SYNOPSIS

Understanding the biology of heat shock protein 90 in opportunistic fungal pathogens.

Heat shock protein 90 is one of the most abundant and evolutionary conserved class of molecular chaperones present throughout the biological kingdom. It is a specialist chaperone as its function extends beyond protein homeostasis. It is known to chaperone a specific set of proteins which lies at the interface of important cellular processes such as growth, signal transduction and developmental networks. It is believed that Hsp90 also functions as a stress responsive storehouse of genetic variation and thereby plays a key role in evolution. Another novel facet of Hsp90 was uncovered when the function of this chaperone was found to be important for pathogenesis of infectious disease-causing agents. Hsp90 was found to regulate stage transition in clinically important protozoan parasites such as *Plasmodium, Giardia, Entamoeba* and *Leishmania.*

Fungal pathogens cause substantial morbidity and mortality worldwide, however, the true impact of this group of pathogens on human health is not widely appreciated. Majority of fungal infections are often concealed under major comorbid conditions such as AIDS, cancer and other immunosuppressive conditions. Diagnosis and treatment of fungal infections is notoriously challenging due to emerging pathogens and drug resistance. Prospects of targeting Hsp90 to curb fungal pathogens has been explored in *Candida albicans* and *Aspergillus fumigatus*. Studies have revealed that Hsp90 governs one of the classical virulence traits of *C. albicans*, the yeast to hyphal transition. Additionally, the role of Hsp90 in potentiating antifungal resistance has been examined by genetic and inhibitor based studies. In this study, I have explored novel facets of Hsp90 in two clinically important fungal pathogens: *Candida* and *Cryptococcus. Candida* and *Cryptococcus* are the among the chief cause of death in cancer and AIDS patients. Furthermore, there is a surge in nosocomial fungal infections worldwide. I have investigated the hierarchy of *Candida* species implicated in invasive infections and found a clinical misdiagnosis of a multidrug resistant pathogen, *Candida auris*. Further, I have explored the role of Hsp90 in aiding resistance and thermotolerance of this emerging fungal pathogen.

Comparative analysis of Hsp90s of *Candida* and *Cryptococcus*

As mentioned previously, despite the great interest in examining the potential of Hsp90 to serve as a drug target to treat fungal infections, the basic biochemical properties of Hsp90s from fungal pathogens have not been studied previously. With this view, in Chapter 3, I have compared the biochemical properties of Hsp90 of these two fungal pathogens. Functionally, Hsp90 is an ATPase and the N-terminal domain of the protein harbours the nucleotide binding pocket. ATP hydrolysis is essential for its chaperoning ability. Bioinformatic analysis of the binding pocket of both *Candida* Hsp90 (CaHsp90) and *Cryptococcus* Hsp90 (CnHsp90) revealed subtle differences in the amino acid residues implicated in ATP binding, despite general conservation at the level of its primary structure. Many pharmacological inhibitors of Hsp90 such as geldanamycin and radicicol also bind to the nucleotide binding pocket and inhibit its activity. Therefore, we carried out *in vitro* ligand and inhibitor binding studies as well as ATPase activity studies using recombinant, bacterially expressed proteins obtained by cloning and purification. Both fungal Hsp90s i.e. CaHsp90 and CnHsp90 showed high affinity to ATP with a k_d of 125.4 µM and 497.05 µM respectively. Further, the catalytic efficiencies of these Hsp90s was determined by measuring ATP hydrolysis rate using γ^{32} P ATP as a tracer. Catalytic efficiency of CaHsp90 was found to be 11.6×10^{-5} $min^{-1}\mu M^{-1}$ whereas CnHsp90 showed an efficiency of 6.39×10^{-5} $min^{-1}\mu M^{-1}$, indicating that CaHsp90 is a more active ATPase as compared to the *Cryptococcus* counterpart.

