

Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease

Leah E. Cowen^{a,1}, Sheena D. Singh^a, Julia R. Köhler^b, Cathy Collins^a, Aimee K. Zaas^c, Wiley A. Schell^c, Hamza Aziz^c, Eleftherios Mylonakis^d, John R. Perfect^{c,e}, Luke Whitesell^f, and Susan Lindquist^{f,1}

^aDepartment of Molecular Genetics, University of Toronto, Toronto, ON M5S 1A8, Canada; ^bDivision of Infectious Disease, Children's Hospital, Boston, MA 02115; ^cDepartment of Medicine, Duke University Medical Center, Durham, NC 27710; ^dHarvard Medical School, Massachusetts General Hospital, Division of Infectious Diseases, Boston, MA 02114; ^eDepartment of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27710; and ^fWhitehead Institute for Biomedical Research, The Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA 02142

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Invasive fungal infections are a leading cause of mortality among immunocompromised individuals. Treatment is notoriously difficult with the limited armamentarium of antifungal drugs, whose efficacy is compromised by host toxicity, a limited activity spectrum, or the emergence of drug resistance. We previously established that the molecular chaperone Hsp90 enables the emergence and maintenance of fungal drug resistance. For the most prevalent fungal pathogen of humans, *Candida albicans*, Hsp90 mediates resistance to azoles, which inhibit ergosterol biosynthesis and are the most widely deployed antifungals in the clinic. For the emerging opportunistic pathogen *Aspergillus terreus*, Hsp90 is required for basal resistance to echinocandins, which inhibit $\beta(1, 3)$ -glucan synthesis and are the only new class of antifungals to reach the clinic in decades. Here, we explore the therapeutic potential of Hsp90 inhibitors in fungal disease using a tractable host-model system, larvae of the greater wax moth *Galleria mellonella*, and a murine model of disseminated disease. Combination therapy with Hsp90 inhibitors that are well tolerated in humans and an azole rescued larvae from lethal *C. albicans* infections. Combination therapy with an Hsp90 inhibitor and an echinocandin rescued larvae from infections with the most lethal mold, *Aspergillus fumigatus*. In a murine model of disseminated candidiasis, genetic compromise of *C. albicans* HSP90 expression enhanced the therapeutic efficacy of an azole. Thus, harnessing Hsp90 provides a much-needed strategy for improving the treatment of fungal disease because it enhances the efficacy of existing antifungals, blocks the emergence of drug resistance, and exerts broad-spectrum activity against diverse fungal pathogens.

antifungal | *Aspergillus fumigatus* | azole | *Candida albicans* | drug resistance

Invasive fungal infections are a leading cause of human mortality worldwide, especially among immunocompromised individuals. The frequency of fungal infections continues to increase in concert with the growing immunocompromised patient population, including individuals infected with HIV as well as those undergoing chemotherapy, major surgery, or transplantation of solid organs or hematopoietic stem cells (1, 2). The economic burden of invasive fungal disease is staggering, with conservative estimates of \$2.6 billion annually in the United States alone (2). Chief among the opportunistic fungal pathogens are *Candida albicans* and *Aspergillus fumigatus*, the leading causal agents of mycotic death in patients with cancer and neutropenia (3). *C. albicans* is the fourth most common cause of hospital-acquired infectious disease and the primary cause of systemic candidiasis, with mortality rates approaching 50% (4). *A. fumigatus* is the primary causal agent of invasive aspergillosis and reigns as the most deadly mold, with mortality rates up to 90% (5), and remaining at 40% with state-of-the-art antifungal therapy (6).

Treatment of invasive fungal infections is notoriously difficult. There are a limited number of antifungal drugs with highly selective toxicity to fungi, in large part because of the close evolutionary relationships fungi share with their human hosts (7–9). Three of the 5 classes of antifungals in clinical use target the biosynthesis or function of ergosterol, the major sterol in fungal membranes (8–10). One class inhibits synthesis of nucleic acids, while the remaining class inhibits synthesis of $\beta(1, 3)$ -glucan, a key component of the fungal cell wall (8–10). This paucity of targets is problematic, given the prevalence of cross-resistance to all drugs with a common target. The efficacy of most antifungals currently deployed is compromised by static rather than cidal activities that block fungal growth but do not eradicate the pathogen population. Therapeutic challenges are exacerbated by the frequent emergence of drug resistance in pathogen populations (8–10), demanding the development of new therapeutic strategies for fungal infectious disease.

