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Yeast species-specific, differential inhibition of β-1,3-glucan synthesis by poacic acid and caspofungin

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ABSTRACT

The rise of widespread antifungal resistance fuels the need to explore new classes of inhibitory molecules as potential novel inhibitors. Recently a plant natural product poacic acid (PA) was shown to inhibit β-1,3-glucan synthesis, and to have antifungal activity against a range of plant pathogens and against *Saccharomyces cerevisiae*. As with the echinocandins, such as caspofungin, PA targets the synthesis of cell wall β-1,3-glucan and has potential utility in the treatment of medically important fungi. However, the antifungal activity of PA against human pathogenic *Candida* species has not been explored and the precise mode of action of this compound is not understood. Here, we show that PA sensitivity is regulated by the calcineurin pathway and that susceptibility to PA varied significantly between *Candida* species, but did not correlate with *in vitro* β-glucan synthase activity, cell wall β-glucan content or the sensitivity of the species to caspofungin. Strains with point mutations (S645Y or S645P) in the hotspot1 region of the β-1,3-glucan synthase subunit Fks1, had decreased sensitivity to caspofungin but increased sensitivity to PA. *C. guilliermondii*, *C. orthopsilosis*, and *C. parapsilosis* were more sensitive to PA than *C. albicans*, *C. dubliniensis*, *C. tropicalis*, and *C. glabrata*. These observations suggest that there are significant differences in the mode of action of PA and caspofungin and that PA or PA analogues are not likely to have broad spectrum activity in the treatment of *Candida* infections.

Introduction

The fungal cell wall is a dynamic organelle that is essential for its viability. The fungal cell wall is a promising antifungal target for the therapeutic treatment of human fungal pathogens because the major components - chitin, glucan, and mannan, are absent from the human body. Depending on the fungus and growth conditions, the proportion and structural composition of cell wall components varies considerably (Free, 2013; Erwig et al., 2016; Gow et al., 2016; Gow et al., 2017). However, to date all fungi examined have β-1,3-glucan in their cell wall and this plays critical roles as a physical barrier, as a scaffold for the attachment of other cell wall components and in maintaining cell shape (Gow et al., 2017). This makes β-1,3-glucan synthesis an ideal broad-spectrum target for antifungal drugs. However, the cell wall is dynamic and can alter its structure depending on the environment and carbon source the fungi encounter, and in response to cell wall stress. Activation of the Ca^{2+}/calcineurin, HOG and PKC pathways all occur in response to cell wall damage and result in the induction of compensatory mechanisms such as the synthesis of chitin (Munro et al., 2007; Munro, 2013; Gow et al., 2017). Failure to maintain cell wall integrity compromises cell viability and ultimately results in cell death (Negishi et al., 2010; Rodríguez-Pena et al., 2010; Walker et al., 2013a,b), therefore, cell wall integrity is constantly monitored via sensors located in the cell wall and membrane and at critical cell cycle check points (Roberts et al., 1983; Suzuki et al., 2004; Côte et al., 2009; Negishi et al., 2010; Gow et al., 2012; Negishi et al., 2016).

