

The Toxicity of a Novel Antifungal Compound Is Modulated by Endoplasmic Reticulum-Associated Protein Degradation Components

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In a search for new antifungal compounds, we screened a library of 4,454 chemicals for toxicity against the human fungal pathogen *Aspergillus fumigatus*. We identified sr7575, a molecule that inhibits growth of the evolutionary distant fungi *A. fumigatus*, *Cryptococcus neoformans*, *Candida albicans*, and *Saccharomyces cerevisiae* but lacks acute toxicity for mammalian cells. To gain insight into the mode of inhibition, sr7575 was screened against 4,885 *S. cerevisiae* mutants from the systematic collection of haploid deletion strains and 977 barcoded haploid DAMP (decreased abundance by mRNA perturbation) strains in which the function of essential genes was perturbed by the introduction of a drug resistance cassette downstream of the coding sequence region. Comparisons with previously published chemogenomic screens revealed that the set of mutants conferring sensitivity to sr7575 was strikingly narrow, affecting components of the endoplasmic reticulum-associated protein degradation (ERAD) stress response and the ER membrane protein complex (EMC). ERAD-deficient mutants were hypersensitive to sr7575 in both *S. cerevisiae* and *A. fumigatus*, indicating a conserved mechanism of growth inhibition between yeast and filamentous fungi. Although the unfolded protein response (UPR) is linked to ERAD regulation, sr7575 did not trigger the UPR in *A. fumigatus* and UPR mutants showed no enhanced sensitivity to the compound. The data from this chemogenomic analysis demonstrate that sr7575 exerts its antifungal activity by disrupting ER protein quality control in a manner that requires ERAD intervention but bypasses the need for the canonical UPR. ER protein quality control is thus a specific vulnerability of fungal organisms that might be exploited for antifungal drug development.

The burden of fungal infections in the human population is very high, with an estimated 1.5 million annual deaths worldwide, despite antifungal prophylaxis (1–3). The evolutionary proximity between mammalian and fungal cells creates a challenge for the identification of selective drug targets. Consequently, there are only a few mechanistically distinct classes of antifungal agents. The major antifungal drugs in clinical use disrupt membrane homeostasis by targeting ergosterol (4), impair cell wall integrity by inhibiting β-1,3-glucan synthase (5), or perturb nucleic acid synthesis via a fluorinated nucleotide analogue (6). The limited number of therapeutic options impedes effective management of invasive fungal infections, particularly when resistance to a drug is either emerging or an intrinsic characteristic of the fungal pathogen.

The identification of novel drugs and their targets can follow several strategies, ranging from the inhibition of a known protein target with a panel of inhibitors to the analysis of mutant strain sensitivity to toxic compounds (7). Chemical genomic screens analyze large collections of genetically defined mutant strains for their sensitivity to chemical libraries in a systematic manner. Data from these screens can provide insight into candidate targets for a given drug, as well as the cellular pathways required to buffer drug toxicity (8–11). The interpretation of chemogenomic screens depends on the type of mutant collection utilized for the analysis. For example, the absence of a general dosage compensation mechanism in yeast (12) allows heterozygous deletion strains to be used as tools to determine how a reduction in the level of a gene product impacts drug sensitivity. However, since heterozygous deletion

strains retain some level of gene function, compensatory mechanisms could mask changes in drug sensitivity. Haploid deletion strains can circumvent this problem and increase the sensitivity of the screen. The resulting chemogenomic profile, or pattern of mutants that are affected by a given compound, is predictive of the mechanism of action and has been successfully applied to drug lead identification and target classification in yeast (13). Further insight into pathways that are modulated by a compound can be obtained by developing *in silico* comparison tools that link the results of a chemogenomic analysis to the data of other published

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large-scale chemogenomic or genetic interaction screens (for examples, see references 14 and 15).

Most of the large chemogenomic data sets currently available have investigated nonessential gene deletions in haploid strains (10, 16). Essential genes, representing about one-sixth of all *Saccharomyces cerevisiae* genes, are more difficult to study in haploid or heterozygous deletion strains, so an alternative approach is the use of DAmP (decreased abundance by mRNA perturbation) strains. DAmP strains contain a drug resistance marker inserted into the 3' untranslated region (UTR) of a gene, resulting in defects in mRNA stability that can create hypomorphic alleles for phenotypic analysis of essential gene function (17). These strains have provided important insights into gene function as well as the response of cells to stress (for example, see reference 18).

In this study, we report the identification of sr7575, a small molecule with fungistatic activity against *S. cerevisiae* and three genera of human fungal pathogens: *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Candida albicans*. We employed a genome-wide approach to characterize the mode of action of sr7575, using a systematic determination of *S. cerevisiae* deletion and DAmP mutant sensitivity to the drug, combined with extensive *in silico* comparisons with large-scale data sets from published chemogenomic and genetic interaction screens. The strategy led to the conclusion that *S. cerevisiae* and *A. fumigatus* mutants that are deficient in endoplasmic reticulum-associated degradation (ERAD), a degradative pathway that disposes of misfolded proteins that arise in the ER membrane or lumen (19), are hypersensitive to sr7575. Collectively, these data implicate ER protein quality control as the target of sr7575 toxicity in evolutionarily distant fungi and suggest that further analysis of compounds that disrupt ER homeostasis may provide novel avenues for antifungal drug development.

MATERIALS AND METHODS

Screening procedure of the CERMN chemical library. All robotic steps were performed on a Tecan Freedom EVO platform. Compounds were transferred from mother plates into clear, flat-bottom, barcoded tissue culture 96-well plates (Greiner Bio One): 1 μ l of a dimethyl sulfoxide (DMSO) solution containing 3.3 mg/ml of each compound was spiked into dry wells of daughter plates (80 compounds per plate). For each plate, columns 1 and 12 served as controls: 8 positive controls spiked with DMSO alone provided the reference as 100% growth, and 8 negative controls contained the antifungal drug amphotericin B at 15 μ g/ml to kill all cells. A mixture (130 μ l) containing 10 volumes of a conidial suspension of 10^5 conidia/ml (in RPMI with 0.1% Tween 20) and 3 volumes of 0.01% resazurin was added to each well. After 48 h of incubation at 37°C, the absorbances at 570 nm (measurement wavelength) and 604 nm (reference wavelength) were measured on a Safire2 (Tecan) microplate reader. The data were normalized using the following formula: % viability = $100 \times (\text{sample value} - \text{average value of negative controls}) / (\text{average of positive controls} - \text{average of negative controls})$.

For analysis of toxicity to human cells, compounds were added to HeLa cells at a concentration of 10 μ M (2.8 μ g/ml for sr7575) and the release of cytoplasmic lactate dehydrogenase was measured using the enzyme-linked immunosorbent assay (ELISA)-based cytotoxicity detection kit (Roche) according to the manufacturer's recommendations. Mutagenic activity was tested in the bacterial reverse mutation test, either in the presence or in the absence of a rat metabolizing system (performed by CiToxLAB Safety and Health Research Laboratories). To determine acute mouse toxicity, groups of four NMRI mice were given a single dose of sr7575 (100 mg/kg of body weight) by intraperitoneal (i.p.) injection, and mortality was monitored for 3 days.

Yeast strains, growth, and media. All strains used in this study are described in Table S4 in the supplemental material. The pooled haploid deletion library (MATa) contained deletions in 4,885 nonessential genes along with DAmP modifications of 977 essential genes (20, 21). Wild-type (WT) *S. cerevisiae* strain BY4741 was routinely maintained on YPD agar (YPDA; consisting of 1% yeast extract, 2% peptone, 2% dextrose, and 2% Bacto agar).

