Pharmacological targeting of Ire1 of *Candida albicans*

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Abstract

The common fungal pathogen Candida albicans primarily affects immunocompromised individuals and the rise in antifungal resistance underscores the urgent need for new treatment options. In this study, we investigate the potential of STF-83010, a human Ire1 inhibitor, as a treatment for candidiasis. Our findings reveal that STF-83010 effectively inhibits Hac1 splicing and increases susceptibility to DTT-induced ER stress. Additionally, the inhibitor significantly reduces biofilm formation and blocks the yeast-to-hyphal transition, two critical factors in C. albicans pathogenicity.

Further analysis indicates that STF-83010 impairs thermotolerance and reduces the expression of virulence-related genes. Our findings suggest that STF-83010 can be used as an antifungal treatment to target the UPR pathway in C. albicans.

Keywords: *Candida albicans*, *HAC1* splicing, Ire1, STF-83010, UPR.

Introduction

The endoplasmic reticulum (ER) plays a key role in essential cellular processes, particularly in the folding, modification and synthesis of proteins destined for secretion or integration into organelles. This complex process is finely regulated and is crucial for maintaining ER homeostasis. When this balance is disturbed, it leads to a condition known as ER stress. To manage this, cells activate an adaptive mechanism known as the unfolded protein response (UPR). The UPR is a homeostatic pathway that senses the buildup of misfolded or unfolded proteins in the ER and adjusts the ER's capacity to restore proper protein folding. UPR is activated by ER-resident proteins and consists of three main branches in mammalian cells, each controlled by a specific ER sensor: i) Ire1, ii) ATF6 and iii) PERK[8,16,17]. Together, these branches work to alleviate ER stress and maintain cellular function.

Among the three branches of the UPR signalling network, the Ire1 branch is the only branch present in fungus that offers a crucial platform for determining cell fate in ER stress[20]. Ire1 is a type I transmembrane protein with an N-terminal ER luminal domain that detects misfolded protein and a C-terminal cytosolic region that contains both kinase and endoribonuclease (RNase) domains[7]. Upon the accumulation of unfolded proteins, the ER chaperone BiP is released from ER luminal domain, triggering the activation of Ire1[2,5]. Once activated, Ire1's endonuclease domain catalyses the splicing of mRNA encoding the bZIP transcription factor *XBP-1* in humans and *HAC1* in yeast, independently of the spliceosome[1,43]. After splicing, *HAC1* get activated and plays a vital role in restoring ER homeostasis[4,9].

The distinctive structure and function of Ire1 play a crucial role in UPR activation[26,50]. While Ire1's fundamental structure and function remain conserved from yeast to humans, slight structural modifications and downstream signalling execution account for the differing activation mechanisms between these taxa[10,32,50]. Researchers have focused on developing drugs that target different domains of Ire1 for therapeutic purposes. Additionally, downstream signalling components of Ire1 have also been of interest for drug development.

Various inhibitors of the kinase and nuclease domains have been utilised to investigate the role of UPR in human diseases and physiology[22,25,44,47]. Notably, salicylaldehyde-based inhibitors have shown significant potency in directly blocking the RNase domain of Ire1[46]. Earlier studies have revealed that these inhibitors form a stable Schiff base with a lysine residue in the RNase domain of Ire1[11,12,46]. The salicylaldehyde-based inhibitor, namely STF-83010 (a human Ire1 inhibitor), has been extensively discussed[13,19,35]; however, their effects on *C. albicans* remain unexplored. In this study, we demonstrate that a human *IRE1* inhibitor, STF-83010 can also effectively block the unfolded protein response (UPR) and can reduce pathogenicity in *Candida albicans.*

Material and Methods

Strain and growth condition: *Candida albicans* (SC5314) was cultured in YEPD medium composed of 1% yeast extract, 2% glucose, 2% bacto-peptone solidified with 2% agar. STF-83010 and fluconazole were purchased from Sigma-Aldrich, while DTT was obtained from Roche.

Drug susceptibility assays: The *C. albicans* was initially cultured overnight on YEPD plates. Afterwards, they were re-suspended in 0.9% saline solution to reach an OD_{600} of 0.1. From this suspension, each strain was spotted onto YEPD plates in five-microliter volumes across four successive dilutions (ranging from 5 × 10^3 to 5 × 10^5 cells). These spots were plated both with and without drugs/inhibitor. Following a 48-hour incubation period at 30° C, growth differences were observed on the plates.

