A Human IRE1 Inhibitor Blocks the Unfolded Protein Response in the Pathogenic Fungus Aspergillus fumigatus and Suggests Noncanonical Functions within the Pathway

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ABSTRACT - The unfolded protein response (UPR) is a signaling network that maintains homeostasis of the endoplasmic reticulum (ER). In the human-pathogenic fungus Aspergillus fumigatus, the UPR is initiated by activation of an endoribonuclease (RNase) domain in the ER transmembrane stress sensor IreA, which splices the downstream mRNA hacAu into its active form, hacAi, encoding the master transcriptional regulator of the pathway. Small-molecule inhibitors against IRE1, the human ortholog of IreA, have been developed for anticancer therapy, but their effects on the fungal UPR are unexplored. Here, we demonstrate that the Ire1 RNase inhibitor 4μ8C prevented A. fumigatus from increasing the levels of hacAi mRNA, thereby blocking induction of downstream UPR target gene expression. Treatment with 4μ8C had minimal effects on growth in minimal medium but severely impaired growth on a collagen substrate that requires high levels of hydrolytic enzyme secretion, mirroring the phenotype of other fungal UPR mutants. 4μ8C also increased sensitivity to carvacrol, a natural compound that disrupts ER integrity in fungi, and hygromycin B, which correlated with reduced expression of glycosylation-related genes. Interestingly, treatment with 4μ8C was unable to induce all of the phenotypes attributed to the loss of the canonical UPR in a ΔhacA mutant but showed remarkable similarity to the phenotype of an RNase-deficient IreA mutant that is also unable to generate the hacAi mRNA. These results establish proof of principle that pharmacological inhibition of the canonical UPR pathway is feasible in A. fumigatus and support a noncanonical role for the hacAu mRNA in ER stress response.

IMPORTANCE The unfolded protein response (UPR) is a signaling pathway that maintains endoplasmic reticulum (ER) homeostasis, with functions that overlap virulence mechanisms in the human-pathogenic mold Aspergillus fumigatus. The canonical pathway centers on HacA, its master transcriptional regulator. Translation of this protein requires the removal of an unconventional intron from the cytoplasmic mRNA of the hacA gene, which is achieved by an RNase domain located in the ER transmembrane stress sensor IreA. Here, we show that targeting this RNase activity with a small-molecule inhibitor effectively blocked UPR activation, resulting in effects that mirror the consequences of genetic deletion of the RNase domain. However, these phenotypes were surprisingly narrow in scope relative to those associated with a complete deletion of the hacA gene. These findings expand the understanding of UPR signaling in this species by supporting the existence of noncanonical functions for the unspliced hacA mRNA in ER stress response.

KEYWORDS A. fumigatus, UPR, 4μ8C, ER stress, Ire1, IreA, XBP1, Hac1, HacA, secretion, RNase, STF-083010, Ire1, Xbp1
several species that infect plants, animals, or humans have uniquely adapted this stress response pathway to support the expression of pathogenicity traits, such as antifungal drug resistance, nutrient acquisition, host-temperature adaptation, cell wall or membrane homeostasis, and effector protein secretion (2–12). The pathway detects and responds to the accumulation of unfolded proteins, which is a situation that arises when the demand for secretion exceeds the protein folding capacity of the ER or when the cell encounters stimuli that disrupt ER homeostasis. The resulting accumulation of unfolded proteins activates the cytosolic RNase domain of the ER transmembrane stress sensor IreA, which removes an intron from the uninduced mRNA hacA, converting it into the induced mRNA hacA, which is translated into the bZIP transcription factor HacA that coordinates a transcriptional program to augment ER folding capacity. The small-molecule inhibitor 4μ8C blocks the activity of the IreA RNase domain required for hacA mRNA processing. (B) Alignment of the RNase domain of human Ire1 with the corresponding region in A. fumigatus, C. albicans, and C. neoformans. The arrow indicates the conserved lysine residue targeted by 4μ8C. Black shading indicates identity to the column consensus and gray indicates similarity.

**FIG 1** Schematic representation of the UPR pathway in *A. fumigatus* and alignment of the amino acid sequence of the RNase domain of human Ire1 with orthologs in fungi. (A) The UPR is activated by ER stress, which occurs when the demand for secretion exceeds the protein folding capacity of the ER or when the cell encounters stimuli that disrupt ER homeostasis. The resulting accumulation of unfolded proteins activates the cytosolic RNase domain of the ER transmembrane stress sensor IreA, which removes an intron from the uninduced mRNA hacA, converting it into the induced mRNA hacA, which is translated into the bZIP transcription factor HacA that coordinates a transcriptional program to augment ER folding capacity. The small-molecule inhibitor 4μ8C blocks the activity of the IreA RNase domain required for hacA mRNA processing. (B) Alignment of the RNase domain of human Ire1 with the corresponding region in *A. fumigatus*, *C. albicans*, and *C. neoformans*. The arrow indicates the conserved lysine residue targeted by 4μ8C. Black shading indicates identity to the column consensus and gray indicates similarity.
for the translation of the bZIP transcription factor HacA (1, 3, 5) (Fig. 1A). This master transcriptional regulator of the canonical UPR pathway helps to buffer physiological fluctuations in ER stress by orchestrating a transcriptional program that augments the folding of proteins trafficking through the secretory pathway (3, 13, 17).