For *S. cerevisiae* serial dilution spot assays, fresh colonies from plates were used to inoculate overnight cultures in YPD. The next morning, cultures were washed once and diluted to an optical density at 600 nm (OD_{600}) of 1 in phosphate-buffered saline (PBS), and serial 10-fold dilutions were carried out in a 96-well plate. Ten microliters of each dilution was spotted onto SC medium (containing 6.7 g/liter yeast nitrogen base with ammonium sulfate [BD Difco], with all amino acids, 2% dextrose, and 2% Bacto agar) lacking or supplemented with 0.25 μ g/ml sr7575. Plates were incubated at 30°C, and growth was monitored every 24 h over 3 days. Spot assays on SC supplemented with the analog sr7576 were conducted in a similar fashion.

The same serial dilution spot assay used to assess sensitivity in *S. cerevisiae* was used for *C. albicans* and *C. neoformans*, with the exception that the plates were supplemented with sr7575 between 1 and 8 μ g/ml and were incubated at 37°C.

Verification of *S. cerevisiae* mutant strains. The following mutants were extracted from the gene deletion library maintained in the 96-well format (8): YBR283C (*SSH1*), YCL045C (*EMC1*), YDL020C (*RPN4*), YDL226C (*GCS1*), YER019C-A (*SBH2*), YER090W (*TRP2*), YKL126W (*YPK1*), YKL207W (*EMC3*), YML105C (*SEC65*; DAmP strain), YMR022W (*UBC7*), YMR264W (*CUE1*), YOL013C (*HRD1*), YOR008C (*SLG1*), YOR153W (*PDR5*), YPR060C (*ARO7*), YHR079C (*IRE1*), YFL031W (*HAC1*), YBR201W (*DER1*), YLR207W (*HRD3*), YIL030C (*SSM4/DOA10*), YDL190C (*UFD2*), YGL013C (*PDR1*), YNL181W (DAmP strain), and YML125C (*PGA3*; DAmP strain). Genomic DNA was extracted using phenol-chloroform followed by ethanol precipitation. Mutants were verified by PCR amplification using a common forward primer annealing to the KanMX cassette (KaniF) and gene-specific reverse primers (oligonucleotides are listed in Table S5 in the supplemental material).

Complementation tests in *S. cerevisiae*. Complementation tests were performed with plasmids from the Molecular Barcoded Yeast ORF collection (MoBY-ORF) (22). *URA3* plasmids carrying open reading frame (ORFs) corresponding to genes *ARO7*, *CUE1*, *EMC1*, *EMC3*, *HRD1*, *RPN4*, *SSH1*, and *UBC7* (see Table S4 in the supplemental material) were recovered from *Escherichia coli* grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% Bacto agar) supplemented with chloramphenicol (60 μ g/ml) and kanamycin (50 μ g/ml) (22). Five hundred nanograms of each plasmid was transformed into the appropriate yeast deletion parent following the lithium acetate protocol (23), and *URA3*-expressing transformants were selected on SC medium lacking uracil (–URA). The resulting transformants were purified by passaging onto fresh SC (–URA) medium, and four clones of each transformant set were screened by colony PCR using a gene-specific primer pair (see Table S5 in the supplemental material), generating product sizes ranging between 500 and 1,000 bp. The deletion parent was always included as a negative control. Complemented strains were screened in parallel with the parental deletion strains in spot assays.

Overexpression tests in *S. cerevisiae*. 2 μ m-based *LEU2* plasmids from the systematic overexpression library (24) corresponding to regions of the yeast genome that contain the ORFs *PDR1*, *PDR5*, and *PDR12* and a control lacking intact genes (see Table S4 in the supplemental material) were recovered from *E. coli* DH10B cultures grown in LB medium supplemented with kanamycin (50 μ g/ml) and transformed into wild-type BY4741, and transformants were selected on SC lacking leucine (–LEU) plates. Transformants were purified by passaging onto fresh SC (–LEU) plates. Overexpressing strains were screened by serial dilution spot assays on SC medium supplemented with increasing concentrations of sr7575.

Chemogenomic profiling. Concentrations of sr7575 that inhibit WT growth by 10 to 20% in liquid culture were determined using the haploid strain BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). Single colonies from fresh YPDA plates were inoculated into 10 ml YPD and incubated at 30°C for 14 h. Cultures were diluted to an OD₆₀₀ of 0.01 and grown to an OD₆₀₀ of 0.05 prior to the addition of increasing concentrations of sr7575 (0.0625 μg/ml to 0.5 μg/ml). DMSO was used as a vehicle control, but there was no observable difference in growth rate between the no-vehicle and DMSO-treated cultures. Growth was monitored by measuring the OD₆₀₀ every hour, starting from 0 h until 10 h (see Fig. S2A in the supplemental material).

Pooled 400-ml cultures of the haploid deletion library were grown for 12 generations in the presence of sr7575 at 0.125 μg/ml or DMSO vehicle control. Amplified TAG products from the pooled cultures were hybridized to Agilent barcode-specific microarrays (GEO platform GPL18088) as previously described (14). Images obtained with a GenePix 4200AL scanner were annotated by using GenePix Pro 7 (Molecular Devices, CA, USA). Gpr files were normalized separately for the UP and DOWN barcodes and aggregated for each mutant. Raw and normalized data were deposited in the GEO database (see below). Only results for which data were obtained in two independent biological replicates were further considered for analysis. A total of 4,909 mutants, including both deletion and DAmP-modified strains, showed consistent growth measurements (see Table S6 in the supplemental material), with a Pearson correlation coefficient between the two series of log-transformed values of 0.78.

Gene set enrichment analysis and correlations. Overrepresentation of gene ontology (GO) terms in the chemogenomic screen results was analyzed using the web interface at <http://go.princeton.edu/cgi-bin/GOTermFinder> to the GO Term finder program (25). This program identifies enriched GO terms by calculating the frequency with which one expects to encounter a number of genes having the same annotation in a subset of genes (hypergeometric distribution). Correlations with published large-scale data sets were computed using the R project (<https://cran.r-project.org/>) function “cor.test,” using either “pearson” or “spearman” as comparison methods. Treatments or gene deletion perturbations were ranked in decreasing order of calculated correlation coefficients.

A. fumigatus strains, growth, and media. WT *A. fumigatus* strain kuA and deletion mutant *derAΔ*, *hacAΔ*, *hrdAΔ*, and *hrdAΔ derAΔ* strains were maintained on malt slants (2% malt extract, 2% Bacto agar) while *ireAΔ* and *hacAΔ derAΔ* strains were maintained on *Aspergillus* minimal medium (MM) with 5 mM ammonium tartrate as the nitrogen source and osmotically stabilized with 1.2 M sorbitol (26). G418 was obtained from Invitrogen, and Sigma-Aldrich was the source for ampicillin, kanamycin, and chloramphenicol.

Conidia from *ireAΔ* and *hacAΔ hrdAΔ* strains were recovered from *Aspergillus* MM plus 1.2 M sorbitol slants, while those of the parental strain kuA and the remaining deletion strains were recovered from 10-day-old malt slants in 0.05% Tween. Conidia were diluted to 10⁷ conidia/ml, and serial 10-fold dilutions were carried out in a 96-well plate prior to spotting 10 μl of each dilution onto MOPS (morpholinepropanesulfonic acid)-buffered RPMI 1640, pH 7.0, plates in the presence or absence of 5 μg/ml sr7575. The analog sr7576 precipitated out of solution in RPMI 1640 medium and was therefore not included in the analysis. Plates were incubated at 37°C, and growth was monitored over 4 days.

