obtain a stock solution of 10 mg L^{-1}. All drugs were stored at −20 °C until use. Drug concentration ranged from 0.125 to 64 mg L^{-1} of FLC and PCZ and from 0.0313 to 16 mg L^{-1} of POS, VRC, and AND; MIC determination was repeated at least twice.

In vitro induction experiments were performed as described by Borst *et al.* (2005) and Pinto e Silva *et al.* (2009). It was carried out daily for 90 days keeping a constant subinhibitory concentration of PCZ. MIC of PCZ was determined every ten days throughout the 90 days of assay. Whenever a marked MIC increase was observed (fourfold the initial PCZ MIC), the MIC values of clinical antifungals were determined as well.

To assess the stability of the developed elevated MIC, the induced strains were afterward subcultured for an additional ninety days in the absence of antifungal and MIC values re-determined, as previously described in the induction assay.

RNA was extracted as described by Köhrer & Domdey (1991). For each real-time quantitative PCR (RT-qPCR) analysis, three replicates for each species, of the initial and final strain, were included: for *C. albicans CDR1*, *CDR2*, *MDR1,* and *ERG11*; for *C. parapsilosis MRR1*, *MDR1*, *UPC2*, *NDT80*, *ERG6*, and *ERG11*; for *C. glabrata PDR1*, *CDR1*, *PDH1*, *YOR1*, *SNQ2,* and *ERG11* (Kanafani & Perfect, 2008). The signal obtained for each gene was normalized with the *ACT1* for *C. albicans* and *C. glabrata* and with *TUB4* for *C. parapsilosis.*

In vitro induction assays were performed with three isolates of each species with similar results within the same species – one isolate representative of each species was shown in the results. The three species developed a progressive increment of PCZ MIC value in comparison to the initially determined value. After 10 days of induction, all *Candida* species developed a 32–64 times higher PCZ MIC value. In addition, a concomitant increase of the MIC of FLC, VRC, and POS was observed, but only for *C. glabrata*; regarding POS, cross-resistance was well established after 60 days of induction. No cross-resistance was registered regarding AND (Table 1).

Table 1. Susceptibility profile of *Candida* species tested to PCZ, triazoles, and anidulafungin

Species	Time of exposure to PCZ (days)	MIC (mg L^{-1})				
		PCZ	FLC	VRC	POS	AND
C. albicans	0	0.5	0.25	0.03	0.03	0.015
	10	16	0.25	0.06	0.5	0.03
	30	16	0.5	0.06	0.5	0.03
	60	16	0.5	0.06	0.5	0.03
	90	16	1	0.06	0.5	0.03
	Ø90	16	0.5	0.06	0.5	0.03
C. parapsilosis	0	2	0.5	0.03	0.03	1
	10	64	1	0.06	1	1
	30	64	1	0.06	1	1
	60	64	1	0.06	2	2
	90	64	1	0.06	2	2
	Ø90	64	1	0.06	2	2
C. glabrata	0	1	1	0.25	0.03	0.06
	10	64	16	0.5	16	0.125
	30	64	16	0.5	16	0.125
	60	64	32	1	16	0.125
	90	64	64	1	16	0.125
	Ø90	64	64	0.25	16	0.125

PCZ, prochloraz; FLC, fluconazole; VRC, voriconazole; POS, posaconazole; AND, anidulafungin; Ø, MIC after 90 days of culture in the absence of PCZ.

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The *in vitro* developed high MIC values of PCZ for all species were stable in the absence of the inducing antifungal PCZ as well as the cross-resistance observed in *C. glabrata*.

No significant difference was obtained between the initial and final strain, regarding gene expression in *C. albicans* or *C. parapsilosis*. *C. glabrata* was the only species that consistently overexpressed genes previously associated with azole resistance due to upregulation of efflux pumps (Bennett *et al.*, 2004). Meanwhile, *ERG11* was found to be downregulated (0.141-fold and $P = 0.775$). PCZ exposure triggered overexpression of ATP Binding Cassette (ABC) multidrug transporters *PDH1*, *YOR1*, *CDR1,* and *SNQ2*. The first two genes were found to be 48.5-fold ($P < 0.001$) and 66.8-fold ($P < 0.001$) overexpressed, respectively; *CDR1* and *SNQ2* showed an expression level of 2.9-fold ($P = 0.008$) and 1.3-fold ($P = 0.193$), respectively. These multidrug transporters are regulated by *PDR1* encoded transcription factor, which was also found to be overexpressed – 6.8-fold ($P < 0.001$) (Fig. 1). Therefore, both *C. glabrata* transcriptional factor and related efflux genes were upregulated following the *in vitro* induction assay. To determine whether resistance was associated with mutations: *ERG 11* and *PDR1* genes were sequenced for a single *C. glabrata* isolate. DNA products were sequenced in an ABI Prism 3130 genetic analyzer (Applied Biosystems). A G727A point mutation in *PDR1* gene was found, leading to an aspartic acid to asparagine amino acid substitution at codon 243. *ERG11* analysis revealed several synonymous single nucleotide polymorphisms (SNPs) (Supporting information, Fig. S1).