Selective inhibitors of the kinase or RNase domain of human IRE1 have been developed as tools to experimentally dissect the contribution of the UPR to cell physiology and human disease, particularly with respect to cancer (18–24). Among these compounds, salicylaldehyde-based inhibitors have shown high efficacy by directly blocking the RNase domain (25). The compounds 4µ8C and STF-083010 are two well-described examples of this class (26–29), but their effects on a fungal pathogen are still unexplored. Here, we demonstrate that 4µ8C (30) is an inhibitor of the canonical UPR pathway in A. fumigatus, effectively blocking the accumulation of hacA mRNA and downstream target gene induction. We found that treatment with 4µ8C failed to recapitulate the entire collection of phenotypes associated with a ΔhacA deletion mutant but was similar to phenotypes displayed by an IreA RNase domain mutant that is unable to process hacA mRNA into hacA'. These data demonstrate the feasibility of pharmacological disruption of the canonical UPR pathway in a fungal pathogen and support emerging evidence that both the unspliced and spliced hacA mRNAs have functions in ER stress responses.

RESULTS

The human IRE1 RNase inhibitor 4µ8C blocks the canonical UPR of A. fumigatus. We previously showed that the canonical UPR pathway of A. fumigatus involves a linear order of molecular events triggered by the activation of IreA in the presence of ER stress (3). Upon activation, the endoribonuclease domain of IreA splices an intron from the uninduced cytoplasmic mRNA hacA, converting it to its induced form, hacA', which is subsequently translated into the encoded transcription factor HacA (Fig. 1A). Subsequent studies revealed that deleting the ireA gene, or the ortholog in other fungal species (5, 31), is more deleterious than deleting the gene encoding the downstream transcription factor, suggesting that additional branches of the pathway emanating from IreA contribute to the ER stress response independently of HacA (Fig. 1A). To further examine the possibility of more complexity in the pathway, we focused in this study on the RNase domain, incorporating both small-molecule and genetic inhibition approaches.

The synthetic coumarin derivative 8-formyl-7-hydroxy-4-methylcoumarin (abbreviated as 4µ8C) was initially identified in a high-throughput screen for selective inhibitors of the human IRE1 RNase (30). The molecule forms a Schiff base with a lysine residue in the active site of the RNase domain, which prevents IRE1 from splicing its target mRNA (XBP1” in humans). Since the homologous IRE1 is conserved in A. fumigatus IreA (Fig. 1B), we hypothesized that 4µ8C could be used to modulate the processing of hacA mRNA into its activated form, hacA' (Fig. 1A). To test this, we used quantitative reverse transcription-PCR (RT-qPCR) analysis to monitor the induction of hacA' levels during acute ER stress. Dithiothreitol (DTT) is the most widely used approach to experimentally induce ER stress because it reduces the disulfide bridges that stabilize many proteins in the secretory pathway, resulting in a rapid increase in the level of unfolded proteins that activate the IreA/RNase sensor (32). Consistent with previous reports (33), DTT treatment of A. fumigatus hyphae induced accumulation of hacA mRNA, reflecting activation of the IreA RNase domain (Fig. 2A). Since the hacA gene promoter is a target of the HacA transcription factor (34), this surge in hacA' mRNA levels in the presence of DTT is due to a positive feedback loop that involves three linked events: first, initial activation of the IreA RNase converts basally expressed hacA” mRNA into hacA’; second, translation of hacA’ mRNA into the HacA transcription factor increases transcription from the hacA gene promoter itself, thereby replenishing the pool of hacA” mRNA; and third, splicing of hacA” into hacA’ continues as long as the IreA RNase remains activated (5, 35). We found that incorporation of 4µ8C into the medium prevented DTT from triggering an increase in hacA’ mRNA abundance, dem-
onstrating that the canonical UPR is not responsive to an acute ER stress stimulus in the presence of this compound. The lowest concentration for blocking the UPR under these conditions was determined to be 10 mg/liter (see Fig. S1 in the supplemental material), so this concentration was used for all subsequent experiments in this study.

We have previously reported that low levels of hacAi are also detectable in the absence of added ER stress, representing a basal UPR that functions to buffer fluctuations in protein folding that arise during normal vegetative growth (5). We found that treatment with 4μ8C also reduced the steady-state levels of hacAi mRNA under these conditions (Fig. 2B), consistent with the ability of the compound to impair basal IreA activation. In contrast, expression of the sod1 gene, which does not depend on the UPR for its expression (36), was unaffected by 4μ8C treatment (Fig. 2B), suggesting that downregulation of hacAi mRNA levels by 4μ8C is not due to a general downregulation of transcription. Moreover, since the effects of DTT on cell physiology are not limited to ER protein folding (37, 38), we also compared the effects of 4μ8C on the expression of Afu8g05720, representing a gene that we have previously shown to be induced by DTT independently of the UPR (5). As shown in Fig. 2C, 4μ8C did not block the induction of this gene by DTT, illustrating specificity of 4μ8C treatment for the inhibition of UPR target genes.

Since 4μ8C effectively blocks hacAi induction during acute ER stress (Fig. 2A), the compound should also prevent the upregulation of UPR target genes that are under the control of the HacA transcription factor. To test this, we compared the expression levels of three genes that are established UPR targets across multiple species: the Hsp70 chaperone gene bipA, the protein disulfide isomerase gene pdiA, and the oxidoreductase gene eroA (34, 39). As expected, all three of these UPR markers were strongly induced by DTT in A. fumigatus (Fig. 2D). However, no induction was detected in the

![FIG 2](image-url)
presence of 4μ8C, consistent with the ability of this compound to prevent the accumulation of hacA mRNA (Fig. 2D). The compound also blocked the ability of DTT to induce the expression of srcA and pmrA (Fig. S2A), encoding ER/Golgi P-type Ca2++ ATPases that we have recently identified as targets of the UPR in A. fumigatus (33). Interestingly, we found that a ΔsrcA/ΔpmrA double deletion mutant was hypersensitive to the effects of 4μ8C relative to its parental strain (Fig. S2B). Since the absence of these Ca2++ ATPases exacerbates ER stress (33), the increased sensitivity of this mutant to 4μ8C is likely to be due to the ability of the compound to prevent the canonical UPR from mounting an adaptive response to low Ca2++ levels in the secretory pathway caused by the absence of these Ca2++ pumps. We conclude that 4μ8C is able to enter the fungus and reach the IreA sensor, thereby inhibiting the RNase activity that is required for hacA mRNA processing and downstream activation of UPR target genes.