MIC determination. Determination of the MIC for yeast strains was carried out by the CLSI M27-A3 broth microdilution method (27). Growth inhibition of *Aspergillus* strains was monitored using a colorimetric test described earlier (28). The MIC of the *A. fumigatus* strain that constitutively expresses DsRed fluorescent protein (29) was determined following growth for 24 h at 37°C by measuring fluorescence using a Biotek Synergy fluorescent microplate reader with an excitation wavelength of 254 nm and emission filter set at 291 nm. The relative fluorescence units were plotted against the compound concentrations to determine the MIC.

Measurement of fungistatic or fungicidal activity. For yeast, freshly growing YPD cultures were diluted to an OD₆₀₀ of 0.001. sr7575 was added at 0.625 μg/ml, amphotericin B was added at 0.5 μg/ml, and DMSO was used as a vehicle control. One hundred-microliter aliquots were recovered for plating on YPDA. Cultures were grown for 16 h, cells were washed once in 1× PBS, and 100-μl volumes of serially diluted samples were plated. Colonies were counted following 48 h and normalized to the OD₆₀₀.

For *A. fumigatus*, 50-ml RPMI cultures with a starting cell number of 1 × 10⁵ conidia/ml were set up in the presence or absence of 5 μg/ml sr7575 (in duplicate). One hundred-microliter aliquots were recovered for enumeration of CFU. Following 16 h of growth, mycelia from one pair of flasks were filtered and mycelial dry weight was estimated. From the second pair, 100 μl from the drug-treated flask was serially diluted and plated to assess viability.

qRT-PCR. *A. fumigatus* conidia were inoculated into YG medium (0.5% yeast extract, 2% glucose) and incubated overnight at 37°C, 200 rpm. The mycelium was treated with the indicated concentrations of sr7575 or dithiothreitol (DTT), along with appropriate vehicle controls, for 1 h. The mycelia were harvested by filtration and lysed by crushing in liquid nitrogen. RNA was isolated using the TRIzol reagent, treated with DNase to remove traces of DNA, and reverse transcribed using Moloney murine leukemia virus (M-MuLV) reverse transcriptase (NEB) together with an oligo(dT) primer. Quantitation of *bipA* and *tigA* mRNA expression was performed by reverse transcriptase quantitative PCR (qRT-PCR), as previously described (30).

Microarray data accession number. Raw and normalized data obtained in this study were deposited in the GEO database under identifier GSE60934.

RESULTS

Identification of a new inhibitor of fungal growth. In a search for new antifungals, we tested the toxicity of 4,454 chemicals from the CERMN compound library against *A. fumigatus* using the strategy outlined in Fig. 1A. The CERMN library is part of the French national collection of chemicals (31) and has been developed since 1998 to be used in the framework of partnerships with public research laboratories. The dynamic range and degree of separation between positive and negative controls in the screen were evaluated by computing the Z' score (32). The average Z' value was 0.92 ± 0.03, indicating a robust and reliable assay. Data analysis identified 76 hits showing greater than 90% fungal growth inhibition, which were clustered into 7 chemical families and 29 singletons (see Table S1 in the supplemental material). Compounds with known effects on human physiology (33) or that showed cytotoxicity for HeLa cells in a lactate dehydrogenase release assay were eliminated from further consideration. The compound sr1810 was active against *A. fumigatus* and was selected for further analysis. Since sr1810 consisted of a mixture of two isomers, 75% of sr7575 (1) (Fig. 1A) and 25% of sr7576 (2), we synthesized each isomer (see Fig. S1A and B in the supplemental material) and found that it was only sr7575 that was responsible for the antifungal activity. The sr7575 compound showed no mutagenic activity in the bacterial reverse mutation test, and no acute toxicity was observed in mice at a dose of 100 mg/kg.

To gain insight into the structural basis for sr7575 antifungal activity, we prepared 30 analogues using aniline derivatives with different substitutions in the first reaction (compounds 3 to 32; see Tables S2 and S3 in the supplemental material and the details of synthesis in the supplemental text). Growth inhibition tests with these compounds showed that at least two features of sr7575 were required for its antifungal potency: the chlorine at position 4

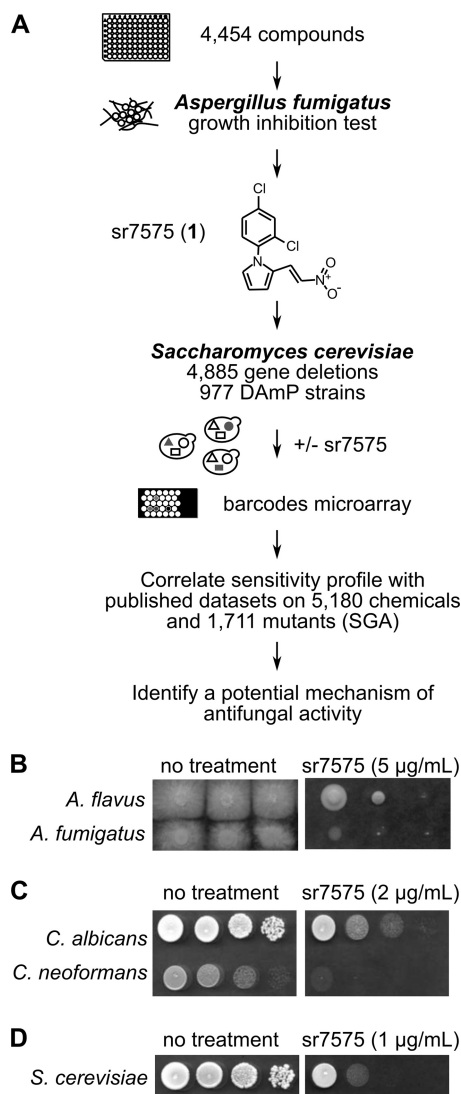


FIG 1 Identification of a compound with broad antifungal activity. (A) Selection of a new antifungal, sr7575, through a chemical library screen of *A. fumigatus* growth inhibition was followed by chemogenomic profiling in *S. cerevisiae* to identify a potential mechanism of action. (B to D) sr7575 inhibited growth of various fungi, including *A. flavus* (48 h, 37°C, RPMI medium, 5 µg/ml) (B), *C. albicans* and *C. neoformans* (24 h, 37°C, SC medium, 2 µg/ml) (C), and *S. cerevisiae* (48 h, 30°C, SC medium, 1 µg/ml) (D).

of the phenyl group and the positioning of the nitro group in relation to the pyrrole moiety (see Table S2 in the supplemental material).

In addition to its effects on *A. fumigatus*, sr7575 was active against *Aspergillus flavus* (Fig. 1B), *C. neoformans*, *C. albicans* (Fig. 1C), and *S. cerevisiae* (Fig. 1D) on plates and in liquid medium at inhibitory concentrations ranging from 0.6 to 10 µg/ml (see Fig. S2A, B, C, and D in the supplemental material). More than 90% of either *S. cerevisiae* or *A. fumigatus* cells were able to resume growth after 16 h of incubation in the presence of sr7575 (0.625 µg/ml and 5 µg/ml, respectively) indicating that the compound exerts a fungistatic effect.