We previously established that Hsp90, an essential molecular chaperone that regulates the form and function of many key signal transducers (11–13), enables the emergence and maintenance of drug resistance in diverse fungal species (14, 15). For the most prevalent fungal pathogen of humans, *C. albicans*, Hsp90 mediates resistance to the azoles, which inhibit ergosterol biosynthesis and are the most widely used class of antifungals in the clinic. Pharmacological inhibition of Hsp90 blocks the emergence of azole resistance and abrogates resistance of laboratory mutants and strains that evolved resistance in a human host (14, 15). For the emerging opportunistic pathogen *Aspergillus terreus*, separated from *C. albicans* by ≈ 1 billion years of evolution (16), Hsp90 inhibitors reduce resistance to echinocandins, which inhibit $\beta(1, 3)$ -glucan synthesis and are the only new class of antifungal drug to reach the clinic in decades (15). Hsp90's role in drug resistance is to enable specific cellular signaling required for crucial responses to cellular stress induced by drug exposure, such as changes in the composition of cell membranes and cell walls (8). Several small-molecule Hsp90 inhibitors are currently in clinical or late-stage preclinical investigation for anticancer activity (17–19). Our *in vitro* studies demonstrate that at concentrations that are well tolerated in humans, Hsp90 inhibitors prevent the emergence of drug resistance and render existing antifungals more efficacious (14, 15).

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¹To whom correspondence may be addressed. E-mail: leah.cowen@utoronto.ca or lindquist_admin@wi.mit.edu.

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improved survival of larvae relative to those receiving monotherapy with either agent ($P < 0.0001$).

Genetic Compromise of HSP90 Expression Enhanced the Therapeutic Efficacy of FL in a Murine Model of Disseminated *C. albicans* Disease.

Evaluating the therapeutic potential of Hsp90 inhibitors requires careful testing in a mammalian model to address complexities of immune responses, drug metabolism, and potential toxicity issues. Initial experiments in mice indicated that inhibition of host Hsp90 is not well tolerated in the context of an acute fungal infection (data not shown). Because fungal-specific Hsp90 inhibitors have not yet been identified, we turned to genetic regulation of *C. albicans* HSP90 to establish proof of principle.

We engineered a strain in which the only HSP90 allele is under the control of a tetracycline-repressible promoter (*tetO*). In the absence of tetracycline, this strain has comparable Hsp90 levels to a strain in which the only HSP90 allele is under the control of the native stress-inducible promoter (data not shown). While expression of HSP90 from the native promoter is induced in response to an increase in temperature or in response to FL exposure, expression of HSP90 from the *tetO* promoter is not (Fig. S2). The *tetO-HSP90/hsp90Δ* strain is hypersensitive to FL in vitro compared to a strain with its only HSP90 allele controlled by the native promoter (Fig. 4A). Restoring a wild-type HSP90 allele to the *tetO-HSP90/hsp90Δ* strain complements the FL-hypersensitivity (see Fig. 4A).

For maximal sensitivity to detect antifungal drug efficacy, we used a well-established murine model in which fungal inoculum is delivered by tail-vein injection and progresses from the bloodstream to deep-seated infection of major organs, such as the kidney, coupled with quantitative analysis of kidney fungal burden (23). Mice infected with the *tetO-HSP90/hsp90Δ* strain demonstrated significantly reduced fungal burden relative to those infected with a strain expressing wild-type HSP90 levels ($P < 0.001$, Kruskal-Wallis) (Fig. 4B). There was no significant difference in fungal burden between mice infected with a strain expressing wild-type HSP90 levels or an HSP90 heterozygote (data not shown). Treatment of mice with FL reduced fungal burden in all cases ($P < 0.001$) (see Fig. 4B). FL was significantly more efficacious in mice infected with the *tetO-HSP90/hsp90Δ* strain compared to the wild type, with a 3.6-fold and 1.4-fold reduction in fungal burden compared to untreated counterparts, respectively (see Fig. 4B) ($P < 0.01$, for posthoc comparison between *tetO-HSP90/hsp90Δ* with and without FL, versus $P > 0.05$ for posthoc comparison between wild type with and without FL, Dunn's Multiple Comparison). Tetracycline-mediated transcriptional repression of HSP90 resulted in complete clearance of the infection (Shapiro RS, Uppuluri P, A.K.Z., C.C., Senn H, J.R.P., Heitman J, and L.E.C., submitted). These findings establish proof of principle in a mammalian model that Hsp90 provides an attractive therapeutic target for fungal disease.