Quantitative real-time PCR: *C. albicans* was grown overnight in YEPD medium and subsequently subcultured into fresh YEPD at a starting OD_{600} of 0.2. The cultures were incubated at 30° C until reaching an OD_{600} of 1.0. At this point, the inhibitor was added to the media and the cultures

were allowed to grow for an additional hour. Cells were harvested by centrifugation at 5,000 rpm for 5 minutes at 4° C from both treated and untreated control samples. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and DNA contamination was removed with DNase I treatment (Thermo Scientific). cDNA was synthesized following the manufacturer's instructions using the RevertAid H Minus First Strand cDNA synthesis kit (Biorad).

All real-time PCR reactions were carried out in a 25 µl volume using Thermo Scientific Maxima SYBR Green mix in a 96-well plate. Gene expression was quantified using the comparative CT method, where the fold change was calculated as $2^{-\Delta\Delta CT}$. The qPCR primers used in this study were designed with Primer Express 3.0 and are listed in table 1.

***HAC1* splicing:** *C. albicans* cells were exposed to DTT(Dithiothreitol) for one hour followed by the extraction of total RNA as per previously described protocols. The cDNA synthesis was performed using the RevertAid H Minus First Strand cDNA synthesis kit (Biorad). To assess *HAC1* mRNA splicing, we conducted reverse transcriptase-PCR (RT-PCR) with *HAC1*-specific primers, HAC1SP (F) and HAC1SP (R), as detailed in table 1. The resulting PCR products were analyzed using a 4% agarose gel. *ACT1* was utilized as an internal control.

Table 1
Primer List

Primer	Sequences
HAC1 SP F	AGACGCTTTTTGGAATTACCCATCACCA
HAC1 SP R	TCAAAGTCCAACTGAAATG
ACT1 F	GAAGCCCAATCCAAAAGAGG
ACT1 R	CTTCTGGAGCAACTCTCAAT
HWP1 F	GACCGTCTACCTGTGGGACAGT
HWP1 R	GCTCAACTTATTGCTATCGCTTATTACA
PLB1 F	GGTGGAGAAGATGGCCAA
PLB1 R	AGCACTTACGTTACGATGCAACA
ALS2 F	CCGGTATCGTCTGATTCATTAAG
ALS2 R	AATTGTCTGTTCTGGCCTGACA
ALS3 F	CCCCAACTTGGAATGCTGTT
ALS3 R	TGTATCTCCCGGACTTGCACTA
SAP2 F	GAATTAAGAATTAGTTTGGGTTCAGTTGA
SAP2 R	CCACAAGAACATCGACATTATCAGT

Morphogenesis assays: For the filamentation assay in liquid media, cells from an overnight YEPD culture were subcultured to an initial OD_{600} of 0.3 in fresh filamentation-inducing spider medium. The cultures were incubated at 37° C with continuous shaking for 4–5 hours. Aliquots were collected at different intervals over a 12-hour period, washed with 1X PBS and observed under a light microscope to monitor changes in filamentation patterns.

Biofilm assays: *In vitro* biofilm assays were performed using a spider medium, cultivating the biofilm directly at the bottom of 96-well polystyrene plates, following established protocols. *Candida albicans* strains were cultured overnight in YEPD at 30°C for 12–14 hours, then diluted to an OD_{600} of 0.5 in spider medium. The inoculated plates were covered with breathable films and incubated at 37° C for 90 minutes with shaking at 250 rpm to promote initial cell adhesion. After adhesion, the cells were washed with 200 µl of 1X PBS and 200 µl of fresh spider medium was added. The plates were then covered with new breathable films and incubated at 37° C for additional 24 hours with shaking at 250 rpm to facilitate biofilm formation. Post-incubation, the breathable films and medium were removed and the OD_{600} was measured using a standard plate reader to evaluate the extent of biofilm formation. A well containing only the medium served as a contamination control. Statistical significance was determined using a one-tailed paired Student's t-test with p-values as follows: * <0.05.

