

# Exploring Small Heat Shock Proteins (sHSPs) for Targeting Drug Resistance in *Candida albicans* and other Pathogenic Fungi

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## Abstract

Fungal infections have predominantly increased worldwide that leads to morbidity and mortality in severe cases. Invasive candidiasis and other pathogenic fungal infections are a major problem in immunocompromised individuals and post-operative patients. Increasing resistance to existing antifungal drugs calls for the identification of novel antifungal drug targets for chemotherapeutic interventions. This demand for identification and characterization of novel drug targets leads to the development of effective antifungal therapy against drug resistant fungi. Heat shock proteins (HSPs) are important for various biological processes like protein folding, posttranslational modifications, transcription, translation, and protein aggregation. HSPs are involved in maintaining homeostasis of the cell. A subgroup of HSPs is small heat shock proteins (sHSPs), which functions as cellular chaperones. They are having a significant role in the many cellular functions like development, cytoskeletal organization, apoptosis, membrane lipid polymorphism, differentiation, autophagy, in infection recognition and are major players in various stresses like osmotic stress, pH stress, etc. Studies have shown that fungal cells express increased levels of sHSPs upon antifungal drug induced stress responses. Here we review the important role of small heat shock proteins (sHSPs) in fungal diseases and their potential as antifungal targets.

**Keywords:** *Candida albicans*, Drug resistance, Small heat shock proteins, Antifungals

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## INTRODUCTION

Fungi are the most common human pathogen that causes severe cutaneous infections to life-threatening systemic infections. In the United States, hospital born *Candida* is a major pathogen and cause frequent systemic infections in the patients. It also causes frequent oral infection in immunocompromised individuals. There are several virulent factors like an expression of adhesion molecules, secretion of hydrolytic enzymes, biofilm formation and micro-environment conditions like pH, nutrients which are responsible to convert non-virulent yeast form to virulent hyphal form (Polymorphism).

In major surgeries, severe cases of superficial and systemic infections of *C. albicans* are reported in immunocompromised patients<sup>1</sup>. Several groups of antifungal drugs attack or disrupt cell membrane of *C. albicans* e.g. echinocandins, polyenes, and azoles etc.<sup>2</sup>, which act as stress to the pathogen. The severity of stress condition depends upon dose and type of drug. Various receptors on the membrane of pathogenic fungi are involved to perceive this stress by eliciting various conserved signaling pathways like MAP kinase (MAPK) and calcineurin signal transduction pathways etc.<sup>3,4</sup>

In drug resistant pathogenic fungi, several counter mechanisms exist to minimize or nullify the stress produced due to exposure of antifungal agents. Fungi static drugs that inhibit growth at the minimum growth-inhibitory concentration (MIC) did not kill fungal pathogen because of the drug tolerance phenomenon<sup>5</sup>, whereas fungicidal drugs that are been used at a higher concentration than MIC did not kill drug resistant pathogens. Prolonged exposure to different fungistatic drugs can cause genetic mutation in fungal cells that leads to various mechanisms for drug resistance like overexpression of membrane efflux pump proteins, mutations in drug target enzymes, and upregulation of stress-response genes etc.<sup>6-8</sup>.

Extensive use of these anti-fungal drugs in the clinics has increased resistance and ineffectiveness against *C. albicans* infections<sup>9,10</sup>. Therefore it is an emergency to come up with new therapeutic targets that can be explored for developing novel antifungal agents against *C. albicans*<sup>10,11</sup>. Occurrences of Heat shock proteins (HSPs) are integral to almost every organism and

are also highly diverse and widely distributed among fungal groups. It is been shown that under thermal stress, these proteins are getting upregulated. Expression of the heat shock proteins under heat stress is regulated by the heat shock transcription factor 1 (Hsf1)<sup>12</sup>. In *C. albicans*, Hsps are important not only for the growth but also for developing infection and virulence<sup>13-15</sup>. Stress conditions are inducive to promote the expression of heat shock proteins. Hsps interact with various cellular signaling pathways like MAPK, calcium-calcineurin, cell cycle control signaling, etc. regulating homeostasis and virulence in *C. albicans*<sup>14,16,17</sup>. It is been reported that inhibiting or disrupting Hsps causes growth inhibition of *C. albicans*, which leads to reverse tolerance to available antifungal drugs. Studies have shown their involvement to confer resistance to antifungal agents by modulating HSPs associated signaling pathways in *C. albicans* and other pathogenic fungi.