4μ8C impairs growth on a polymeric protein substrate. Mutants of A. fumigatus that harbor deletions of the hacA gene grow relatively well on medium containing simple carbon and nitrogen sources (3). Similarly, we found that the growth rate of the A. fumigatus parental strain KU70 on solid minimal medium supplemented with 4μ8C was only slightly reduced, comparable in magnitude to the mild growth inhibition displayed by a KU70-derived mutant lacking the hacA gene (Fig. 3A). In liquid culture, both KU70 and the unmodified wild-type strain CEA10 showed only a 10% reduction in metabolic activity in the presence of 4μ8C (Fig. 3B), making it unlikely that the inhibitory effect of 4μ8C on UPR gene expression is due to broad inhibition of fungal metabolism. In addition, although a slight reduction in spore germination rate was observed in the presence of 4μ8C, the total biomass accumulation after 24 h of incubation in liquid culture was indistinguishable in the presence or absence of the compound (Fig. S3). We conclude that pharmacological inhibition of the IreA RNase domain has minimal growth-impairing effects on A. fumigatus, consistent with the relatively normal growth characteristics of the ΔhacA mutant in the absence of stress.

The ability of 4μ8C to block the induction of hacAi mRNA and downstream UPR target gene activation under ER stress predicted that treatment with this compound would have similar effects as deleting the hacA gene under conditions that require UPR activity. Filamentous fungi that lack the hacA gene grow poorly on polymeric substrates because their secretory pathways are unable to meet the demand for hydrolytic enzyme secretion (3, 9, 40, 41). To determine how 4μ8C would impact utilization of a complex protein substrate, conidia were inoculated into a solution of type I collagen as the only source of carbon and nitrogen. Although both KU70 and CEA10 isolates grew well on this medium, the inclusion of 4μ8C strongly impaired growth (Fig. 3C). The ΔhacA mutant also grew poorly on this substrate, indicating that loss of the canonical UPR, either by genetic deletion of hacA or by treatment with 4μ8C, impairs the ability to grow on a protein substrate that requires enzymatic breakdown by secreted hydro-lases prior to absorption.

FIG 3 Growth on a complex protein polymer is impaired in the presence of 4μ8C. (A) Conidia were spot inoculated onto the center of plates containing AMM in the presence/absence of 10 mg/liter 4μ8C, and colony diameter was measured after 6 days at 37°C. (B) Overnight cultures in liquid AMM at 37°C were treated with 4μ8C at the indicated concentrations for 1 h, and metabolic activity was determined using resazurin. (C) Conidia from the indicated strains were inoculated onto the surface of a collagen gel matrix containing 10 mg/liter 4μ8C. Images of mycelial growth were captured after 72 h at 37°C. Bars show mean values ± SD of the results from three biological replicates per strain and condition (****, P < 0.0001; ns, not significant [one-way ANOVA with Tukey’s post hoc test]).
4µ8C increases sensitivity to the antimicrobial agents carvacrol and hygromycin. The inability of *A. fumigatus* to activate the canonical UPR in the presence of 4µ8C suggests that the fungus would be hypersensitive to a drug that causes ER stress. To test this, the effects of 4µ8C on growth were compared in the presence of carvacrol, a plant-derived compound that disrupts ER homeostasis in *Candida albicans* and triggers UPR intervention (42). As shown in Fig. 4A, carvacrol induced accumulation of the hacAi mRNA in the CEA10 strain, confirming that this compound has similar adverse effects on ER homeostasis in *A. fumigatus* as it does in *C. albicans*. In addition, the ΔhacA mutant was hypersensitive to carvacrol relative to its parental strain KU70, indicating a role for the canonical UPR in protecting against carvacrol-induced ER stress (Fig. 4B). We found that treatment with 4µ8C prevented carvacrol-induced hacAi accumulation (Fig. 4A), which was associated with increased susceptibility to carvacrol in both the KU70 and CEA10 backgrounds (Fig. 4B). An isobolographic analysis (43) in liquid culture confirmed that the combination of 4µ8C and carvacrol treatment had synergistic toxicity toward the fungus (Fig. 4C). We conclude that preventing the canonical UPR from responding to carvacrol-induced ER stress, either by chemical treatment with 4µ8C or by genetic deletion of hacA, is toxic to *A. fumigatus*.