Results and Discussion

Conserved lysine residue is present in the Ire1 of *C. albicans*: The small molecule inhibitors have demonstrated remarkable efficacy in inhibiting the accumulation of Hac1 mRNA by forming a Schiff base with a critical lysine residue located within the active site of the RNase domain, as observed in both *H. sapiens* and *A. fumigatus*[43,46]. We extended our investigation to the RNase domain of Ire1, specifically probing for the presence of the conserved lysine. Using Clustal Omega, we aligned the Ire1 sequences of humans, *Aspergillus fumigatus* and *C. albicans*. Our findings confirmed the conservation of amino acid K1158 across these species (Figure 1A). The binding of STF-83010 with the lysine residue initiates the formation of a Schiff base, which crucially disrupts *HAC1* splicing, as demonstrated by previous research[11,46]. This suggests that STF-83010 may also inhibit Ire1 in *C. albicans*.

Docking and post-docking analysis: As already discussed, Ire1 is composed of different domains including the endonuclease domain, which is especially targeted by the STF-83010. Employing the CD-DOCK web server, we conducted docking studies of the protein-ligand interaction which exhibits binding affinity to Ire1's endonuclease domain, securing a docking score of -8 kcal/mol. The interaction interface encompassed residues Y1153, R1163, R1151, N1167, D1174, K1168, H1171, Y1153, R1151, Y1150, L1147 and N1144 (Figure 1B).

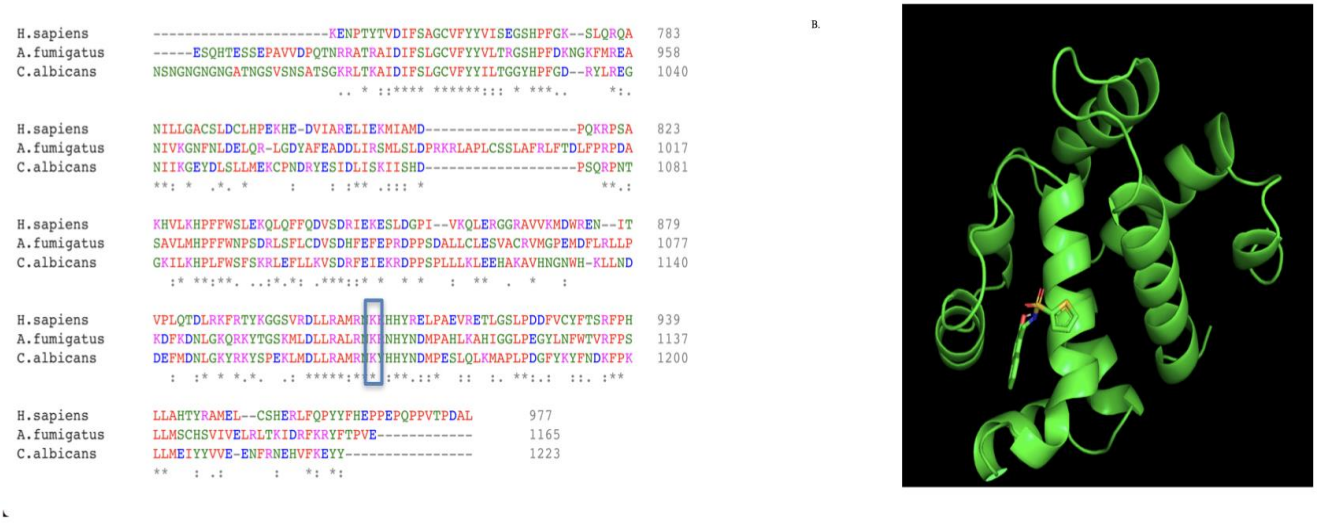

Figure 1: *C. albicans* has conserved lysine in active site of the RNase domain of Ire1. (A) Alignment of the RNase domain of human Ire1 with the corresponding region in *A. fumigatus and C. albicans*. The box indicates the conserved lysine residue targeted by STF-83010. (B) Molecular docking confirms the binding of STF-83010 with conserve lysine(1168Lys) of Ire1, The Ire1 structure is shown in cartoon model (green colour) while the STF-80310 is shown in stick model (red and green colours).