ERAD-deficient mutants of *S. cerevisiae* are hypersensitive to sr7575. To gain insight into the mechanism by which sr7575

perturbs fungal physiology, the effect of sr7575 was tested on the growth rate of each of 4,885 haploid yeast deletion strains in the systemic deletion collection (20). In addition, sr7575 activity was measured on 977 locus-tagged barcoded DAmP (17) mutants of essential genes that were previously generated in our laboratory (21). This collection of gene knockout and DAmP strains contains molecular barcodes to facilitate detection and quantitation of DNA by custom Agilent microarrays (34, 35). Following the strategy outlined in Fig. 1A, the normalized ratio of the hybridization signal in the presence or absence of treatment was used as an estimate of relative growth rates in pools of mutants. Only a fraction of mutant strains showed hypersensitivity to sr7575, as indicated by the left tail of the distribution for sensitivity values (Fig. 2A). The strain that showed the most dramatic increase in sr7575 sensitivity harbors a deletion of the *PDR1* gene, encoding the main regulator of multidrug resistance in yeast (36). *Pdr1* is a transcriptional activator for xenobiotic efflux transporter genes, thereby governing resistance to numerous toxic compounds. It is likely that the effect of *PDR1* deletion on sensitivity to sr7575 is mediated through the plasma membrane ATP-binding cassette (ABC) transporter *Pdr5*, since *PDR5* is a known target of *Pdr1* (37) and the *pdr5* mutant was ranked 6th among deletion strains that were most affected by sr7575.

To identify cellular pathways or protein complexes that allow cells to counteract sr7575 effects, we used a gene set enrichment analysis on 89 mutant strains that showed an average increase in generation time of at least 10% relative to the WT in the presence of sr7575. The most overrepresented pathway in the data set was ER-associated protein degradation (ERAD), specifically the GO term “ER-associated ubiquitin-dependent protein catabolic process” (GO:0030433), with a *P* value corrected for multiple-hypothesis testing of 1.5×10^{-6} . This set included *CUE1*, *UBC7*, *HRD1*, *HRD3*, *UFD2*, *UBX4*, *SSM4* (*DOA10*), *DSK2*, and *UBX2*, encompassing one-fifth of the total number of genes annotated to this term (Fig. 2B). The second most overrepresented GO term was “aromatic amino acid family biosynthetic process” (GO:0009073). However, strains deficient in this pathway are known to exhibit a multidrug response signature (9), so the study of the corresponding strains was not pursued further.

Cellular component enrichment analysis was used to determine whether any of the 89 proteins selected in the screen were linked to the same protein complex or intracellular location. The ER membrane protein complex (EMC) was the most overrepresented group by this analysis, with a *P* value of 2×10^{-7} . In addition to gene deletions directly affecting *EMC1*, *EMC3*, *EMC4*, and *EMC5*, deletions affecting dubious ORFs that overlap *EMC2* (*YJR087W*) and *EMC1* (*YCL046W*), which are independent mutants of these genes, were also present in this data set. Members of the EMC complex are required for efficient protein folding in the ER (38), potentially through roles in phospholipid metabolism at the ER membrane (39). Other components of the ER membrane showed enrichment, including 10 of 58 genes annotated as “intrinsic components of the ER membrane” (GO:0031227) and two DAmP-modified essential genes of uncharacterized function (*YNL181W* and *PGA3*). In addition, several genes encoding components of the signal recognition particle (SRP) involved in cotranslational targeting of proteins into the ER showed enrichment: *sec65*-DAmP, *srp21*-DAmP, *shr3*-DAmP, *ssh1Δ*, and *sbh2Δ*. Taken together, these findings indicate that sensitivity to the inhibitory effects of sr7575 is exacerbated by defects in the ERAD

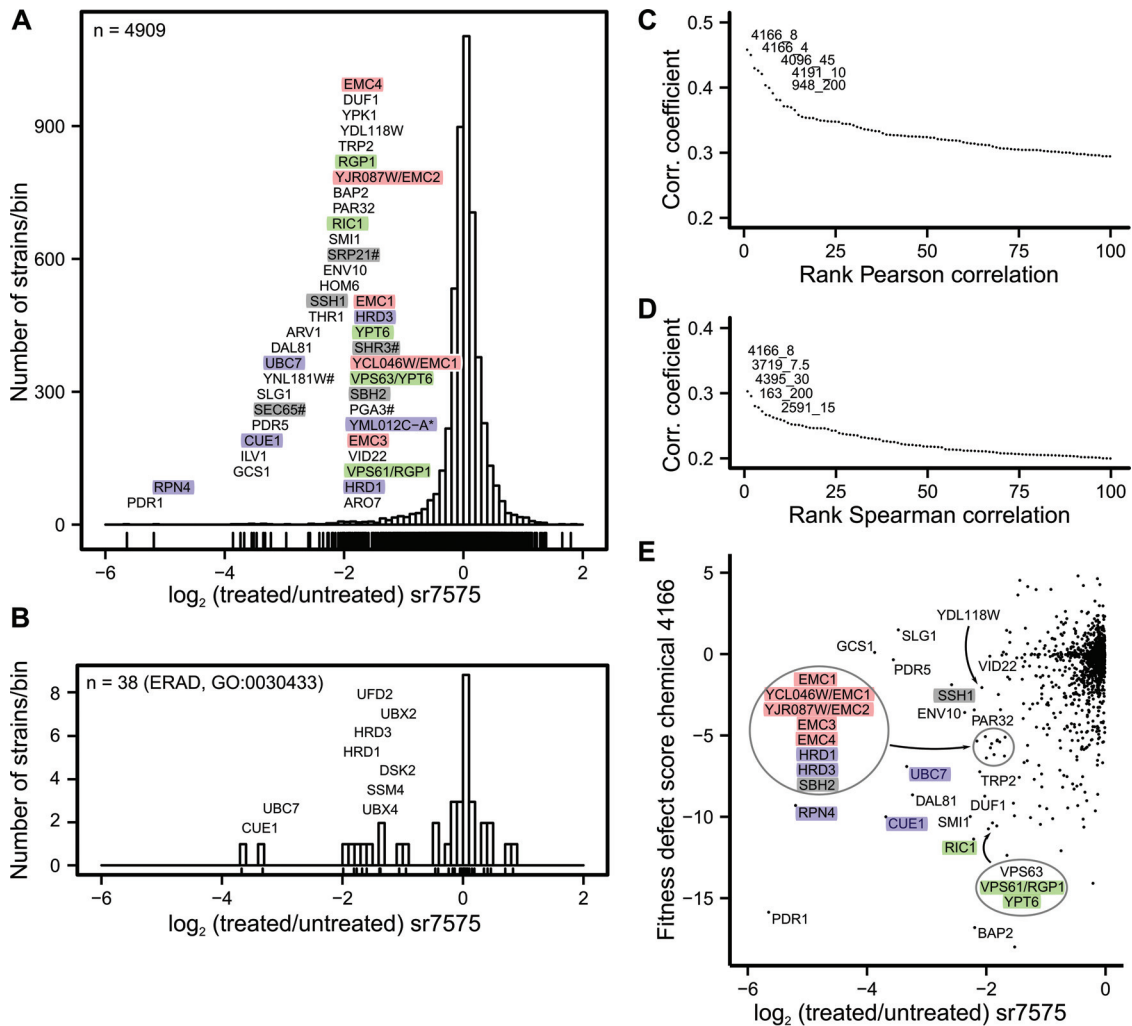


FIG 2 Chemogenomic profiling reveals an ERAD-enriched signature for sr7575 toxicity. (A) Distribution of relative growth values for *S. cerevisiae* mutant strains grown in the presence of sr7575. Colors indicate functional categories from the pooled library with genes annotated as ERAD (violet), protein translocation (gray), ER membrane complex (EMC; pink), and vesicular traffic (green) showing the most sensitivity to sr7575 when mutated. Note that *YML012C-A** overlaps *UBX2*; #, DAMP strain. (B) Distribution of sensitivity values for deletion strains affected for genes annotated with the GO term 0030433, ERAD. (C) Pearson correlation coefficients between results obtained with sr7575 and a published large-scale chemogenomics data set identifies chemical 4166 as having a profile that is most similar to sr7575. Only the scores for the top 100 correlated treatments are displayed. (D) Same as panel C, but with the Spearman rank correlation. (E) Comparison of the fitness defect scores between sr7575 and chemical 4166; gene names are color coded as described for panel A.

