## Summary

*Candida* species are normally present as commensals in healthy humans. However, in immunocompromised patients it turns into an aggressive pathogen and causes invasive infections. *Candida albicans* is responsible for the fourth most common nosocomial bloodstream infections in the USA with 40% mortality rate (Pfaller et al., 2015). The cost of treating such infections is also very expensive (Frieden, 2013). Developing novel antifungals against unique fungal targets is quite difficult, since humans and fungi being eukaryotes share many biosynthetic pathways. Nevertheless, echinocandin class of drugs target cell wall biosynthesis that is unique to fungi. Similarly, ergosterol biosynthetic pathway of fungi is partially different from human cholesterol pathway, and has served as target for azole, allylamine, and morpholine class of antifungals (Onyewu et al., 2003a). Ergosterol is also the target of polyene antifungal like AmB to which they preferentially bind compared to cholesterol. Antifungal therapy is challenging, since besides heritable drug resistance due to mutations, certain stages of fungi, such as biofilms, are inherently resistant to most antifungals.

AmB is one of the priority polyene antifungal that is still considered as a gold standard for the treatment of invasive *Candida* infections in the Asian continent (Saravolatz et al., 2003). However, high dose of AmB is associated with severe side effects such as kidney failure and hepatic toxicity. On the other hand, liposomal formulations of AmB are less toxic but are very costly (Laniado-Laborin and Cabrales-Vargas, 2009). The primary mechanism of AmB is thought to be due to binding to, and extraction of ergosterol from the plasma membrane. Formation of pores by AmB is considered a secondary effect contributing to its fungicidal action (Gray et al., 2012). Mutation at certain steps of the ergosterol biosynthetic pathway results in AmB resistance; mutations in *ERG2*, *ERG6*, and

both *ERG3* and *ERG11* result in to the accumulation of other sterols that can provide the functions of ergosterol, but presumably have poor affinity towards AmB thereby conferring AmB resistance (Eddouzi et al., 2013; Vincent et al., 2013).

Phenotypic switching is a heritable change in the phenotype that occurs at high frequency compared to spontaneous mutation rate (Sonneborn et al., 1999). Previously Miller et al. (2006) have shown that changes in colony colour morphology of *C. lusitaniae* strains on plates containing copper sulphate or phloxine B is associated with AmB resistance. We have observed colonies of different colour arising on these plates, but there was no change in their AmB resistance or sensitivity. We have also selected for AmB resistant spontaneous mutants from *C. lusitaniae* strains and found no relation between colony colour and AmB resistance. Sterol absorption profile of two resistant mutants showed that they lack ergosterol, suggesting that they are mutated in the ergosterol biosynthetic pathway. We sequenced the ERG2 genes of these strains and found that they have got two different missense mutations at conserved regions of Erg2 protein.

The molecular chaperone Hsp90 is known to play an important role in modulating resistance to azoles and echinocandins (Cowen et al., 2009b; Robbins et al., 2011; Singh et al., 2009). AmB resistant mutants *erg2*Δ/Δ, *erg6*Δ/Δ and *erg3*Δ/Δ-*erg11*Δ/Δ are very sensitive to Hsp90 inhibitor geldanamycin, indicating that emergence of AmB resistance is also dependant on Hsp90 (Vincent et al., 2013). This role of Hsp90 was claimed to be mediated through Hog1 and calcineurin proteins (Vincent et al., 2013). We have found that the AmB resistant mutants obtained from a *C. lusitaniae* strain are also sensitive to geldanamycin, as well as cyclosporin A, an inhibitor of calcineurin. Vincent et al. (2013) have claimed that in Hog1 is necessary for AmB resistance. However, we have found that *hog1*Δ/Δ *erg2*Δ/Δ double mutant is 4-fold more resistant to AmB compared to *hog1*Δ/Δ

deletant, which is comparable to the fold resistance conferred by *erg2*Δ/Δ deletion in the wild-type background, suggesting that absence of Hog1 does not prevent cells becoming AmB resistant. As far as calcineurin requirement is concerned, deletion of its gene *CNB1* is synthetic lethal with deletions in *ERG2* or *ERG6* genes. Thus, the apparent requirement of calcineurin for emergence of AmB resistance is perhaps due to this synthetic lethality, than calcineurin or Hsp90 facilitating AmB resistance. Nevertheless, the hypersensitivity of AmB resistant mutants to geldanamycin remains to be explained.

*PDR16* gene of *S. cerevisiae* was earlier identified in our lab as conferring increased AmB resistance upon overexpression, but the mechanistic basis was not fully explored. Pdr16p is a phosphotidyl transfer protein that is present mainly in lipid bodies involved in regulation of lipid synthesis (Ren et al., 2013). Since absence of ergosterol is known to increase AmB resistance, we checked if *PDR16* overexpression modulates ergosterol levels, but we found that it did not have any such effect. *PDR16* was also able to increase AmB resistance in *erg2*Δ, *erg3*Δ, *erg6*Δ, *erg4*Δ, *and erg5*Δ mutants, confirming that ergosterol biosynthetic pathway is not critical for AmB resistance conferred by *PDR16*. It was also not dependant on Hsp90 or Hog1 for providing this phenotype. Since AmB binds and sequesters ergosterol from the plasma membrane, we have checked ergosterol distribution by staining with filipin (Beh and Rine, 2004). Distribution of the membrane ergosterol was highly reduced in *PDR16* overexpression strain in comparison to the wild type after the treatment with AmB. Moreover, *PDR16* overexpression strain has more lipid bodies compared to the wild type strain, which gets further enhanced by the AmB. This suggests that membrane ergosterol was moved to the lipid bodies and stored there in the form of sterol ester in the *PDR16* overexpression strain, thereby making it unavailable for AmB to bind and cause damage. Lem3, involved in phospholipid translocation, and Osh3 having sterol transporter

activity, were found to be essential for *PDR16* mediated AmB resistance, consistent with the view that ergosterol relocation in *PDR16* overexpression strain could be responsible for increased AmB resistance. Sac1, Fen1 and Sur4 were also found to be important for AmB resistance conferred by *PDR16*, implying that sphingolipids are involved in modulating Pdr16 function.

AmB, in spite of its severe side effects, is considered as a lifesaving drug due to its low rate of resistance and broad spectrum of activity against pathogenic fungi (Saravolatz et al., 2003). However, emergence of strains resistant to AmB, also having cross resistance to azole drugs is life threatening in nosocomial infections (Pfaller and Diekema, 2007). In this study we have screened a library of 2320 drug and drug-like compounds against wild-type and AmB resistant mutants. It was observed that 35 compounds, which are not presently used as antifungals, were quite active against AmB resistant mutants. Moreover, 9 compounds showed additive effect in combination with AmB. Out of these two were active against AmB resistant mutants. Taken together, combination therapy of these compounds with AmB, besides suppressing possible emergence of AmB resistant mutants, may also reduce AmB dose needed.