Ire1 inhibitor increases *Candida* sensitivity toward the ER stressor: To assess the impact of the STF-83010 on *C. albicans* growth, we conducted experiments where the yeast was grown in the presence of the inhibitor alone as well as in combination with ER stressors. Previous research had shown that the *ire1* mutant exhibited heightened sensitivity to azoles and ER stressors[14]. In this study, the effects of the inhibitor with the genetic deletion of Ire1 were compared. The results were striking; treating *C. albicans* with STF-83010 severely affected its growth under ER stress conditions, similar to the growth pattern observed in the *ire1* mutant. These findings suggest that the compound's inhibition of the UPR pathways may significantly impact the yeast's ability to cope with ER stress (Figure 2 A).

Deleting *IRE1* of *C. albicans* increased its sensitivity towards the azole[40]. This prompted us to check the effect of STF-83010 on the azole stressor. We grew *C. albicans* in the presence of fluconazole alone and in combination with STF-83010. We found that STF-83010 is not working synergistically with fluconazole (data not shown).

Ire1 inhibitor inhibits the canonical UPR pathway in *C. albicans*: Ire1 plays a pivotal and conserved role by interacting with unfolded proteins to initiate the Unfolded Protein Response (UPR), as highlighted by Hollien et al[17]. Under conditions of endoplasmic reticulum (ER) stress, Ire1 targets the unspliced form of *HAC1*, catalyzing its conversion to the spliced, active form by excising a 19-base pair intron[14,40]. To examine the impact of STF-83010 on *HAC1* splicing in *C. albicans*, we utilized reverse transcription-PCR (RT-PCR) to monitor *HAC1* splicing levels. Dithiothreitol (DTT) was used to induce ER stress in our experiments. Previous studies have shown that ER stress triggers *HAC1* splicing in *C. albicans*[14]. Once spliced, *HAC1* acts as a transcription factor, activating the transcription of UPR target genes which helps alleviate ER stress. Our findings reveal that adding STF-83010 to the medium inhibits *HAC1* splicing in *C. albicans* in a dose-dependent manner (Figure 2 B).

Ire1 inhibitors inhibits the virulence traits of *C. Albicans*: *C. albicans*, a commensal organism, naturally resides in diverse human body parts. It exists in polymorphic forms and its transition from yeast to hyphal states is a significant pathogenic trait[49]. Other pathogenic characteristics include biofilm formation, genome plasticity and cell-surface variation[23,29,31,41]. The versatility of *C. albicans* to alter its form from yeast to hyphae, its capability to form biofilms and its proficiency to thrive at elevated temperatures, constitute vital attributes that contribute to its successful infection within the human host.

The yeast-to-hyphae transformation plays a pivotal role in the development of infections. While both forms are present during infections, they fulfil distinct roles. The yeast form is implicated in the initial stages of infection such as adhesion and dissemination, while the hyphal form is associated with invasion[24,34].

Due to its crucial involvement in infections, filamentation has garnered significant attention among various candidiasis virulence factors and is considered a prime target for treatment[45]. Mutants lacking the ability to filament are non-virulent in murine models of invasive *C. albicans* infection[37]. Yeast and hyphal forms differ in physiology, cell wall composition and surface proteins. They exhibit distinct roles in immune cell recognition and pathogenesis and respond differentially to host immune cells[45].

We hypothesised that inhibiting filamentation development in *C. albicans* could serve as a valuable target for drug development. To explore this, we assessed the effect of STF-83010 on hindering the yeast-to-hyphae transition of *C. albicans. C. albicans* was cultured overnight at 30° C in a YEPD liquid medium. Subsequently, we initiated secondary cultures with 10^6 cells. We evaluated the effect of STF-83010 at time intervals of 2.5 hours, 5 hours, 7.5 hours and 12 hours under conditions conducive to high filamentation, namely spider medium at 37° C.

Our findings revealed that the inhibitor effectively impedes the morphological transition of *C. albicans* (Figure 3A). These findings prompted us to examine the impact of this inhibitor on the biofilm formation of *C. albicans*. Given the pivotal role of biofilm in facilitating successful infections by shielding the fungus from both host immune responses and antifungal drugs, our results point towards the inhibitory effect of STF-83010 on biofilm formation (Figure 3 B).