Other plant-derived compounds that are structurally related to carvacrol have been shown to enhance the toxicity of drugs that target translation fidelity, such as aminoglycosides (44). Aminoglycosides are a therapeutically important class of antimicrobials that reduce the discrimination between cognate and near-cognate tRNAs, causing the incorporation of incorrect amino acids that promotes the accumulation of toxic protein aggregates (45). As shown in Fig. 4A, the aminoglycoside hygromycin B (HygB) induced hacAi in *A. fumigatus*, which was blocked by treatment with 4µ8C (Fig. 4A), suggesting a role for the UPR in countering protein folding stress associated with HygB-induced translational errors. In support of this, we found that the ΔhacA mutant was hypersensitive to HygB and that treatment of either KU70 or CEA10 strains with 4µ8C increased...
sensitivity to this compound (Fig. 4B). An alternative explanation for this increased HygB susceptibility stems from the observation that yeast glycosylation mutants are hypersensitive to this aminoglycoside, a phenomenon that is attributed to one or more HygB resistance proteins that require glycosylation for optimal activity (46). Our previous demonstration that the ΔhacA mutant is deficient in the expression of genes involved in N-glycan biosynthesis (5) would be consistent with a glycosylation defect that enhances HygB sensitivity. This predicts that UPR inhibition by 4μ8C would also reduce the expression of glycosylation-related genes. To test this, we compared the expression levels of glycosylation genes that were previously shown to be downregulated in the ΔhacA mutant (5): mdsS (encoding an α-mannosidase) (47), the DTT-inducible gene Afu5g08970 (WBPI in Saccharomyces cerevisiae, an oligosaccharyl transferase gene), and Afu5g03500 (ROT2 in S. cerevisiae, a glucosidase I gene). Similarly to ΔhacA (Fig. 5A), all three genes were downregulated in A. fumigatus after treatment with 4μ8C as demonstrated by RT-qPCR analysis (Fig. 4D). As expected, the induction of WBP1 and ROT1 upon ER stress with 1 mM DTT was blocked in the presence of the inhibitor (Fig. 4D). We conclude that the canonical UPR is an integral part of the cellular response to HygB in A. fumigatus and that preventing UPR activation, either by treatment with 4μ8C or by genetic deletion of hacA, enhances the toxicity of this aminoglycoside against the fungus.

4μ8C reveals unexpected complexity in the canonical UPR pathway. The ΔhacA mutant of A. fumigatus exhibits increased sensitivity to both the triazole and echinocandin classes of antifungals, as well as to the ER stress agents DTT and tunicamycin (3). Although blocking hacA mRNA induction by 4μ8C treatment increased carvacrol and hygromycin B sensitivity, we were surprised to find that the compound had minimal effects on the sensitivity of the fungus to DTT, tunicamycin, or itraconazole (Fig. 5A). A fundamental difference between the ΔhacA mutant and chemical inhibition of hacA processing is that the ΔhacA mutant lacks both hacA and hacA mRNAs due to the absence of the hacA gene, whereas the 4μ8C-treated organism still expresses hacA (Fig. 2B). This raises the possibility that residual hacA levels in cultures treated with 4μ8C are sufficient to provide some protection against ER stress. We therefore examined a mutant that harbors an inactivating deletion in the RNase domain of ireA but still retains the hacA gene (ireAΔRNase). As observed with 4μ8C treatment, no hacA mRNA could be detected by RT-qPCR in the ireAΔRNase mutant, even in the presence of a strong ER stress stimulus, confirming that this strain is incapable of mounting a canonical UPR response (Fig. 5B). The levels of hacA were notably elevated in the ireAΔRNase mutant (Fig. 5B), consistent with a failure to process basally expressed hacA mRNA into hacA. Interestingly, and similar to what we observed with 4μ8C treatment, the ireAΔRNase mutant showed no hypersensitivity to DTT, tunicamycin, or itraconazole (Fig. 5A).

We have previously shown that the ΔhacA mutant has a lower MIC to caspofungin using an MIC test strip and that the normally fungicidal effects of caspofungin toward A. fumigatus became fungicidal in the absence of hacA (Fig. 5C) (3). In contrast, caspofungin remained fungistatic to the ireAΔRNase mutant and the 4μ8C-treated parental strain, with no decrease in the MIC (Fig. 5C). In addition, we found that neither the ireAΔRNase mutant nor treatment with 4μ8C increased thermal stress sensitivity as previously reported for the ΔhacA mutant (Fig. 5D) (3). However, the ireAΔRNase mutant was hypersensitive to carvacrol and grew poorly on a collagen substrate (Fig. 5F and F), similar to the effects of 4μ8C on the control strain (Fig. 3C and Fig. 4B). Together, these findings indicate that blocking hacA accumulation under conditions of ER stress by either a chemical or genetic approach is less detrimental to the fungus than the complete absence of the hacA gene, suggesting that there is additional complexity in the pathway that involves functions for the unspliced hacA mRNA that are distinct from the spliced mRNA.

DISCUSSION

Selective inhibitors of the kinase and/or RNase domains of human IRE1 are under development for the purpose of creating toxic levels of unfolded proteins in human tumor cells, particularly when used in combination with drugs that exacerbate ER stress. 4μ8C inhibits the Aspergillus fumigatus UPR.
These compounds are well tolerated in animal models and have been associated with favorable therapeutic outcomes (21–24, 50). However, their effects on a fungal pathogen have not been reported. Here, we demonstrate that the human IRE1 RNase inhibitor 4μB8C impaired the ability of A. fumigatus to maintain basal levels of hacA mRNA under vegetative growth conditions, in addition to preventing the accumulation of hacA under conditions of acute ER stress induced by treatment with DTT or the natural product carvacrol. This correlated with reduced expression levels of known UPR target genes, reflecting inhibition of the canonical UPR. STF-083010 is another inhibitor of the human IRE1 RNase and is structurally related to 4μB8C (21). We found that this compound also impaired hacA mRNA induction and sensitized the fungus to carvacrol-induced ER stress (see Fig. S5 in the supplemental material), providing additional support for the notion that disabling the fungal UPR is achievable by small-molecule inhibition. However, in contrast to 4μB8C, STF-083010 was only partially effective, so it was not pursued further in this study. Our data on 4μB8C provide the first validation of small-molecule inhibition of the fungal UPR in A. fumigatus, which could provide a valuable tool for future studies into how this stress response pathway integrates with other cellular circuitry to coordinate adaptive responses in fungi. It is also worth mentioning that UPR activity impacts one of the bottlenecks that limit the secretion of proteins by filamentous fungi used in the biotechnology industry (51).