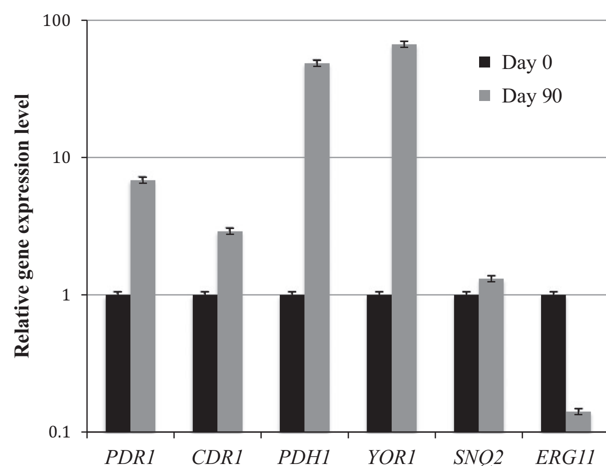

Fig. 1. Gene expression alterations triggered by PCZ exposure in *C. glabrata*. Black bars represent the susceptible initial strain – day 0; gray bars represent the same strain after the induction assay, day 90. Comparative gene expression profile between the initial, day 0, susceptible strain and the strain after the induction assay, day 90. Gene expression is expressed as average with standard deviation of three independent experiments. Each mean value was normalized with the *ACT1* gene.

The concept that the use of azoles in agriculture would not only influence plant pathogenic species but also impair susceptible species of opportunistic human pathogens has gained relevance; such drugs may also have an impact in saprophytic fungal species found in human microbial communities (Snelders *et al.*, 2009, 2012; Verweij *et al.*, 2009; Bowyer & Denning, 2014). In fact, such an imbalance might affect the endogenous population and medically important pathogens. It is generally accepted that a persistent antimicrobial pressure on a complex microbial population will lead to selection of resistant clones. Systemic infections due to *C. glabrata* are characterized by a high mortality rate; they are difficult to treat due to the intrinsically low susceptibility of this species to azole drugs (Pfaller *et al.*, 2003). In addition, *C. glabrata* easily develops fluconazole resistance during patient treatment. In fact, it is now common to find azole-resistant *Candida* isolates from patients not previously exposed to clinical antifungal agents (Pfaller & Diekema, 2004; Pfaller *et al.*, 2004). In our study, in all the three species PCZ MIC value increased from 32- to 64-fold compared to the initial value. However, neither *C. albicans* nor *C. parapsilosis* developed cross-resistance. Anidulafungin activity was not impaired following the selective pressure of an agricultural azole compound, which is not surprising considering that echinocandins have a different mechanism of action. Our results suggest a different perspective on the way *C. glabrata* species develop stable resistance to medical triazoles. Drug efflux, resulting from the increased expression of ABC transporter proteins, is the predominant mechanism by which *C. glabrata* mediates resistance to a wide range of antifungal compounds. Also Pdr1, as the principal regulator of ABC transporter gene expression, has been found to be a key player in such resistance (Bennett *et al.*, 2004; Tsai *et al.*, 2006; Vermitsky *et al.*, 2006; Ferrari *et al.*, 2009). These genetic alterations may transform an intrinsically susceptible to a permanently resistant phenotype. In fact, haploid fungal cells – as is the case of *C. glabrata* – might be more prone to such events (Brockert *et al.*, 2003). We assessed the most common associated genes with azole resistance and found that all ABC transporters were upregulated, as well as their regulatory transcription factor. To our knowledge, this is the first time that *YOR1* was found to have such high expression in a *C. glabrata* azole-resistant strain – it was 66.8-fold overexpressed. Also, previous reports addressing genes involved in azole resistance in *C. glabrata* state that the predominant basis for acquired azole resistance is the constitutively upregulated expression of multidrug transporter genes *CDR1*

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and *PDH1* (Bennett *et al.*, 2004; Sanguinetti *et al.*, 2005; Ferrari *et al.*, 2009). Borst *et al.* (2005) reported a rapid and stable acquisition of azole resistance by *C. glabrata* after an induction assay with FLC; the same ABC transporters were found overexpressed while no contribution of *ERG11* was verified. As previously described, a single-point mutation was found at *PDR1* while *ERG11* only showed the existence of several synonymous SNPs suggesting that this gene was not involved in *C. glabrata* azole resistance in the isolate examined (Sanguinetti *et al.*, 2005; Ferrari *et al.*, 2009) Certainly, additional studies are necessary to address the involvement of such genes in the development of azole cross-resistance triggered by the selective pressure of an agricultural drug.

In conclusion, apart from very few speculative reports published some years ago, there is still no evidence for a clear correlation between the agricultural use of azoles and the increasing clinical azole resistance (Müller *et al.*, 2007; Serfling *et al.*, 2007; Hof, 2008). Nevertheless, our results strongly suggest such possibility and have the merit to put in evidence the molecular mechanisms triggered by such an exposure.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Single Nucleotide Polymorphisms (SNPs) found in *Candida glabrata PDR1* and *ERG11* genes.

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