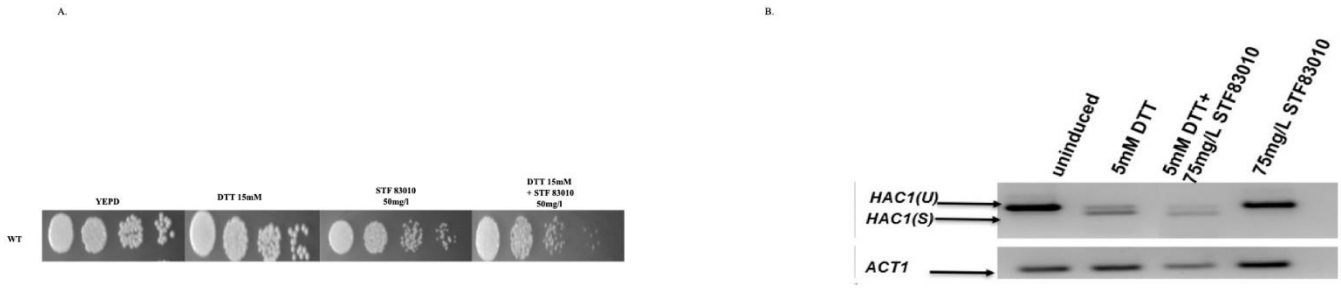

Figure 2: Ire1 inhibitor inhibits the canonical UPR pathway. (A) Five-fold serial dilutions of cell suspensions were spotted on YEPD plates in the absence (Growth control) and in the presence of the compound of interest. Growth differences were recorded following incubation of the plates for 48 h at 30 ºC. (B) For *HAC1* splicing pattern analysis during ER stress,cDNA was obtained from the *C. albicans* after treatment with 5mM DTT or STF-83010 75mg/ml and both for 1 h, PCR amplification was done using primers flanking the *HAC1* intron and the product was analysed on a 4% agarose gel.