FIG 5 The compound 4μB8C reveals additional complexity in the canonical UPR pathway. (A) Serial dilutions of conidia from the indicated strains were spotted onto AMM plates containing DTT, tunicamycin (Tn), or itraconazole (ITZ) in the presence or absence of 10 mg/liter 4μB8C and incubated for 48 h at 37°C. (B) Fold change in the expression of hacA and hacA mRNAs by RT-qPCR after treatment of the indicated strains for 1 h with 1 mM DTT. Cultures inoculated at 10⁶ conidia/ml were grown in AMM for 16 h at 37°C. (C) The caspofungin sensitivities of the ireAΔRNase and ΔhacA mutants were compared to that of the parental strain KU70 in the presence or absence of 4μB8C using the MIC test strip method. (D) Colony diameters on minimal medium at 37°C and 45°C. Conidia from the indicated strains were spot inoculated onto the center of plates containing AMM, and colony diameter was measured after 6 days at 37°C. (E) Serial dilutions of conidia from the indicated strains were spotted onto AMM plates containing carvacrol and incubated for 72 h at 37°C. (F) Conidia from the indicated strains were inoculated onto the surface of a collagen gel matrix, and mycelial growth was photographed after 72 h at 37°C. Values in panels B and D represent the mean ± SD of the results from three biological replicates per strain and condition (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; nd, not detected; ns, not significant [one-way ANOVA with Dunnett’s (B) or Tukey’s (D) post hoc test]).
suggesting that 4μ8C could have broader utility as a way to understand how the UPR affects industrial secretion processes.

It is well known that filamentous fungi possess a high capacity to secrete large quantities of hydrolytic enzymes that allow them to break down complex substrates (52). Minimal medium that is optimized for the growth of A. fumigatus contains simple forms of carbon and nitrogen that are readily assimilated by the fungus. Treatment with 4μ8C had only a minor inhibitory effect on growth in this medium, similar to the mild growth impairment displayed by a ΔhacA mutant under the same conditions (Fig. 3). In contrast, either deletion of hacA or treatment with 4μ8C severely impaired the ability of A. fumigatus to grow on a collagen substrate. This is consistent with the importance of the UPR in supporting the accurate folding of secreted hydrolytic enzymes that are necessary to break down complex biopolymers (Fig. 3C).

Carvacrol is an essential oil from the oregano plant with in vitro antifungal activity against a variety of fungal species (42, 53–56) and has been shown to have potential in vivo application as a prophylactic agent for the prevention of avian aspergillosis (57). Although the precise molecular target of carvacrol is unknown, its ability to disrupt ER morphology and induce a UPR transcriptional signature in C. albicans clearly indicates that it causes acute ER stress (42). We found that carvacrol also induced the UPR in A. fumigatus, which could be blocked by treatment with 4μ8C (Fig. 4A). The two compounds showed synergistic toxicity against A. fumigatus, suggesting that 4μ8C prevents the accumulation of hacA mRNA needed to counteract the toxic effects of carvacrol on ER homeostasis (Fig. 4B and C). We also found that 4μ8C increased the susceptibility of C. albicans to carvacrol, suggesting a conserved mechanism of carvacrol between species (Fig. 56). Interestingly, our findings also revealed enhanced toxicity of hygromycin B against A. fumigatus in the presence of 4μ8C. This is likely to be due, in part, to the ability of 4μ8C to prevent the canonical UPR from adequately responding to the ER stress caused by hygromycin-induced errors in protein translation (Fig. 4A). In addition, the adverse effects of 4μ8C on the expression of glycosylation-related genes (Fig. 4D) would be expected to impair glycosylation-assisted protein folding, as well as impair glycosylation-dependent hygromycin B resistance mechanisms that have been well described from studies on yeast glycosylation mutants (46).

We conclude that 4μ8C-induced hypersensitivity to hygromycin B is likely to be multifactorial but mediated in part by UPR signaling through the canonical HacA-directed pathway.

Despite the existence of two overlapping reading frames in all species homologs of the mRNA encoding the UPR transcription factor, it is widely assumed that the spliced mRNA is the most relevant to ER homeostasis because it translates a bZIP transcription factor involved in UPR target gene expression. Interestingly, a phylogenetic analysis of vertebrate homologs of this mRNA has indicated that the evolution of the two overlapping reading frames argues for functionality of the unspliced transcript protein (58). Recent studies in human cells have shown that one function of the protein specified by the unspliced transcript is to target the nascent protein-mRNA-ribosome complex to the ER membrane via the signal recognition particle (SRP) pathway (59–62).