Pharmacological inhibitors of Hsp90 serve as a valuable molecular tool to understand protein function. We measured binding affinities of fungal Hsp90s to 17-AAG and we found that both CaHsp90 and CnHsp90 showed much higher affinity towards 17-AAG as compared to ATP with dissociation constants in range of 3-14 µM. Also, the ATPase activity of these fungal proteins was found to be more sensitive to pharmacological inhibition of Hsp90, indicating the possibility of drug development. Overall, we have carried out systematic biochemical analysis of Hsp90 of these two fungal pathogens.

Cellular functions of Hsp90 in *Cryptococcus neoformans*

In Chapter 4, I have investigated the role of Hsp90 in the pathogenesis and thermotolerance of *C. neoformans*, an environmental fungus that causes meningoencephalitis in humans. The genus *Cryptococcus* comprises over 37 species, most of which are environmental saprobes and non-pathogenic to humans. The pathogen

has two unique virulence factors, melanised cell wall and a polysaccharide capsule which helps to combat host defences. However, many species of *Cryptococcus* such as *C. podzolicus* are equipped with these two pathogenicity armours but are not pathogenic to humans. Only two species which can grow at 37°C are human pathogens implicating the importance of thermotolerance for pathogenesis. The mechanism of growth at elevated temperature with respect to heat shock machinery has long been enigmatic. We found that thermotolerance of *Cryptococcus* critically depends on Hsp90 function as modest inhibition of Hsp90 function led to robust compromise in growth of the fungus at 37°C with little effect at 25°C. This observation also correlated with the finding that *in vitro*, 17-AAG showed a more potent inhibition of ATPase activity at 37°C. Interestingly, indirect immunofluorescence analysis using an antibody specific to CnHsp90 revealed cell surface localization via ER-Golgi classical secretory pathway. Furthermore, inhibition of Hsp90 function led to decrease in capsular volume and also improved the natural resistance of *C. neoformans* to cell wall targeting inhibitors echinocandins. Thus, Hsp90 dictates important virulence determinants of this pathogen.

Misdiagnosis of *Candida auris*

Numerous studies have linked Hsp90 to the pathogenic potential of fungi including yeast to hyphal transition, drug resistance and biofilm formation. However, these studies have been done in *C. albicans* because it is the leading fungal pathogen in the western countries. However, several epidemiological studies have reported the preponderance of non albicans *Candida* species in India. Therefore, we first sought to investigate the hierarchy of *Candida* species implicated in invasive infections. To address this, we collaborated with Manipal hospital, Bangalore, India. We found that *C. tropicalis* and *C. parapsilosis* were highly prevalent in this part of the world. During our study period, we noted the emergence of a new species called *Candida haemulonii.* Identification of the isolates was done by a commercial, automated system called Vitek2 by the hospital. Surprisingly, molecular studies in the lab revealed that these group of clinical isolates completely differ from the *C. haemulonii* wild type isolate. We further sequenced the whole genome of one of the clinical isolate Ci6684 and we found that it is *Candida auris* and was misdiagnosed to be *C. haemulonii.* In this study, we have developed a specific PCR to resolve the diagnostic dilemma between *C. auris* and *C. haemulonii*. Given that *C. haemulonii* genome is not sequenced, we have used the gene sequence for mating factor alpha as the candidate gene since it is known to be unique for each *Candida* species.

In 2016, *C. auris* outbreaks have been reported from many countries across the globe, including South Korea, India, Israel, South Africa, Japan, Canada, Germany, Colombia, Kenya, Spain, Norway, the United Kingdom, Pakistan and United States. We speculate that *C. auris* infections may also have occurred in other countries, however, the actual prevalence is underreported since the commercial automated systems used in clinics routinely misidentifies *C. auris*. This highlights the importance of early diagnosis of invasive candidiasis to initiate prompt treatment as delay in the administration of appropriate therapy increases mortality.