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The three echinocandins caspofungin (CSF), micafungin, and anidulafungin, are the newest class of antifungal agents on the market and have been clinically approved by the US Food and Drug Administration since 2001 (Pappas et al., 2009; Pound et al., 2010; Pfaller, 2012; Perlin, 2015b). They are non-competitive inhibitors of β-1,3-glucan synthase (Douglas et al., 1997; Odds, 2010; Nett et al., 2016) and therefore are not substrate analogues that bind to the enzyme active site. Indeed, the physical binding site of echinocandins to the Fks1 target protein has not been precisely defined. A number of clinical cases of echinocandin resistance have been reported (Imtiaz et al., 2012; Arendrup et al., 2014; Perlin, 2015b; Sanglard, 2016) which is most commonly due to amino acid substitutions in Fks1, one of three major "hotspots" regions in the predicted external face of the β-1,3-glucan synthase transmembrane protein (Kurtz et al., 1996; Park et al., 2005; Garcia-Effron et al., 2008; Garcia-Effron et al., 2009a; Johnson et al., 2011; Johnson and Edlind, 2012; Perlin, 2015b; Kolaczkowska et al., 2016; Prasad et al., 2016). For example, in *C. albicans* the serine^{645} to proline or tyrosine amino acid substitution at of Fks1 is frequently found in echinocandin-resistant strains (Perlin, 2015a; Perlin, 2015b). These mutations are often, but not always, accompanied by elevated cell wall chitin content (Ben-Ami et al., 2011; Ben-Ami et al., 2012; Lee et al., 2012; Perlin, 2015a, 2015b Perlin et al. 2015; Walker et al., 2013a,b). However, resistance to CSF has also been described which is not associated with amino acid substitutions in Fks1. In addition, *C. albicans* strains that have an elevated chitin content in the cell wall are significantly less susceptible to CSF *in vivo* and *in vitro* (Walker et al., 2008; Lee et al., 2012; Walker et al., 2015). Paradoxical growth (also called the "Eagle effect") of *C. albicans* at which growth still occurs at supra-MIC concentrations of the echinocandins has also been shown to be correlated with up-regulation of chitin synthesis (Stevens et al., 2006; Stevens, 2009). Furthermore, a number of CSF resistant isolates are found in other "non-*albicans*" species (Garcia-Effron et al., 2010; Perlin, 2011; Perlin, 2015b), including the emerging pathogen *Candida auris*, where as many as one third of strains are echinocandin resistant or cross-resistant (Lockhart et al., 2017a,b). These health challenges highlight the need for new antifungal agents to augment the antifungal armamentarium.

Poacic acid (diferulate, 8-5-DC), PA, is a natural plant metabolite found in the lignocellulosic hydrolysates of grasses and has been characterised as a promising antifungal agent that targets β-1,3-glucan synthesis (Piotrowski et al., 2015). This compound inhibited growth of *Saccharomyces cerevisiae*, *Alternaria solani* and the oomycete *Sclerotinia sclerotiorum in vitro*. Lesion development on soybean leaves by *S. sclerotiorum* was also inhibited by PA *ex vivo*. PA apparently acts by directly binding to β-1,3-glucan polysaccharide, and therefore differs from the mode of action of echinocandins which directly target the β-1,3-glucan synthase enzyme. In this study we contrast the modes of action of two β-glucan synthesis inhibitors, poacic acid (PA) and caspofungin and show that they have distinct mode of actions and different host range activities. We determined the activity of PA against a range of clinically important fungal pathogens and demonstrate that PA is differentially active against a range of *Candida* species and examine the relationship between species-specificity, β-1,3-glucan synthesis and its sensitivity to PA. Our findings demonstrate that PA and echinocandins inhibit β-1,3-glucan synthesis in different ways and that PA-based pharmacophores may not be suitable as pan-*Candida* inhibitors.

Results

Differential sensitivity of *C. albicans* and *S. cerevisiae* to PA. First we investigated the antifungal activity of PA against the *C. albicans* reference strain SC5314, and *S. cerevisiae* S288C (shown previously to be sensitive to PA) as well as the echinocandin resistant *C. albicans* NR3 strain. As shown in Fig. 1A, *S. cerevisiae* was 5- to 10-fold more sensitive (inhibitory concentration, IC_{50} = 110 μg/ml) to PA than both *C. albicans* strains, NR3 (IC_{50} = 515 μg/ml) and SC5314 (IC_{50} > 1000 μg/ml). The measured sensitivity against *S. cerevisiae* corresponds with that cited previously (Piotrowski et al., 2015). Therefore *C. albicans* SC5314 was significantly less sensitive to PA despite being echinocandin sensitive (Fig. 1C). Unexpectedly, the CSF resistant *C. albicans* NR3 strain was significantly more sensitive to PA, than SC5314. When the cells from the MIC assays were re-plated on YPD agar plates in the absence of drug the PA-treated *C. albicans* SC5314 and NR3 strains grew normally, demonstrating that PA had a fungistatic, rather than a fungicidal effect on growth (Fig. 1B and D).