If the sole purpose of the protein encoded by the unspliced mRNA in A. fumigatus is bringing the hacA mRNA in the proximity of the IreA RNase in order to optimize splicing into hacA, one would predict that preventing hacA accumulation by treatment with 4μ8C or by IreA RNase domain mutation would have the same effects as deleting the hacA gene (Fig. 1A). However, our data show that the effects of deleting hacA are broader in scope relative to chemical or genetic RNase inhibition (Fig. 5), suggesting that there is unexplained functionality of the unspliced hacA mRNA that goes beyond serving as the precursor for hacA. In Cryptococcus neoformans, the majority of the phenotypes associated with ire1 deletion could be complemented with the spliced transcription factor gene but not by the unspliced version (10). However, in that study complementation was performed in the complete absence of Ire1; in our study Ire1 was still present but rendered incapable of inducing the canonical pathway due to chemical or genetic inhibition of the Ire1 RNase. Ire1 is also present in the ΔhacA mutant of A.
MATERIALS AND METHODS

Reagents. Aliquots of 4 μg/ml (EMD Millipore; 412512) of 25 mg/ml were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C until use. Dithiothreitol (DTT; Thermo Scientific; R0862) was dissolved in water at 1 M prior to use. Tunicamycin (Cayman Chemical; 11445) and itraconazole (Sigma; 16657) were dissolved in DMSO at 10 mg/liter and stored until use at −20°C or −80°C, respectively. Hygromycin B (RPI; H75020) was dissolved in water at 100 mg/ml and stored at −80°C prior to use. A 6.4 M stock solution of carvacrol (Sigma-Aldrich; W224511) was diluted to 0.64 M (96.74 g/liter) in DMSO. Dithiothreitol (Sigma-Aldrich; D4059) was dissolved in water at 100 mg/ml and stored at −20°C or −80°C. 2.6% (wt/vol) KCl, 2.6% (wt/vol) MgSO4 heptahydrate, 7.6% (wt/vol) KH2PO4, 5% (vol/vol) salt solution (2.6% [wt/vol] glucose, 1% [vol/vol] NH4 tartrate, and 2% (vol/vol) trace-element solution) and hygromycin B (RPI; H75020) were dissolved in water at 100 mg/ml and stored at −80°C prior to use. A 6.4 M stock solution of carvacrol (Sigma-Aldrich; W224511) was diluted to 0.64 M (96.74 g/liter) in DMSO prior to immediate use.

Strains and culture conditions. The A. fumigatus strains used in this study are summarized in Table S1 in the supplemental material. Conidia were harvested from cultures grown on OSM plates supplemented with 0.8% (wt/vol) agarose (UltraPure agarose; Invitrogen) and incubated at 37°C and photographed after 48 h. For analysis of germination rates, a total of 1 × 10³ conidia were inoculated into liquid AMM containing the indicated concentrations of 4 μg/ml and incubated at 37°C. Controls with DMSO were run in parallel. The number of germinated conidia was then quantified microscopically (90 to 183 per well) and their final concentration was 25 mg/ml were prepared in dimethyl sulfoxide (DMSO). The reaction was performed with the same cycle parameters described above for RT-qPCR. Since expression results obtained with SYBR green were similar to those obtained with a TaqMan probe, the SYBR green method was employed for the results presented in this study, using the same primer concentrations used for TaqMan. For hacAu and hacAi mRNA detection, the cycle conditions were 20 s at 95°C, 40 cycles of 3 s at 95°C, and 20 s at 66°C. For detection of other genes, the cycle conditions were 20 s at 95°C, 40 cycles of 3 s at 95°C, and 30 s at 60°C. The melting curve was monitored to verify the specificity of the amplification reaction. The 18S rRNA was used as a housekeeping gene. Fold change in mRNA levels was determined in comparison to untreated cultures.
Analysis of metabolic activity and antifungal susceptibility. For analysis of metabolic activity, conidia were inoculated at a concentration of 1 × 10⁶ conidia/ml in 150 μl of liquid AMM in a 96-well plate and incubated for 16 h at 37°C. The plates were washed three times, and the indicated concentrations of 4μB8 were added to the plates before incubating for an additional hour at 37°C. The final concentration of DMSO was 0.5% (vol/vol) in each well, including the growth controls. The cultures were then washed three times, and the medium was replaced with AMM containing 0.02 mg/ml of the oxidation-reduction metabolic indicator resazurin (14322; Cayman Chemicals). After incubating for 1 h at 37°C, the fluorescence was measured (excitation, 535 nm; emission, 590 nm) in a microplate reader (Synergy H1; BioTek). For analysis of synergy between carvacrol and 4μB8, conidia were inoculated into liquid AMM containing 0.02 mg/ml of resazurin at a concentration of 2.5 × 10⁶ conidia/ml. Serial 2-fold dilutions of 4μB8 were then dispensed from column 2 in the 96-well plate to column 9. Additionally, 2-fold dilutions of carvacrol were dispensed from row A to H into each well containing 4μB8. Columns 1 and 11 were used as growth and sterility controls, respectively. Columns 10 and 12 were used to display the individual MICs of carvacrol and 4μB8. The plates were incubated at 37°C for 24 h, after which the fluorescence was measured as described above. The MIC was defined as the lowest concentration required to inhibit 90% of fungal metabolic activity after 24 h of incubation. An isobologram representation was used to determine if the combination of 4μB8 and carvacrol had synergistic activity. The diagonal line in Fig. 4C connects the individual MICs for each compound (shown by the solid circles on the axis lines), and the remaining circles represent the MICs for different combinations of the two drugs. Synergistic, additive, and antagonistic effects are represented by solid circles that fall below the diagonal line, on top of the diagonal line, or above the diagonal line, respectively. The fractional inhibitory concentration (FIC) index was determined as (MIC carvacrol in combination with 4μB8/MIC carvacrol alone) + (MIC 4μB8 in combination with carvacrol/MIC 4μB8 alone). The interaction between the drugs was defined as synergistic (FIC < 0.5), indifferent (0.5 < FIC ≤ 4.0), or antagonistic (FIC > 4.0). Antifungal susceptibility using the MIC test strip (MTS) method was determined following the instructions of the manufacturer. A concentration of 10⁶ conidia/ml was spread with a sterile cotton swab onto a plate of RPMI 1640 agar with 0.164 M morpholinepropanesulfonic acid (MOPS) and L-glutamine (pH 7.0). RPMI (Synergy H1; BioTek). 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Statistical analysis. Statistical analysis was performed using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, CA, USA). Statistically significant differences were determined using one-way analyses of variance (ANOVA) with Dunnett’s or Tukey’s multiple-comparison tests.