Genome of *Candida auris*

What makes misdiagnosis more alarming is the fact that these group of clinical isolates are not susceptible to the two frontline antifungals fluconazole and amphotericin B. To gain deeper insights into the biology of this misdiagnosed, multidrug resistant pathogen, we have generated the first draft genome of this pathogen which has been discussed in Chapter 6. The assembled draft genome of *C. auris* (clinical isolate Ci6684) consists of 99 scaffolds, 8358 protein coding genes, 189 tRNAs and 7 rRNAs. The draft genome size is 12.49 Mb, GC content 44.53% and 1.327 % Ns were estimated. Comparison of the genome of *C. auris* with other sequenced pathogenic *Candida* species by phylogeny and synteny analysis revealed that it has a highly divergent genome. Among the sequenced *Candida* species, *C. auris* was closest to *C. lusitaniae* as evident by substantial genomic collinearity seen in synteny dot plots and similarity in codon usage patterns. We further mined the genome to make an inventory of functional elements which can be potentially explain the pathogenicity mechanisms of *C. auris*. Enzyme classification analysis revealed that *C. auris* genome is enriched in hydrolases particularly, lipases, phospholipases and aspartyl proteinases. The activity of these enzymes is known to be high during invasive infections. Orthologs of genes implicated in biofilm formation, Rim101 transcriptional pathway and mitogen-activated protein kinase (MAPK) pathways were also conserved in *C. auris*. The draft genome sequence of *C. auris* will facilitate further studies to decipher the biology and virulence of *C. auris*.

Further, I have probed the antifungal resistant profile of clinical isolates of different *Candida* species including *C. auris*, *C. tropicalis* and *C. haemulonii*. Upon investigation of the prevalence of drug resistance in these species, we found that *C. auris* is currently the most resistant pathogen. Considering the conserved role of Hsp90 in antifungal resistance, we examined the effect of pharmacological inhibition of Hsp90 on drug

resistant phenotype of these clinical isolates. Pharmacological inhibition of Hsp90 abrogated resistance in *C. tropicalis* and *C. haemulonii*. Surprisingly, Hsp90 inhibitors had no effect on fluconazole and amphotericin B resistance in *C. auris*. Genomic analysis revealed an enrichment of drug transport and metabolism pathways in *C. auris*. Further, we found that the pathogen is equipped with an arsenal of drug efflux pumps belonging to the ATP binding cassette (ABC) superfamily and major facilitator superfamily. Abundance of multidrug efflux pump genes encoding these drug transporters may explain the intrinsically low susceptibility of *C. auris* to antifungal drugs. Our analysis also predicted a multitude of zinc finger transcription factors which are known to modulate the expression of drug efflux pumps in response to antifungals. Furthermore, we also found that *C. auris* can grow at 42°C making it more thermotolerant as compared to other species. Interestingly, inhibition of Hsp90 led to complete abrogation of thermotolerance as evident by lack of growth at higher temperature. Further studies based on genetic compromise of Hsp90 function are needed to establish whether drug resistance in *C. auris* is Hsp90 independent or the effect seen is merely due to the activity of drug transporters.

Summary

Overall, in the thesis, we show that Hsp90s from two clinically important fungal pathogens differ in terms of their biochemical and cell biological properties. In *Cryptococcus*, Hsp90 was found to hone virulence traits including thermotolerance and capsulation. Interestingly, Hsp90 was also found to be cell surface localised and inhibition of Hsp90 pharmacologically improved anidulafungin tolerance at 37°C. We have also highlighted misdiagnosis of a multidrug resistant, emerging pathogen *Candida auris*. We have generated the first draft genome of this pathogen which facilitated the development of a molecular diagnostic method. The genome of *C. auris* Ci6684 is now the reference genome for this species. Analysis of the genome revealed the core virulence repertoire of *C. auris* which includes hydrolases, mannosyl transferases, adhesins and drug efflux pumps. Further, we found that although Hsp90 inhibition had no effect of on drug resistance of *C. auris*, however, Hsp90 inhibition led to abrogation of thermotolerance indicating the myriad ways by which it can be exploited by fungal pathogens.