stress response, as well as by alterations in ER membrane composition that affect optimal ER protein translocation and folding.

The sr7575 sensitivity profile suggests an unfolded protein response-independent stress response. The effects of sr7575 on haploid yeast deletion strains were compared to profiles obtained from 1,824 different chemicals in a recently published large-scale chemogenomic screen (16). The compound CMB4166 had the highest Spearman correlation coefficient in this comparison ($r = 0.44$) (Fig. 2C) and showed a profile remarkably similar to that of sr7575 (Fig. 2D). Most of the strains showing sensitivity to sr7575 were also sensitive to CMB4166 (Fig. 2E), suggesting that the two compounds trigger similar cellular responses. However, CMB4166 is a macrolide (D. Hoepfner, personal communication) and shares no structural homology to sr7575.

To acquire insights into the specificity of the response to sr7575, we compared its sensitivity profile to published results on 3,356 other chemical compounds (10). The pattern of sr7575 sen-

sitive mutants revealed little to no similarity to profiles obtained from the other compounds in this comparison. For example, the maximum computed Pearson correlation coefficient was 0.27 for the compound k048-0007 (screen SGTC_352; see Fig. S3A in the supplemental material). However, this correlation was due to strains with deletions in *PDR1*, *RPN4*, or *GCS1*, which confer sensitivity to multiple stresses (see Fig. S3C in the supplemental material). To avoid the typically large effect of outliers on the Pearson correlation, we also tested correlation via the Spearman nonparametric test that uses ranks rather than values. The maximum correlation by this approach was also low (0.18), identifying the compound 4245-1575 used in screen SGTC_513 (see Fig. S3B). Most of the correlation in this case could be attributed to the hypersensitivity of the *ubc7* Δ and *cue1* Δ ERAD mutants (see Fig. S3D). Since this second compound was annotated as having an unfolded protein response signature (10), we also tested the correlation between the profile of sr7575 and that of tunicamycin, a

well-known and widely used inducer of the UPR (for reviews, see references 40 and 41). However, no similarity was found (see Fig. S3E). Collectively, these comparisons suggest that while many sensitivity profiles are related and indicate the most frequent types of cellular responses to chemical toxicity (10), the profile obtained for sr7575 was specific, with similarity to only one compound of over 5,000 chemicals analyzed.

Large-scale chemical toxicity screens are complementary to synthetic genetic array analyses (SGA), in which double deletion mutant strains are used to determine functional interactions between genes. We compared the sensitivity profile of sr7575 with the results from 1711 SGA screens (42). The closest hit was the profile shown by a strain harboring a DAmP modification of the essential gene *PGA3* (17) (see Fig. S4A in the supplemental material). The correlation between the sr7575 and *PGA3* profiles was robust, since it also ranked 5th when estimated using the Spearman correlation (see Fig. S4B). Despite the low value of the correlation coefficient (0.23), several strains containing gene deletions were affected by both sr7575 treatment and replacement of *PGA3* with the *pga3*-DAmP allele, including the ERAD-associated genes *cue1* Δ , *ubc7* Δ , *ufd2* Δ , *ssm4* Δ , and EMC complex components (see Fig. S4C). A role in newly synthesized protein trafficking has been proposed for *Pga3* (43), raising the possibility that its presence in our data set is due to a function that impacts ER homeostasis.

The gene deletion that ranked second in terms of correlation with the sr7575 profile involved *CHO2*, encoding a phosphatidyl N-methyltransferase required for phosphatidylcholine synthesis. The absence of *CHO2* renders yeast cells dependent on the UPR for survival (42, 44), suggesting that *CHO2* contributes to ER homeostasis. Consistent with this, *CHO2* deletion shows an aggravating interaction with the loss of EMC genes in terms of yeast growth (39).

A summary of the correlations between sr7575 sensitivity profiles and those derived from published chemogenomic screens is shown in Fig. 3. Since the numerical values reported for genetic and chemogenomic screens are not readily comparable, the ranking of the different mutants in each screen was used to generate a meaningful graphical display. The resulting heatmap (Fig. 3A) highlights the unique ERAD signature of sr7575 relative to currently published screens. A schematic illustrating the ER membrane proteins involved in the ERAD pathway is shown for perspective (Fig. 3B). Since ERAD is known to work in concert with the UPR to relieve ER stress, it is interesting that hypersensitivity to sr7575 was observed for ERAD mutants but not for the UPR-inactivated *ire1* Δ and *hac1* Δ strains. Taken together, these data are consistent with a model in which sr7575 toxicity is counteracted by a functional ERAD machinery, independent of signaling through the UPR pathway.

Specific ERAD deficiencies enhance sr7575 toxicity in *S. cerevisiae*. Seventeen *S. cerevisiae* mutant strains were selected to validate the results of the chemogenomic screen, encompassing strains with deletions in components of the ERAD and proteasome pathways, the EMC, the Ssh1 cotranslocase, the PDR network, aromatic acid biosynthesis, and DAmP modifications of *YNL181W* and *PGA3*. Each strain was analyzed individually for sr7575 susceptibility, using a subinhibitory concentration for the WT (Fig. 4A; see also Fig. S5 in the supplemental material). A strain in which the *ARO7* gene for aromatic amino acid biosynthesis was deleted showed increased sr7575 sensitivity (Fig. 4A),

consistent with the pleiotropic effects of this mutation on stress response.

As predicted by the chemogenomic screen, mutants in the *PDR5* multidrug transporter and its transcriptional activator *PDR1* were hypersensitive to sr7575 (see Fig. S5 in the supplemental material). Conversely, overexpression of *PDR1* and *PDR5*, but not *PDR12*, rendered *S. cerevisiae* cells tolerant to high concentrations of sr7575 (see Fig. S6A). This phenotype was conserved across fungal species, since clinical isolates and laboratory *C. albicans* strains that overexpress *CDR1*, the ortholog of *S. cerevisiae* *PDR5*, were also tolerant to sr7575 (see Fig. S6B).