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.
FIG S1, TIF file, 0.2 MB.
FIG S2, TIF file, 1.3 MB.
FIG S3, TIF file, 2.4 MB.
FIG S4, TIF file, 0.2 MB.
FIG S5, TIF file, 0.4 MB.
FIG S6, TIF file, 0.5 MB.
TABLE S1, DOCX file, 0.02 MB.
TABLE S2, DOCX file, 0.02 MB.

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REFERENCES
1. Preissler S, Ron D. 2019. Early events in the endoplasmic reticulum unfolded protein response. Cold Spring Harb Perspect Biol 11:a033894. https://doi.org/10.1101/cshperspect.a033894.
2. Hampel M, Jakobi M, Schmitz L, Meyer U, Finkernagel F, Doehlemann G, Heimel K. 2016. Unfolded protein response (UPR) regulator Cib1 controls expression of genes encoding secreted virulence factors in Ustilago maydis. PLoS One 11:e0153861. https://doi.org/10.1371/journal.pone.0153861.
3. Richie DL, Hartl L, Aimananda V, Winters MS, Fuller KS, Miley MD, White S, McCarthy JW, Latgé JP, Feldmesser M, Rhodes JC, Askew DS. 2009. A role for the unfolded protein response (UPR) in virulence and antifungal susceptibility in Aspergillus fumigatus. PLoS Pathog 5:e1000258. https://doi.org/10.1371/journal.ppat.1000258.
4. Bitencourt TA, Lang EAS, Sanches PR, Peres NTA, Oliveira VM, Fachin AL, Rossi A, Martinez-Rossi NM. 2020. HacA governs virulence traits and adaptive stress responses in Trichophyton rubrum. Front Microbiol 11:193. https://doi.org/10.3389/fmicb.2020.00193.
5. Feng X, Krishnan K, Richie DL, Aimananda V, Hartl L, Grahl N, Powers-Fletcher MV, Zhang M, Fuller KK, Nierman WC, Lu LJ, Latgé JP, Woollett L, Newman SL, Cramer RA, Rhodes JC, Askew DS. 2011. HacA-independent functions of the ER stress sensor IreA synergize with the canonical UPR to influence virulence traits in Aspergillus fumigatus. PLoS Pathog 7:e1002330. https://doi.org/10.1371/journal.ppat.1002330.
6. Wimalasena TT, Enjalbert B, Guillemette T, Plumridge A, Budge S, Yin Z, Brown AJP, Archer DB. 2008. Impact of the unfolded protein response upon genome-wide expression patterns, and the role of Hac1 in the
stress in the pathogenic fungus Candida albicans. Antimicrob Agents Chemother 59:4584–4592. https://doi.org/10.1128/AAC.00551-15.

43. Gesser PK. 1995. Isobolographic analysis of interactions: an update on applications and utility. Toxicology 105:161–179. https://doi.org/10.1016/0300-488X(95)03210-7.

44. Moreno-Martínez E, Vallerie C, Holland SL, Avery SV. 2015. Novel, synergistic antifungal combinations that target translation fidelity. Sci Rep 5:16700–16710. https://doi.org/10.1038/srep16700.

45. Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V, 2000. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature 407:340–348. https://doi.org/10.1038/35030019.

46. Dean N. 1995. Yeast glycosylation mutants are sensitive to aminoglycoside antibiotics. J Biol Chem 270:1287–1291. https://doi.org/10.1073/pnas.92.5.1287.

47. Li Y, Zhang L, Wang D, Zhou H, Ouyang H, Ming J, Jin C. 2008. Deletion of the msdS/AfmnsDc gene induces abnormal polarity and septation in Aspergillus fumigatus. Microbiology (Reading) 154:1960–1972. https://doi.org/10.1099/mic.0.2008/017525-0.

48. Mimura N, Fulciniti M, Gorgun G, Tai YT, Cirstea D, Santo L, Hu Y, Fabre C, Minami J, Ohguchi H, Kiziltepe T, Ikeda H, Kawano Y, French M, Blumenthal M, Tam Y, Kertesz NL, Malyankar UM, Hokenson M, Pham T, Zeng Q, Patterson JB, Richardson PG, Munshi NC, Anderson KC. 2012. Blockade of XBP1 splicing by inhibition of IRE1α is a promising therapeutic option in multiple myeloma. Blood 119:5772–5781. https://doi.org/10.1182/blood-2011-07-366633.

49. Chalmers F, Mogre S, Son J, Blazanin N, Glick AB. 2019. The multiple roles of unfolded protein response regulator IRE1α in cancer. Mol Carcinog 58:1623–1630. https://doi.org/10.1002/mc.23031.