Discussion

Ideally, a new therapeutic strategy for fungal infectious disease would enhance the efficacy of existing antifungal drugs, block the emergence of drug resistance, have fungicidal activity, and demonstrate broad efficacy against diverse fungal pathogens. We have established that harnessing fungal Hsp90 meets all of these criteria and has profound therapeutic benefits in combination with antifungal drugs in 2 well-established metazoan models of fungal disease.

Hsp90 regulates antifungal drug resistance of the 2 leading fungal pathogens of humans separated by ≈ 1 billion years of evolution. For *C. albicans*, Hsp90's role in regulating cellular signaling required to survive the membrane stress exerted by azoles is now well established (see Fig. 1A and refs. 8, 14, and 15). Compromising Hsp90 function blocks the de novo emergence of azole resistance and abrogates resistance that has

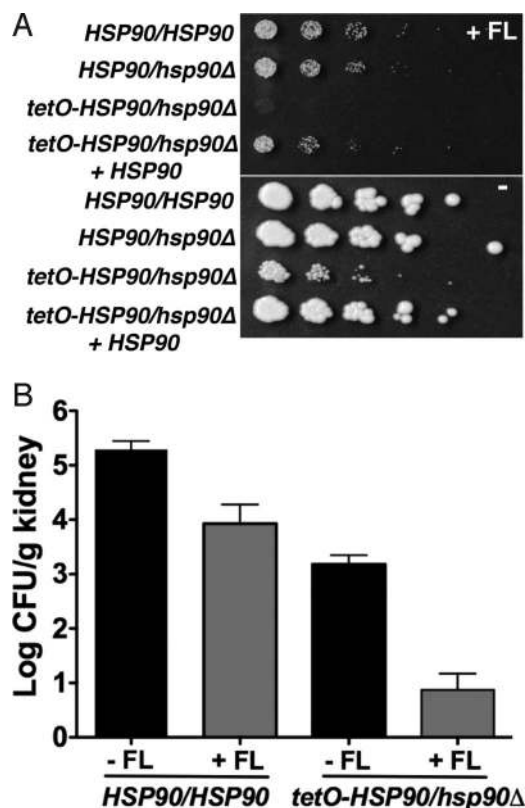


Fig. 4. Genetic compromise of *C. albicans* HSP90 renders FL more efficacious both in vitro and in a murine model of disseminated disease. (A) Compromising HSP90 expression in the *tetO-HSP90/hsp90Δ* strain results in FL-hypersensitivity, even in the absence of tetracycline. Fivefold dilutions of cells (from 1×10^6 /ml) were spotted on YPD with FL (1.5 μ g/ml) (Top) and without FL (Bottom). (B) Compromising *C. albicans* HSP90 expression enhances the therapeutic efficacy of FL in a murine model of disseminated disease. CD1 mice were infected with an inoculum of 200 μ l of 1×10^6 CFU/ml of a strain expressing wild-type HSP90 levels. Inoculum for the strain with its only HSP90 allele regulated by *tetO* was 200 μ l of 1×10^7 cells/ml, corresponding to ≈ 4 -fold higher CFU/ml compared to the wild type (see Materials and Methods). FL was administered at 2-mg/kg i.p. at 1 h after infection and then daily, as indicated.

already evolved. Principal limitations of the clinical efficacy of azoles include their fungistatic action against *Candida* species and their vulnerability to the rapid emergence of resistance in these pathogens. We demonstrate that in combination with Hsp90 inhibition, the fungistatic azoles become fungicidal (see Fig. 1B). This broadens the therapeutic implications of combination therapy with Hsp90 inhibitors to encompass benefits of both reversing antifungal drug resistance and enhancing azole efficacy against drug-sensitive isolates. Consistent with previous findings with *A. terreus*, the most striking synergy with Hsp90 inhibitors against *A. fumigatus* is with the echinocandins (see Fig. 3 and Fig. S1). The specificities of synergistic interactions of Hsp90 inhibitors with antifungal drugs likely reflect the key cellular circuitry underpinning responses to drug-induced stress. A key mediator of Hsp90-dependent azole resistance in *C. albicans* is the protein phosphatase calcineurin, which regulates crucial cellular responses to the membrane stress exerted by azoles (8, 9, 14, 15, 26, 27). With *Aspergillus*, Hsp90's role in echinocandin resistance again tracks with calcineurin's central function in mediating crucial responses to the cell wall stress exerted by echinocandins (28, 29). These findings support a broader paradigm of targeting regulators of signaling and cellular stress responses in the treatment of fungal disease.

