6. SUMMARY

System level understanding of the mode of action these antifungal agents could provide novel insight and novel drug targets which can be exploited to devise new generation of antifungal agents. With this aim we chose to study antifungal agents Fludioxonil and Ciclopirox-olamine to understand their mode of action better. Fludioxonil is a phenylpyrrole group of fungicide which is widely used in agricultural practices. It targets HHK3 but the exact mode of its fungicidal action is not elaborated. The heterologous expression of HHK3 e.g. ClNik1 in *Saccharomyces cerevisiae* confers fludioxonil sensitivity to this yeast and we used this model to carry out screening of the genome wide deletion library to identify genes that are hypersensitive to fludioxonil. This screening allowed us to identify vesicular trafficking and cytokinesis as the main pathways to be affected in *S. cerevisiae* model. It identified that the genes involved in early and late endosomal transport e.g. CORVET tethering complex and its regulators, SNARE proteins could be potential downstream effectors of fludioxonil toxicity. In this study we showed that the adverse effect of fludioxonil on late endosomal trafficking was quite evident as we observed highly fragmented vacuole after drug treatment. Similarly, fludioxonil also impaired the CORVET localization on endosomal dots. The over-expression of *VPS11*, *VPS16* and *VPS18* conferred resistance to fludioxonil which further confirmed the direct involvement of endosomal trafficking. Vps11p, Vps16p and Vps18p are the core subunits of CORVET and HOPS tethering complex. Impairment of cytokinesis was also observed after fludioxonil treatment. Endosomes are a central compartment in the endocytic pathway and therefore any adverse effect on it can affect collective vesicle trafficking capacity of the cell by interfering with recycling endosomes or protein targeting to vacuole for degradation. Endocytic pathway is essential for polarized growth and survival in filamentous fungi. In *C. albicans* model fludioxonil completely inhibited hyphal growth whereas the growth of yeast form of *C. albicans* remained unaffected. The polarised growth in the constitutively hyphal form was severely hampered and there was a prominent pseudo-hyphal morphology. Interestingly, the over expression of some of the components of the CORVET complex e.g. *VPS11*, *VPS16* and *VPS 18* could alleviate the vesicle fusion defect and abrogate the fludioxonil toxicity and therefore indicating their direct role in this process. We have shown that fludioxonil inhibits hyphal growth in *C. albicans* by impeding vesicle trafficking and the over expression of CaVps16 or CaVps18 or Ca Vps11 could reverse

this. The deletions of *VPS11*, *VPS16* and *VPS18* showed synthetic lethality with TOR1 and affect TOR1 signalling (Catlett *et al.*, 2003). Therefore, fludioxonil can inhibit a number of crucial physiological processes through its effect on endosomal trafficking downstream of HHK3 in addition to its effect on HOG pathway. Interestingly, the vesicle trafficking and secretory pathway have been proposed to be the next generation target for developing antifungal agents as few recently discovered fungicides appeared to act through these processes (Neto *et al.*, 2012; Zurita-Martinez *et al.*, 2007). Our study further supports this proposition.

Present study can also be construed as chemical genetic evidence regarding the physiological role of HHK3 in the vesicle trafficking in fungi. Deletion of HHK3 had pleiotropic effects. Concomitant to its established role in osmoadaptation, the HHK3 deletion mutants showed osmo-sensitive phenotype. Besides this, hyphal growth, virulence, conidiation were also found to be highly compromised in the deletion mutants in most species (Hagiwara *et al.*, 2009). Since, the vesicle trafficking has vital role in all these processes we propose that HHK3 regulates hyphal growth, virulence, conidiation through vesicle trafficking. Thus, our study for the first time provides novel insight into the function of HHK3 which can be exploited for developing antifungal agent for therapeutic uses.

Hyphal growth in *C. albicans* has also been attributed as one of the virulence factors in invasive candidiasis (Moyes *et al.*, 2016; Dalle *et al.*, 2010). In our present study we observed that hyphae formation in *Candida* was inhibited and therefore we wanted to explore the role of different TF in hyphal induction in response to fludioxonil. In order to do so we carried out screening with an in-house library of transcription factor mutant strains (Dhamgaye *et al.*, 2012) containing 240 homozygous mutant strains. From this screening it was indicated that RIM101, SSN3, SPP1, GZF3 and Orf19.794 were capable of forming hyphae in response to fludioxonil unlike the wildtype SC5314 which was incapable of forming hyphae in response to fludioxonil. Whereas, Orf19.4545 encoding Swi4 and Orf19.3012 encoding Aro80 were found to be sensitive fludioxonil. RIM101 has been implicated in pH regulated hyphae formation and important for host pathogen interaction (Davis *et al.*, 2000; Davis *et al.*, 2003). Though, the role of SSN3, SPP1, Orf19.794, Orf19.4545 and orf19.3012 in hyphae formation has not been investigated. In our studies in *S. cerevisiae,* deletion of Swi4 (Orf19.4545) have been shown to have sensitive phenotype (Sharma *et al.*, 2014) which corroborates with our

results here. This study also provides the scope of elucidating the role of the mentioned Transcription factors in Fludioxonil toxicity. The inhibition of the Hyphal morphology, a key virulence factor, by fludioxonil makes it a promising antifungal agent. Therefore, if we can establish the key factors involved in yeast to hyphae transition that are targeted by Fludioxonil they can give us key insights into the polarised growth and also their use as drug targets.