50. Ri M, Tashiro E, Oikawa D, Shinjo S, Tokuda M, Yokouchi Y, Narita T, Masaki A, Ito A, Ding J, Kusumoto S, Ishida T, Komatsu H, Shiotzu Y, Ueda R, Iwawaki T, Imoto M, Iida S. 2012. Identification of Toxyacycin, a potent cytoxic for multiple myeloma cells, as a potent inhibitor of ER stress-induced XBP1α mRNA splicing. Blood Cancer J 2:e79. https://doi.org/10.1038/bcj.2012.26.

51. Guillemette T, van Peij HH, Goosen T, Lanthaler K, Robson GD, van den Ijssel P, Sterflinger K, Archer DB. 2007. Genomic analysis of the stress secretion system in the enzyme-producing cell factory Aspergillus niger. BMC Genomics 8:158. https://doi.org/10.1186/1471-2164-8-158.

52. Heimel K. 2015. Unfolded protein response in filamentous fungi—Implications in biotechnology. Appl Microbiol Biotechnol 99:121–132. https://doi.org/10.1007/s00253-014-6192-7.

53. Scalsas D, Mandras N, Roana J, Tardugno R, Cuffini AM, Ghisetti V, Benvenuti S, Tullio V. 2018. Use of Pinus sylvestris (Pinaceae), Origanum vulgare L. (Lamiaceae), and Thymus vulgaris L. (Lamiaceae) essential oils and their main components to enhance iraconazole activity against azole susceptible/not-susceptible Cryptococcus neoformans strains. BMC Complement Altern Med 18:143. https://doi.org/10.1186/s12906-018-2219-4.

54. Rao A, Zhang Y, Mued S, Rao R. 2010. Mechanism of antifungal activity of terpenoid phenols resembles calcium stress and inhibition of the TOR pathway. Antimicrob Agents Chemother 54:5062–5069. https://doi.org/10.1128/AAC.01050-10.

55. Kim J, Chan K, Mahoney N. 2015. Augmenting the activity of monoterpene phenols against fungal pathogens using 2-hydroxy-4-methoxybenzaldehyde that target cell wall integrity. Int J Mol Sci 16:2685–2687. https://doi.org/10.3390/ijms16122988.

56. Niu C, Wang C, Yang Y, Chen R, Zhang J, Chen H, Zhuge Y, Li J, Cheng J, Xu K, Chu M, Ren C, Zhang C, Jia C. 2020. Carvacrol induces Candida albicans apoptosis associated with Ca2+-Calcineurin pathway. Front Cell Infect Microbiol 10:192. https://doi.org/10.3389/fcimb.2020.00192.

57. Tartor VH, Hassan FAM. 2017. Assessment of carvacrol for control of avian aspergillosis in intratracheally challenged chickens in comparison to voriconazole with a reference on economic impact. J Appl Microbiol 123:1088–1099. https://doi.org/10.1111/jam.13557.

58. Nekrutenko A, He J. 2008. Functionality of unspliced XBP1α is required to explain evolution of overlapping reading frames. Trends Genet 24:645–646. https://doi.org/10.1016/j.tig.2008.09.012.

59. Plumb R, Zhang Z-R, Appathurai S, Mariappan M. 2015. A functional link between the co-translational protein translocation pathway and the UPR. Elife 4:2–7. https://doi.org/10.7554/Elife.07426.

60. Kanda S, Yanagitani K, Yokota Y, Eskali Y, Kohno K. 2016. Autonomous translational pausing is required for XBP1u mRNA recruitment to the ER via the SRP pathway. Proc Natl Acad Sci U S A113:ES886–ES889. https://doi.org/10.1073/pnas.1604435113.

61. Aragón T, van Anken E, Pincus D, Serafimova IM, Korennykh AV, Rubio CA, Walter P. 2009. Messenger RNA targeting to endoplasmic reticulum stress signalling sites. Nature 457:736–740. https://doi.org/10.1038/nature07641.

62. Yanagitani K, Kimata Y, Kadokura H, Kohno K. 2011. Translational pausing ensures membrane targeting and cytoplasmic splicing of XBP1u mRNA. Science 331:586–589. https://doi.org/10.1126/science.1197142.

63. Martin D, Li Y, Yang J, Wang G, Margariti A, Jiang Z, Yu H, Zampetaki A, Hu Y, Xu Q, Zeng L. 2014. Unspliced X-box-binding protein 1 (XBP1) protects endothelial cells from oxidative stress through interaction with histone deacetylase 3. J Biol Chem 289:30625–30634. https://doi.org/10.1074/jbc.M114.571984.

64. Zhao G, Fu Y, Cai Z, Yu F, Gong Z, Dai R, Hu Y, Zeng L, Xu Q, Kong W. 2017. Unspliced XBP1α protein targets VSMC homeostasis and prevents aortic aneurysm formation via FoxO4 interaction. Circ Res 121:1331–1345. https://doi.org/10.1161/CIRCRESAHA.117.311450.

65. Zhou B, Xie J, Liu X, Wang B, Pan L. 2016. Functional and transcriptomic analysis of the key unfolded protein response transcription factor HacA in Aspergillus oryzae. Gene 593:143–153. https://doi.org/10.1016/j.gene.2016.08.018.

66. Thibault G, Ismail N, Ng DTW. 2011. The unfolded protein response supports cellular robustness as a broad-spectrum compensatory pathway. Proc Natl Acad Sci U S A 108:20957–20962. https://doi.org/10.1073/pnas.1117184109.

67. Fun XH, Thibault G. 2020. Lipid bilayer stress and proteotoxic stress-induced unfolded protein response deploy divergent transcriptional and non-transcriptional programmes. Biochim Biophys Acta Mol Cell Biol Lipids 1865:158449. https://doi.org/10.1016/j.jbilk.2019.04.009.