A group of heat shock proteins known as small heat shock proteins (sHSPs) with a molecular weight ranging from 12 to 43 kDa gets upregulated upon non-thermal cues like oxidative and heavy metal stress<sup>18,19</sup>. sHSPs, not only act as chaperones but are involved in the many biological vital functions like development, cytoskeletal organization, apoptosis, membrane lipid polymorphism, differentiation, autophagy, and infection recognition<sup>20,21</sup>. These proteins can be used as therapeutic targets in the direction of the development of antifungal agents.

### Heat shock proteins (HSPs) in Fungi

Hsps are integral and conserved among all living species, they respond to heat and non-thermal processes like oxidative stress and starvation that leads to stress to the organism<sup>22</sup>. These stressful conditions cause the loss of three dimensional structures of proteins and their aggregations leading to cell death. Hsps are molecular chaperones and expression of Hsps is a protective mechanism of the cell to combat these changes to ensure cell survival under the stress. There are two groups of Hsps, First is ATP-dependent high molecular weight protein Hsps, having four different families (Hsp104, Hsp90, Hsp70, and Hsp60) and a second ATP-independent family of proteins - Hsp12 and Hsp21 having low molecular weight proteins ranging between 12-42

kDa called Small Heat Shock Proteins (sHSPs) (Table 1)<sup>23</sup>. Hsps are expressed in stress and non-stress conditions while sHsps are mostly expressed under stress conditions<sup>24</sup>. It has been shown that most of the antifungal drugs are perceived as stress by the fungal cell<sup>25</sup>. Thus sHSPs can be explored as the putative targets to combat drug resistance and the development of effective therapeutics to treat fungal infections.

### Roles of Heat Shock Proteins (HSPs) in pathogenic fungi

High molecular weight HSPs are the major proteins that are involved in protecting cellular proteins by acting as chaperons. HSPs in pathogenic fungi are assisting in the process of making and maintaining three dimensional conformations of proteins during various stress and unfavorable conditions to counter the changes in the fungal cells. Expression and functions of different Hsps are variable with the kind of stress to the fungal pathogen.

#### Hsp104

Hsp104 was firstly reported in *Saccharomyces cerevisiae* (*S. cerevisiae*) and induced in elevated temperature<sup>23,26,27</sup>. It also acts as a pro-survival protein under high temperature, suggesting its role as thermal tolerance<sup>26</sup>. Mutant of *hsp104Δ/Δ* have shown morphological defects in hyphae<sup>28</sup>. In *C. albicans*, Hsp104 has a greater role in biofilm formation and virulence<sup>28</sup>. Cytosolic Hsp104 of *C. albicans* is not equivalent to human Hsp104, thus can be used as a promising antifungal target against *C. albicans*.

#### Hsp90

Hsp90 determines antifungal drug resistance in several diseases causing fungi like *Candida albicans*, *A. fumigatus*, and *C. neoformans*<sup>29-31</sup>. Hsp90 is involved in several

cellular functions like development, regulation, homeostasis, and drug resistance in *C. albicans*<sup>16,17</sup>. A recent report showed that mutations in the region of Hsp90, responsible for post-translational modifications affect colony morphology<sup>32</sup>. Inhibitors of Hsp90 function have shown additive effects with fluconazole (FLC) against Fluconazole resistant *Candida albicans*<sup>33,34</sup>. In yeast, the Hsp90 function is mediated by its C-terminal phosphorylation, S-nitrosylation, and acetylation<sup>35-37</sup>. Several findings showed that Hsp90 contributes a vital function in confirming resistance to antimycotic drugs. Thus inhibiting Hsp90 function or development of pathogen specific histone deacetylase inhibitor can be the effective therapeutics to treat candidiasis.