Kinetic analyses revealed a correlation between the inhibition of β-1,3-glucan synthase enzyme activity and echinocandin MIC (Garcia-Effron et al., 2009b). In addition, *S. cerevisiae* β-1,3-glucan synthase activity was reduced when treated with PA and ^{14}C-glucose incorporation in glucan was significantly decreased (Piotrowski et al., 2015). Therefore, we compared the effect of PA on β-glucan synthase activity using microsomal cell membrane preparations as a source of Fks1 enzyme. The inhibitory concentration (IC_{50}^{GS}) was determined for β-1,3-glucan synthases isolated from the total membrane fractions in SC5314, NR3, and S288C. The glucan synthase activity of all three stains was found to be inhibited to a similar extent by PA (Fig. 1E). The PA IC_{50}^{GS} for β-1,3-glucan synthase of SC5314 was 194 μg/ml, which was comparable to NR3 (IC_{50}^{GS} = 220 μg/ml) and S288C (IC_{50}^{GS} = 206 μg/ml) suggesting that this hotspot mutation that confers echinocandin resistance is irrelevant for PA sensitivity. Therefore, in in vitro assays, PA inhibited *C. albicans* and *S. cerevisiae* β-1,3-glucan synthases to the same degree suggesting that the differential sensitivity of the *C. albicans* and *S. cerevisiae* is not due to differences in the direct action of PA on Fks1 activity.

Combinatorial synergy between PA and cell wall synthesis inhibitors. PA could potentially be useful as part of a combinatorial therapy against *Candida* infections. Piotrowski et al. demonstrated that PA and CSF acted synergistically against *S. cerevisiae* (Piotrowski et al., 2015). Therefore, we carried out sensitivity assays using combinations of PA and CSF against *C. albicans*. The results described indicate combination of PA and CSF only had a noticeable inhibitory effect the growth of both *C. albicans* and *S. cerevisiae*, compared to treatment with each inhibitor alone (Fig. 2A). Noticeably, when *C. albicans* SC5314 was treated solely with CSF, it displayed paradoxical growth at > 8 μg/ml (Fig. 2A). This paradoxical growth was abolished when SC5314 was treated with both PA and CSF. Combinatorial effects calculated using the sub-MIC/IC50 values of PA and CSF, indicated mild synergist effects of additions of PA and CSF for the NR3 CSF resistant strain and *S. cerevisiae* S288C (Fig. 2B). However, combinatorial treatments of PA and two other echinocandins, micafungin and anidulafungin, did not demonstrate synergistic inhibition against the *C. albicans* wild type strain (data not shown).

Because chitin synthesis protects cells from β-glucan damage we also tested potential synergies between PA and chitin synthesis inhibitors nikkomycin Z (NKZ) (a competitive inhibitor of chitin synthase) and CFW (which can have a cidal effect against fungi by binding to nascent chitin and disrupting chitin chain maturation) (Roncero et al., 1985; Gaughran et al., 1994). Similar to previous findings with *S. cerevisiae* (Piotrowski et al., 2015), we observed that PA and NKZ showed no synergistic effect against *C. albicans* (Fig. 2C). However, *C. albicans* SC5314 treated with both PA and CFW exhibited a synergistic effect on growth (Fig. 2C). These results indicate that the combination of PA and CFW may enhance the cell wall polysaccharide instability, leading to enhanced killing.

PA has no effect on chitin content of the *C. albicans* wild type cell wall. In fungi, β-glucan damage often leads to the induction of chitin synthesis (Munro et al., 2007; Walker et al., 2008; Lee et al., 2012). Therefore, we assessed the effect of PA on cell wall chitin measured by staining cells with CFW. DMSO treated cells of all three stains tested had no significant effect on chitin stimulation compared to the untreated controls. For wild type *C. albicans*, CSF treatments stimulated chitin synthesis, but PA did not (Fig. 3A and B). However, for the CSF