Deletions of genes coding for EMC members, *EMC1* and *EMC3*, and the cotranslational translocase gene *SSH1* conferred increased sensitivity to sr7575, as suggested by the chemogenomic screen. However, a mutant in *SBH2*, which functions in the Ssh1 translocase complex (45), did not show increased sensitivity, at least at this concentration (see Fig. S5 in the supplemental material). Hypersensitivity to sr7575 was confirmed for components of the ERAD complex, including the Hrd1 E3 ubiquitin ligase, the E2 ubiquitin conjugating enzyme Ubc7, and the ER membrane resident recruiter Cue1 (46–48) (Fig. 4A). Although the ERAD component Der1 (49) was not identified in our chemogenomic screen, a mild increase in sr7575 sensitivity was observed for this mutant (see Fig. S5), consistent with ERAD involvement in sr7575 effects. sr7575 hypersensitivity was also validated for a strain lacking *RPN4*, which encodes a transcriptional activator of proteasome genes (Fig. 4A). Since proteasomal degradation is the final step in the disposal of misfolded proteins by ERAD, this finding is consistent with the notion that sr7575 affects protein quality control in the ER. In conclusion, these findings demonstrate that components of the ERAD pathway are necessary to protect yeast cells from the toxic effects of sr7575 in *S. cerevisiae*, suggesting a mechanism of action that involves perturbation of ER protein quality control.

ERAD protects against sr7575 toxicity in *A. fumigatus* but is UPR independent. The UPR is a stress response pathway that communicates information on ER homeostasis to the nucleus (40, 41). The pathway is triggered by misfolded proteins, which accumulate in the ER when the demand for secretion exceeds ER folding capacity or when the cell encounters adverse environmental conditions. Unfolded proteins are sensed by the ER transmembrane sensor Ire1, which triggers the synthesis of Hac1, a transcription factor. Hac1 translocates to the nucleus and upregulates the expression of chaperones, folding enzymes, and other proteins that support ER function (44, 50). Since ERAD mutants are hypersensitive to sr7575 and ERAD capacity can be regulated by the UPR, we were surprised to find that neither *HAC1* nor *IRE1* was identified in the sr7575 chemogenomic screen. The UPR independence of this response was confirmed by susceptibility testing: yeast *ire1* Δ and *hac1* Δ mutants were not affected by sr7575 at concentrations of up to 0.5 μ g/ml (Fig. 4C). These findings suggest that ERAD protects against sr7575 toxicity through a mechanism that is independent of the UPR in *S. cerevisiae*.

Consistent with the results obtained in *S. cerevisiae*, UPR mutants of *A. fumigatus* that lack either the ER sensor IreA or the transcription factor HacA showed no hypersensitivity to sr7575 (Fig. 5A). As in yeast, the *hrdA* Δ mutant, which lacks the ortholog of *S. cerevisiae* *HRD1*, showed increased sensitivity to sr7575. A *derA* Δ mutant that is deficient in the DerA component of the HrdA ERAD complex showed no increase in sr7575 sensitivity.

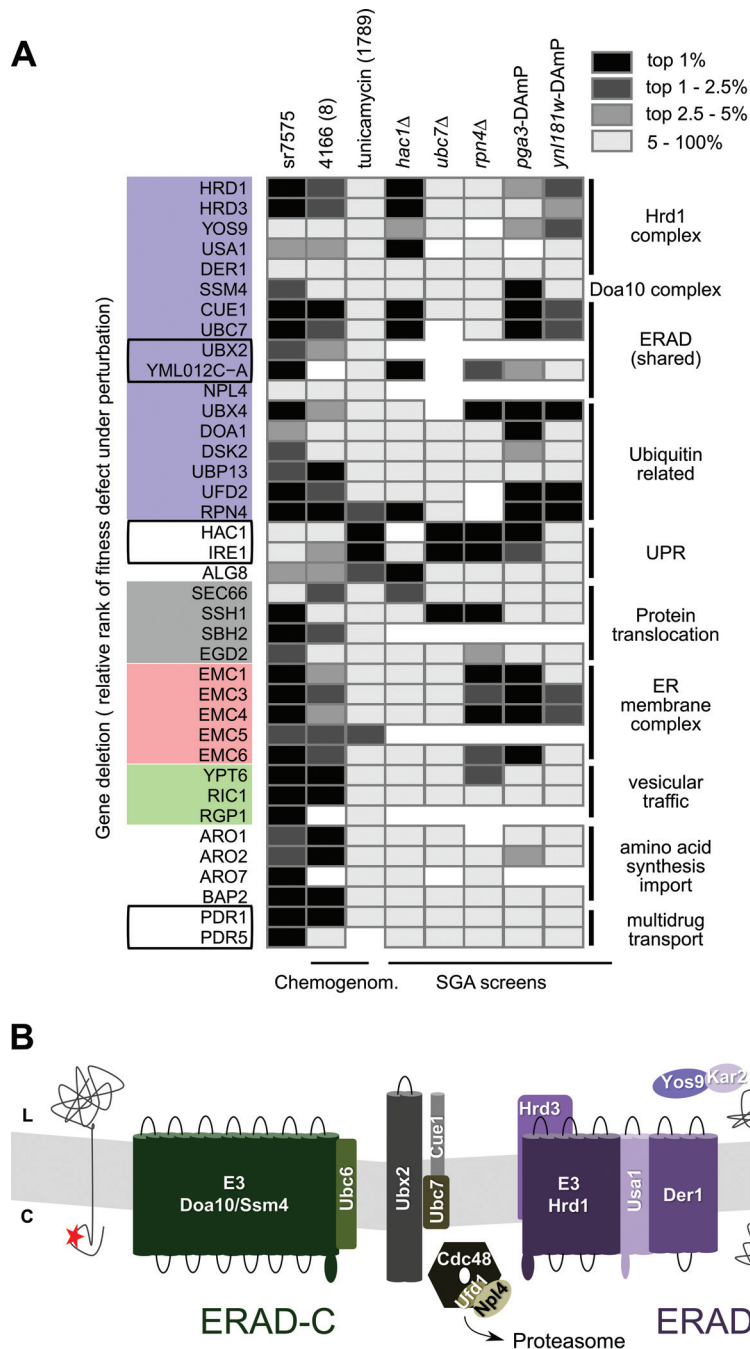


FIG 3 Summary of mutations conferring increased susceptibility to sr7575. (A) Heatmap showing the unique ERAD signature of sr7575 compared with published chemogenomic and SGA growth defect profiles. (B) Model showing the two main pathways responsible for ERAD in fungi: the Doa10 pathway (green) for clearing misfolded proteins with cytosolic lesions and the Hrd1 pathway (violet), which degrades misfolded proteins with luminal or transmembrane lesions. Shared components (Ubx2, Ubc7, Cue1, and the Cdc48 complex) are denoted in gray or black.

However, a double deletion mutant lacking both DerA and HrdA showed greater sensitivity to sr7575 than a mutant lacking HrdA alone, underscoring the importance of the Hrd1 complex in the response to sr7575 toxicity. We conclude that sr7575 action involves the inhibition of an evolutionarily conserved target, necessitating the intervention of the ERAD complex Hrd1/HrdA in both *S. cerevisiae* and *A. fumigatus*.