#### Hsp70

Hsp70 is uniformly present from prokaryotes like bacteria to higher eukaryotes like mammals. Ssa1 and Ssa2 are two important members of the Hsp70 family on the cell surface of *C. albicans*<sup>38,39</sup>. *C. albicans* Ssa1/1 mutant showed altered virulence *in vitro* as well as *in vivo*. Ssa1 and many antimicrobial peptides have Ssa2 as receptors that also have antifungal effects. Hsp70 alone or in combination with Hsp90 plays a major role in morphogenesis and dimorphism. A report have shown that the Hsp70 works with the heat shock transcription factor 1 (Hsf1) to regulate heat shock response in the yeast<sup>40</sup>.

#### Hsp60

Hsp60 encodes a predictive mitochondrial heat shock protein, whose function is not known. A heterozygous mutant of Hsp60 (*hsp60Δ/HSP60*) has been shown to increase sensitivity with increasing temperatures. This is an indication of Hsp60 can be essential to overcome thermal stress<sup>41</sup>. Four fold increased expression of *hsp60* in wild type yeast can resist oxidative stress in comparison to the mutant of *hsp60*. It was because iron/sulfur containing enzymes were protected from oxidative inactivation<sup>42</sup>. Another study showed an increase in the levels of Hsp60 under thermal stress and is important for differentiation, infection, and colonization<sup>43</sup>. Expression of *hsp60* mRNA level increased by 5.9-6.9-fold at 40°C in *A. fumigatus* and *A. terreus*<sup>44</sup>. Hsp60 functions as an immunological trigger and play a role in fungal diseases in humans<sup>45</sup>.

**Table 1.** Different types of heat shock proteins (HSPs)

HSPs (ATP-Dependent High Molecular Weight Proteins)	sHSPs (ATP-Independent Low Molecular Weight Proteins)
Hsp104	Hsp12
Hsp70	Hsp21
Hsp60	

### Role of Small Heat shock proteins (sHSPs) in *C. albicans*

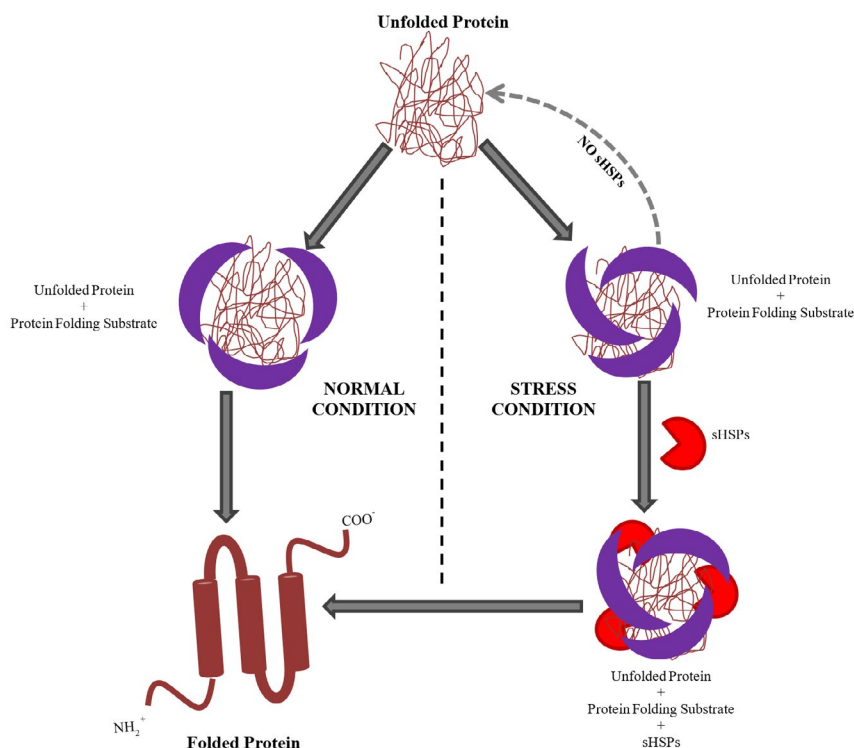
Reports have shown that sHSPs of different species function as molecular chaperones and have conserved the  $\alpha$ -crystallin domain (ACD). sHsps are cellular chaperones, involve in the proper folding of proteins during normal and extreme conditions. Under stress conditions, sHSPs contribute in refolding of partially unfolded proteins. They bind to the irreversible aggregation of denaturing proteins and are prevented in an ATP-independent fashion<sup>46-49</sup>. Exposure to a variety of stresses like elevated temperature or oxidative stress causes unfolding of proteins and form early intermediates that can aggregates. These partially unfolding protein molecules are stabilized by the sHSPs<sup>48,50-52</sup>. Early expression of sHSPs allows rescuing proteins that are getting unfolded under stressed conditions. Completely unfolded proteins and pre-aggregated proteins are not refolded by