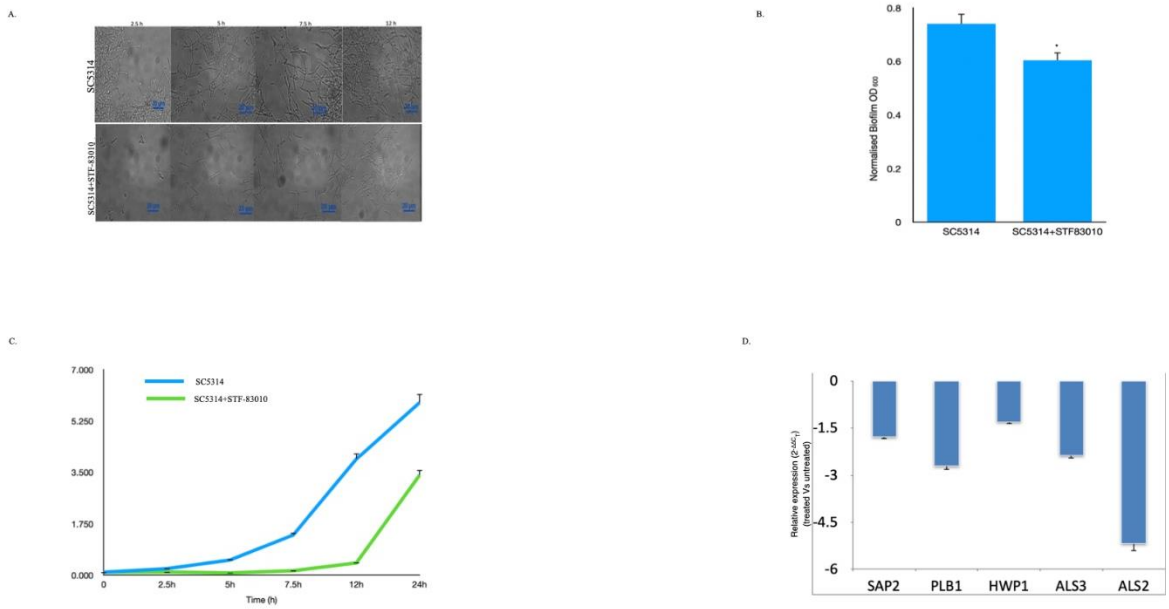

Figure 3: Ire1 inhibitor inhibits virulence traits in *C. albicans* (A) *C. albicans* (SC5314) was grown in filamentation-inducing Spider medium with or without treatment of STF-83010 (50mg/litre); in liquid spider medium at 37 °C and bud-to-hyphae transition was monitored over time by microscopy. (B) In vitro biofilm formation for SC5314, with or without STF-83010 in Spider medium after 24 h of culture growth was monitored. OD_{600} readings were measured for adhered biofilms after removal of the medium. (C) *C.albicans* was grown in YEPD medium with or without treatment of STF-83010 (50 mg/liter) at 42 °C and growth was monitored over time. (D) qPCR analysis for expression of *V*irulence related gene in the presence or absence of 50 mg/liter STF 83010. Fold change is calculated by $2^{-\Delta\Delta CT}$, normalized to *ACT1* (endogenous control). Values are mean ± *SD* derived from three independent experiments. Statistical significance was determined using a one-tailed paired Student's t-test with p-values as follows: * <0.05

The unfolded protein response (UPR) is a regulatory pathway activated in response to misfolded or degraded proteins in the endoplasmic reticulum due to elevated temperatures and other stresses. In *C. neoformans*, Ire1 has been implicated in virulence and thermotolerance[6]. In *C. albicans*, Ire1 serves as a primary sensor of misfolded proteins and triggers UPR[14], essential for growth at high temperatures[34]. Considering that STF-83010 is known to bind to the RNase domain of Ire1, we delved into their impact on impeding the growth of *C. albicans* under thermal stress. We conducted experiments using wild-type strains of *C. albicans*, both with and without the inhibitor, cultivating them at 30° C and 42° C. We monitored their growth over specific time intervals. Intriguingly, our observations revealed that when exposed to higher temperatures, *Candida* cells treated with the inhibitor, exhibited slower growth than the untreated sample (Figure 3 C).

Ire1 inhibitors block the UPR, biofilm formation, filamentation and thermotolerance of *C. albicans*. We were curious to know the effect of STF-83010 on the expression of virulence-related genes. Biofilm formation requires adhesion to host tissues, mediated by adhesin proteins such as agglutinin-like sequences (ALS) and hyphal wall protein (Hwp1)[6,18,26,28,33,36,48]. Additionally, *C. albicans* produces enzymes like secreted aspartyl proteinases (Saps) and phospholipases, which contribute to tissue invasion and damage[28,27,36,38,42]. Our results demonstrate that STF-83010 significantly reduces the expression of ALS, Hwp1, Saps and phospholipase genes (Figure 3D).

Conclusion

Candida albicans is a prevalent human fungal pathogen that primarily affects immunocompromised individuals. The increased usage of antifungal drugs has led to escalating drug resistance. Coupled with limited drug availability, the urgency for novel solutions is evident. Exploring small molecule inhibitors targeting the kinase and RNase domains of human Ire1 to induce unfolded protein levels within tumor cells has garnered attention. Combining these inhibitors with drugs that amplify endoplasmic reticulum (ER) stress, shows promise in targeting tumor cells effectively[20]. While extensively studied in animal models, the impact of the inhibitor on candidiasis remains uncharted.

Our study investigated the potential of STF-83010, human Ire1 inhibitor, in treating candidiasis. Our findings demonstrate that STF-83010 effectively inhibits Hac1 splicing and sensitizes *C. albicans* to endoplasmic reticulum (ER) stress induced by DTT, though it does not exhibit significant synergy with fluconazole. More importantly, the STF-83010 significantly reduces biofilm formation and halts the yeast-to-hyphal transition, both of which are critical for the virulence of *C. albicans*. Furthermore, STF-83010 decreases the expression of virulence-related genes and impairs thermo-tolerance. These results suggest that pharmacological targeting of the unfolded protein response (UPR) pathway, specifically through Ire1 inhibition, may represent a promising strategy to weaken *C. albicans* virulence.

In conclusion, our study highlights the potential of human Ire1 inhibitors such as STF-83010, as novel antifungal agents against *C. albicans*. Their ability to disrupt key virulence factors, including biofilm formation and hyphal development, paves the way for future antifungal therapies aimed at mitigating candidiasis.

Acknowledgement

We would like to extend my gratitude to Dr. Sneh Lata Panwar for her invaluable support.

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(Received 03rd August 2024, accepted 07th October 2024)