The ERAD-enriched signature for sr7575 suggested that some

aspect of ER protein quality control is adversely affected by this compound. However, since UPR-deficient strains of *S. cerevisiae* or *A. fumigatus* showed no increase in sr7575 sensitivity, the results suggest that a UPR-independent mechanism of ERAD activity is involved in the sr7575 response. To confirm UPR independence, qRT-PCR was used to measure mRNA levels for two well-known UPR target genes: the ER chaperone *bipA* and the protein disulfide isomerase *tigA*. As expected, the expression

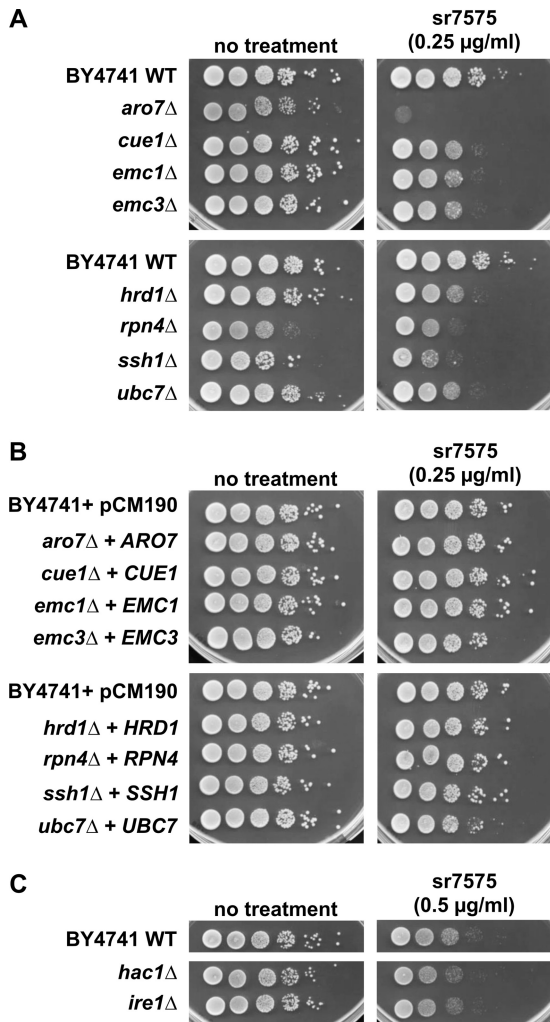


FIG 4 Sensitivity to sr7575 depends on EMC and ERAD components. (A) Serial 10-fold dilutions of the WT and selected haploid deletion mutants were grown on SC plates in the absence or presence of 0.25 $\mu\text{g/ml}$ sr7575 for 48 h at 30°C. (B) Complementation of sr7575 sensitivity for the strains shown in panel A was tested by using single-copy plasmids carrying the corresponding genes. (C) Strains lacking core UPR components *HAC1* and *IRE1* were tested for sensitivity against sr7575 at 0.5 $\mu\text{g/ml}$.

of both genes was strongly induced by treatment with a subinhibitory concentration of dithiothreitol (1 mM), a well-known inducer of the UPR (Fig. 5B). In contrast, no increase in expression was observed following treatment with a subinhibitory concentration of sr7575 (0.1 $\mu\text{g/ml}$). In addition, pretreatment with sr7575 for 1 h prior to DTT exposure failed to block UPR activation. These results indicate that while exposure to sr7575 does not trigger the UPR, it was also unable to prevent UPR activation by DTT. We conclude that sr7575 is unlikely to target UPR signaling for its toxic effects in *A. fumigatus* or *S. cerevisiae*, consistent with the UPR-independent response suggested by the chemogenomic screen.

DISCUSSION

In this study, we describe the identification of a novel antifungal compound, sr7575, that was active against species from four fungal genera. Chemogenomic profiling in *S. cerevisiae* demonstrated

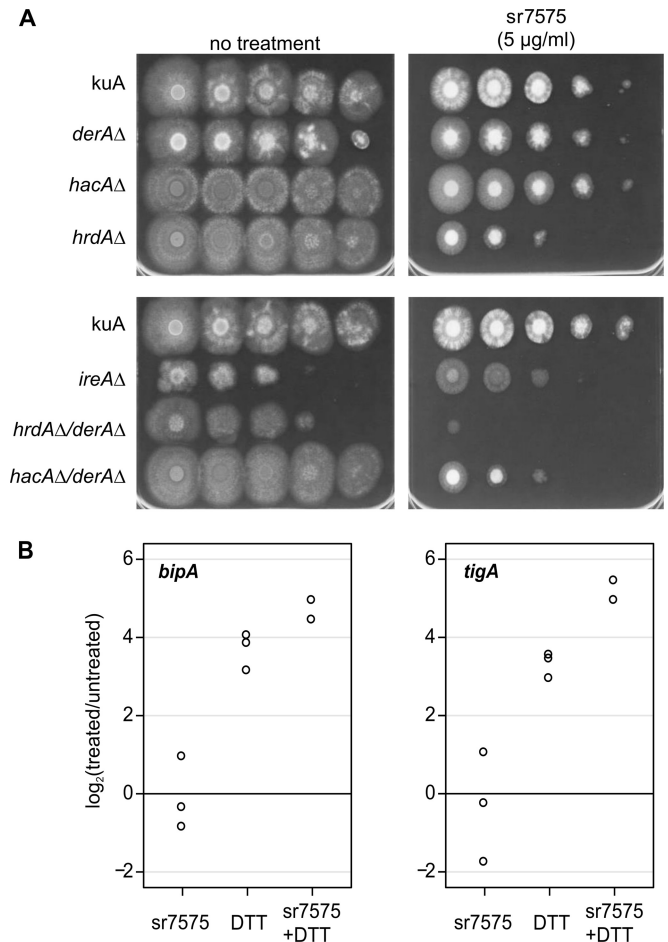


FIG 5 The hypersensitivity of ERAD mutants to sr7575 is conserved in *A. fumigatus*. (A) Conidia from *A. fumigatus* WT and deletion mutants were recovered in 0.05% Tween-water, and serial dilutions were spotted onto sr7575-containing RPMI 1640, pH 7.0. Plates were incubated at 37°C for 72 h. (B) Analysis of UPR target gene expression (*bipA* and *tigA*) by qRT-PCR. Cultures were treated with sr7575, DTT, or sr7575 for 1 h followed by DTT. RNA was extracted and analyzed by qRT-PCR, using *tubA* mRNA for normalization. The results of treated versus untreated samples from three independent experiments are shown.

that the set of genes required for protection against sr7575 was markedly narrow, involving components of the ERAD stress response and other components of the ER membrane. The function of the ERAD pathway is to maintain protein quality control in the ER by eliminating toxic unfolded proteins that may accumulate in the ER during periods of high secretory activity or when the organism encounters adverse environmental conditions. This disposal mechanism centers on a multiprotein complex in the ER membrane that selectively identifies misfolded proteins in the ER lumen or membrane and transports them back into the cytoplasm for degradation by the proteasome. The results from our chemogenomic screen demonstrate that mutants of this complex, either in *S. cerevisiae* or *A. fumigatus*, are hypersensitive to sr7575 inhibition, suggesting that the antifungal effects of this compound involves a disruption of ER protein quality control.