The non-essential gene deletion library screening carried out also identified resistant genes to fludioxonil. Study of the role of Resistant genes indicate the direct targets of the drug. In an attempt to characterise the role of the resistant strains to fludioxonil toxicity deletion strains were re-created to identify Δ*ssd1*, Δ*pet494* and Δ*bsp1* as the main targets of fludioxonil. Furthermore, the overexpression of SSD1, PET494, and BSP1 in the respective deletion background reverted the resistance phenotype. The emergence of sensitivity in the resistance strains upon overexpression of these genes re-establish their role in fludioxonil toxicity. Among these strains Δ*ssd1* was the only strain that did not show cytokinesis defect or vacuole fragmentation or Hog1 phosphorylation in response to fludioxonil as compared to hallmark defects observed in wildtype. Therefore, the role of Ssd1 was further characterised in relation to fludioxonil toxicity. Functional characterisation of different Ssd1 mutants indicated that phosphorylation of the Ssd1 plays a crucial role in fludioxonil toxicity by sequestration of mRNA to P bodies. This was further reinstated by increasingly sequestration of Ssd1-GFP to the foci representing P bodies wherein the Ssd1 bound mRNA is translationally repressed.

Our results also concluded that Cbk1 plays a crucial role in regulating Ssd1 and in polarised growth upstream of HOG pathway in response to fludioxonil. It helps in phosphorylating Ssd1 which helps in expression of the downstream genes regulated by Ssd1 and therefore conferring resistance. Ssd1 carried out the translational repression of many genes related to cell wall biogenesis like chitinase, Cts1 and cell wall remodelling protein like Sun4, Sim1 and Uth1 (Wanless *et al.*, 2014; Hogan *et al.*, 2008). Activation of cell wall integrity pathway in *S cerevisiae* strain expressing in ClNik1 but not in the resistant strain *ssd1*Δ in response to fludioxonil was observed. Therefore, we can conclude *ssd1*Δ cells were able to cope fludioxonil toxicity by not upregulating the CWI or HOG pathway (or by acting upstream of the HOG1 upregulation).

Topical antifungal agent Ciclopirox-olamine belongs to the hydroxypyridone class. The fact that this FDA approved drug has been in extensive use and still not given rise to resistant strains has attracted the attention of the medical practitioners to repurpose the drug to other disease scenarios. We have used the high throughput screening methods of Deletion library screening and RNASeq to have a better understanding of the mode of action of CP. Generation of precursor metabolites and energy, Nucleobase containing small molecule metabolic process, Cellular ion homeostasis, Ion transport, Mitochondrion organisation, Response to chemicals and Cell wall organisation which were over were significantly highlighted in both screenings. *LEU1*, *CCP1*, *ARG4* and *CTT1* were identified as the major factors due to their higher betweenness centrality and these genes appear to contribute to CP toxicity due to the perturbation of their role in multiple pathways.

The pathways identified were further studied in detail. It was observed that cell wall integrity was perturbed due to CP treatment. Further, absence of growth on the non-fermentable carbon sources in the presence of CP indicated the deficient activity of the mitochondria. Further, reduced fluorescence intensity in CP treated cells by Mitotracker Red labelling indicated the reduced mitochondrial activity. Taken together these results reinstate the previously known fact that CP affects mitochondrial activity due to iron chelation (Niewerth *et al.*, 2003). This study is in corroboration with previous studies that established dysfunctional mitochondria or perturbations in iron homeostasis induce abnormal cell wall (CW) composition (Dagley *et al.*, 2011; Yu *et al.*, 2014).

Perturbation of Amino acid metabolism in response to CP was indicated in both the screenings for the first time. It was observed that arginine or leucine supplementation increase the minimum inhibitory concentration to CP. Therefore, higher susceptibility to CP could be due its cytotoxic effect caused by inhibition of the enzymes in the arginine biosynthesis pathway. It is already known the Yeast mTOR are master regulators of morphogenesis and signalling in response to a variety of key stress factors (Hall, 2008). mTOR activation by arginine can also be sensed by two parallel mechanism involving a vacuolar transporter and a sensor for cellular arginine sensor for mTORC1 (CASTOR1) (Chantranupong *et al.*, 2016). mTORC1 can particularly sense leucine and arginine, which were indeed indicated in the RNASeq data (Hara *et al.*, 1998). Therefore, it can be inferred that CP exerts its toxicity by depleting the arginine and leucine concentration which in turn affects the activation of mTOR

pathway. This can additionally be supported by the observation that rapamycin has a synergistic effect with CP. Interestingly, yeast TORC1 was localized on the vacuolar membrane (Binda *et al.*, 2009, Hughes Hallett *et al.*, 2014, Kira *et al.*, 2015) and therefore, it exerts an active role in the transport and sensing of the amino acids, ions and lipids actively being trafficked from the mitochondria (Ben-Sahra *et al.*, 2016). Consequently, severe vacuolar fragmentation in *S. cerevisiae* treated with CP was observed. Taken together our results indicate that CP affects mitochondrial activity. The dysfunctional mitochondria crosstalk with the vacuoles through vCLAMPS along with vacuolar transporters which sense arginine and leucine concentration and inhibit the activation of TORC1. Thus, there is a complex crosstalk between mitochondria, vacuoles involving amino acid sensing via mTOR pathway which is affected by CP. This study also reveals that CP can be used in combination with rapamycin for drug resistant fungi. It is well established from years of clinical use that CP has been an effective alternative antifungal agent for drug resistant fungi and therefore the fact that it can be used combination with rapamycin is another way of tackling the drug resistance menace in fungal species.