sHSPs. Thus, sHSPs protect unfolding proteins that otherwise become irreversible aggregates under stressful conditions to maintain homeostasis within the cell (Fig. 1).

Various studies have shown a link between HSPs and virulence potential of pathogenic microorganisms<sup>18,53-58</sup> including Hsp90 and Hsp70 in *Candida albicans*<sup>33,59</sup>. Biofilm formation is an important virulence phenomenon in *C. albicans* infection and HSPs are required for its progression<sup>14</sup>. In *C. albicans* have reported three sHSPs i.e. Hsp10, Hsp12, and Hsp30/Hsp31.

#### Hsp12

Small heat shock protein (Hsp12) contributes to heat-shock resistance and hybridization of hsp12 mRNA analysis demonstrated its co-regulation with environmental pH and CO<sub>2</sub> in *C. albicans*<sup>60-63</sup>. Intracellular amount of trehalose (Protectant against cell freezing) was minimized in *C. albicans* by TPS1 deletion. TPS1 deletion



**Fig 1.** Model showing working of Small Heat Shock Proteins (sHSPs) system as chaperone. Protein folding is mediated by the cellular chaperon molecules (Protein folding substrate complex) which are active under the normal state of cell. sHSPs are expressed under the stress and they stabilize and assist the protein folding substrate complex to form proper confirmation and folding of the proteins. Loss of function of sHSPs in stress leads to formation of nonfunctional unfolded protein aggregates which can cause cell death, therefore sHSPs are rescuing cell under stress.

showed overexpression of Hsp12 in *C. albicans*. *C. albicans* have shown increased cell adhesion and decrease susceptibility to the quorum sensing molecule, farnesol<sup>64,65</sup>. SSK1 mutant of *C. albicans* was susceptible to several oxidative agents like H<sub>2</sub>O<sub>2</sub> and has also shown a high level of HSP12<sup>66</sup>. This shows Hsp12p has a crucial role in combating various kinds of stresses. Another study showed that increased expression of Hsp12 leads to sensitivity to itraconazole, ketoconazole, and FLC in *C. albicans*<sup>65</sup>.