ER protein quality control is also affected by the UPR, a signaling pathway that counters the accumulation of unfolded proteins in the ER by increasing the expression of chaperones and other

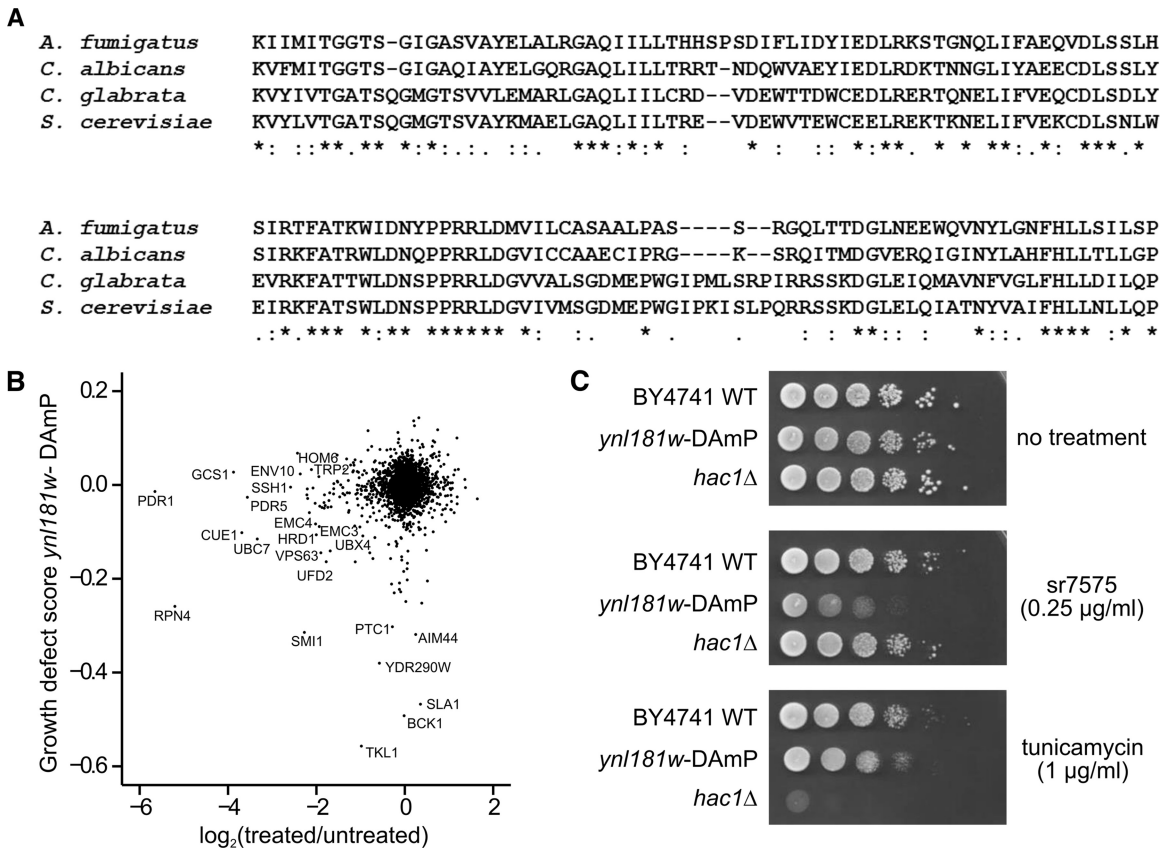


FIG 6 Ynl181w is an ER protein conserved in fungi and involved in adaptation to sr7575. (A) T-Coffee alignment of the conserved short-chain dehydrogenase region within *Sc* Ynl181w (PFAM 54-187) and its orthologs in pathogenic fungi. Gene annotations with a number range indicate the position of the PFAM domain: *A. fumigatus* (Afu5g10790; positions 54 to 205), *C. albicans* (orf19.6233; positions 58 to 204), *C. glabrata* (XP_448202; positions 54 to 193). (B) Scatter plot showing the correlation between sr7575 sensitivity values and the previously published SGA scores for ynl181w-DAmP. (C) Spot assays showing the difference in sensitivity to sr7575 and UPR inducer tunicamycin (TM) for ynl181w-DAmP compared with a strain defective for UPR (*hac1Δ*).

proteins involved in protein folding when the demand for secretion exceeds the folding capacity of the organelle. A tight coordination between the UPR and ERAD pathways was demonstrated in yeast, where UPR mutants have decreased ERAD activity whereas ERAD mutants exhibit constitutive UPR upregulation (50). In addition, although ERAD is sufficient to eliminate misfolded proteins that continually arise during normal growth, it requires the UPR for optimal degradative capacity under conditions of severe ER stress (51). Basal ERAD activity is thus sufficient to handle low levels of unfolded proteins and is UPR independent. However, upregulation of ERAD activity by the UPR is needed when the level of unfolded proteins reaches a critical threshold of toxicity. The connection between these pathways is also evident in *A. fumigatus*, in which mutants deficient in both the UPR and ERAD are less fit than those lacking the UPR or ERAD alone (52).

In view of the link between the UPR and ERAD pathways, we were surprised to find that neither *ire1Δ* nor *hac1Δ* was among the strains most affected by sr7575 in our chemogenomic screen and that these strains showed no increase in sr7575 sensitivity when tested individually. In addition, our experiments revealed that sr7575 did not trigger the UPR, nor did it prevent the UPR from being activated by DTT, a strong inducer of unfolded proteins. These observations suggest that sr7575 does not cause the wide-

spread protein unfolding that is typical of strong ER stress aggravators such as DTT and tunicamycin. Specific ER stress can be induced, for example, by expressing topologically abnormal ERAD-targeted integral membrane proteins without inducing the canonical UPR pathway in yeast (53).

The ability of sr7575 to inhibit the growth of fungi but not human cells raises the possibility that it targets a fungus-specific process. Our chemogenomic screen identified ynl181w-DAmP as one of the top 10 strains most affected by sr7575 toxicity. *YNL181W* encodes an essential ER membrane protein, the function of which is currently unknown but is speculated to involve an oxidoreductase activity (54, 55). The Ynl181w protein is conserved among fungi (Fig. 6A) and has no metazoan ortholog, as defined in the OrthoMCL database (56). Since the protein is essential, a heterozygous *YNL181W/ynl181wΔ* deletion strain was previously used to study its function in chemogenomic investigations (10, 16). We were especially interested in the effects of chemical CMB4166 in these studies because our data revealed that the strain sensitivity profile for that compound (16) most closely resembled that of sr7575 (Fig. 2C, D, and E). A striking finding from this comparison was that the heterozygous deletion strain of *YNL181W* was among the strains most affected by CMB4166 (Fig. 3A), indicating that the absence of Ynl181w sensitizes yeast to both sr7575 and CMB4166.

A DAmP modification of *YNL181W* has been previously combined with a large-scale genetic screen to identify mutations that synergize with loss of *YNL181W* function (42). A comparison of the sr7575 sensitivity profile with the results of this genetic screen identified several ERAD mutants that were affected both by DAmP modification of *YNL181W* and by treatment with sr7575, including *UBC7*, *CUE1*, and *RPN4* (Fig. 6B). The connection to *UBC7* was particularly remarkable because a similar synergistic growth defect associated with *ynl181w*-DAmP and the *ubc7Δ* mutation was observed in two other large-scale studies (17, 57). The correlation between the *Ynl181w* mutation and sr7575 sensitivity obtained by chemogenomics was confirmed on plates by showing that a *ynl181w*-DAmP mutant was hypersensitive to sr7575 (Fig. 6C). We speculate that *Ynl181w* could be involved in processes that are targeted by sr7575.

In conclusion, we report the identification of a novel compound that has activity against both *S. cerevisiae* and *A. fumigatus*. The data are consistent with a model for sr7575 action in which the compound disrupts the structure of one or more proteins in the ER lumen or membrane, resulting in a situation that necessitates ERAD intervention to eliminate the abnormal protein(s) but does not require UPR activation. These findings underscore the importance of ER homeostasis to the growth fungi and suggest the presence of fungus-specific ER processes that could represent new opportunities for antifungal intervention.

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