### Hsp21

Small heat shock protein 21 (Hsp21p) is another protein which plays an important role under various environmental stress for *C. albicans*<sup>61,67-72</sup>. It was found that Hsp21 promotes virulence of *C. albicans*. Tolerance to heat and oxidative stress requires Hsp21 in *C. albicans*<sup>73</sup>. Activation of the mitogen activated protein (MAP) kinase and normal filamentation require Hsp21. Hsp21 mutant and chemical inhibitors have shown a correlation in inhibiting germ tube formation and filamentation at the initial time point<sup>73</sup>. *In-vitro*, analysis of *hsp21*  $\Delta/\Delta$  mutant strain showed an inability to damage endothelial and oral epithelial cells<sup>73</sup>. Growth of Hsp21 mutant was significantly reduced when treated with membrane perturbing agents that target ergosterol biosynthetic pathway i.e. terbinafine, caspofungin<sup>73</sup>. At high temperatures, it is involved in maintaining homeostasis of glycerol, glycogen, and trehalose<sup>73</sup>. Susceptibility to various antifungal drugs was seen in the deletion mutant of *hsp21* of *C. albicans*<sup>74</sup>. Hsp21 can be an effective target to develop a treatment strategy for *C. albicans* infection.

### Hsp10

Hsp10p is present in association with Hsp60p and assisting Hsp60 function<sup>75</sup>. *In-vivo* Hsp60p and Hsp10p did not always act as a single functional unit *in-vitro*. Hsp10p is crucial for cell survival and acting as a co-chaperon to assist the folding of the proteins in the mitochondrial matrix<sup>76</sup>. Functionally defective protein aggregation was seen in the mutant of *Hsp10*. It imparts in the sorting of the RieskeFeS protein during the transport from the matrix to the intermembrane space<sup>77</sup>.

### Hsp30/31

In *C. albicans*, oxidative stress upregulates

Hsp30p besides other heat shock proteins<sup>61</sup>. A gene of *A. nidulans*, *Hsp30* is homologous to the *Hsp26* gene of *Saccharomyces cerevisiae* and found upregulated in numerous stress conditions including low pH<sup>18,78</sup>. In another study, Iron deprivation is sensed as the nutritional deficiency in *Candida* cells, leading to the upregulation of Hsp30p<sup>79</sup>.

Targeting small heat shock proteins (sHSPs) can be an effective combat strategy to overcome drug resistance in pathogenic fungi. Sequence alignment analysis of sHSPs showed no similarity in the nucleotides or protein sequences with that of humans (data not shown). Understanding sHSPs and its interactive partners not only allow us to study drug resistance but also can be the promising lead therapeutic molecules. It will be an interesting study to see their expression pattern during the progression of drug resistance in pathogenic fungi.

## DISCUSSION

Pathogenic fungi are a major cause of secondary infection and hospital-acquired infections. Drug resistance in fungi is the major setback in the treatment of these infections. Several studies have shown the promising role of sHSPs as novel targets to develop an effective treatment against these drug resistant fungal infections. A study showed that potential antifungal treatment of *C. albicans* is achieved by over-expressing Hsp12<sup>65</sup>. Role of Hsp10 and Hsp30/Hsp31 are still not very clear, while on the other hand it will be fascinating to find the crucial function of Hsp21 in the understanding of antifungal drug resistance development. Hsps are not only playing vital roles in many major cellular pathways, such as calcium-calcineurin, MAPK, Ras1-cAMP-PKA, and cell cycle control signaling but several client proteins of Hsps are signaling molecules. Moreover, different groups have reported the involvement of Hsps to confer antifungal drug resistance by modulating these signaling pathways in *C. albicans*. Thus, developing new effective antifungal targets are required to investigate HSPs and other signaling molecules of HSPs-associated pathways in *C. albicans*. Therefore, the study of HSPs expression and the functional role will help us better in exploring

not only their roles as chaperones but also their indulgent in disease development and progression caused by fungal parasites.

## CONCLUSION

This review summarizes some of the explored functions of sHSPs in direction of drug resistance in fungal biology and indicating that available information is insufficient to understand the in-depth role of these sHSPs in drug resistance. Hence sHSPs provide a new horizon to explore the unexplored heat shock molecules to understand their regulation and function in the interactome of cell molecules and could be promising targets to develop new therapeutics to combat drug resistance in pathogenic fungi. Moreover sHSPs have not been given enough attention for a long time to understand their biological roles and targeting them for therapeutic use for overcoming fungal drug resistance.

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## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

## ETHICS STATEMENT

Not applicable.

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