Paradoxically, it is Hsp90's role as a key regulator of cellular signaling and stress response that positions it as an ideal therapeutic target and that complicates its utility in therapeutic interventions. Hsp90 is essential in all eukaryotes tested and highly conserved, such that the human protein can complement deletion of genes encoding Hsp90 in yeast (30). While structurally diverse Hsp90 inhibitors are in clinical development as anticancer agents and are well tolerated in humans (17–19), Hsp90 is intimately connected with cellular circuitry regulating host immune and stress responses important in the context of microbial infection. For example, drugs such as cyclosporin A and FK506, which target the Hsp90 client protein calcineurin, are potent immunosuppressants; they abrogate fungal drug resistance but render patients more susceptible to fungal infection, limiting their utility in antifungal therapy (29).

We found that the challenge of translating *in vitro* results into model systems *in vivo* was coupled with complexity of the system. As predicted by *in vitro* data, combination therapy with an Hsp90 inhibitor and an antifungal drug rescues lethal *C. albicans* and *A. fumigatus* infections in the invertebrate host *G. mellonella* (see Figs. 2 and 3). The lack of complications because of host toxicity may reflect the relative simplicity of this organism's immunological function and physiology. Preliminary experiments in a mouse model of disseminated *C. albicans* infection using 2 structurally unrelated Hsp90 inhibitors revealed significant toxicity associated with inhibition of host Hsp90 in the context of acute fungal infection and precluded detection of a benefit to combination therapy on survival. Consequently, we turned to a genetic system to regulate *HSP90*. Compromising *C. albicans HSP90* expression renders FL more efficacious both *in vitro* and in a mouse model of disseminated disease (see Fig. 4). Hypersensitivity to FL is not a general characteristic of strains with reduced growth rates. Thus, these results establish that genetic compromise of *C. albicans Hsp90* expression renders FL more efficacious. Further genetic depletion of *C. albicans HSP90* results in complete clearance of the fungal infection in the mouse model of disseminated disease.

We note that a recombinant antibody against *C. albicans Hsp90* demonstrated therapeutic benefits in a clinical trial in combination with the antifungal drug amphotericin B, which targets ergosterol (31). However, this must work via a mechanism distinct from pharmacological inhibition: the antibody would not be able to cross the fungal cell wall and gain access to the fungal cytosol where the Hsp90 inhibitors exert their effects. Instead, Hsp90 antibodies likely affect some aspect of host immune responses to the pathogen. Indeed, heat-shock proteins provide immunodominant antigens for recognition of many pathogens and play a central role in mediating both innate and adaptive immune responses (32, 33).

Given its function in chaperoning key regulators of the cellular circuitry governing many aspects of organismal biology, Hsp90 has center stage as a therapeutic target for diverse diseases, including cancer and neurodegeneration. In the context of cancer, the clinical development of Hsp90 inhibitors is moving rapidly. New chemotypes with improved pharmacology have been identified and their activity is being tested in clinical trials either alone or in combination with other agents against diverse malignancies (17–19). Our findings in mice that current broadly active Hsp90 inhibitors can have adverse effects in the context of acute *C. albicans* infection highlight the need to identify fungal-selective inhibitors and advise great caution in the treatment of patients with active fungal infection. In the process of developing anticancer Hsp90 inhibitors, extensive compound collections have been generated that could provide a rich, as yet unexploited resource for the identification of potentially fungal-selective inhibitors of the classical Hsp90 N-terminal ATPase site. Such selectivity could be achieved based on divergence between fungal and human Hsp90 orthologs in terms of sequence,

ATPase activity, or equilibrium of conformational states (34). Indeed, we recently conducted a screen in *Saccharomyces cerevisiae* for Hsp90 pathway inhibitors and recovered compounds that block maturation of diverse Hsp90 clients in yeast but not mammalian cells. Biochemical analysis revealed a unique mechanism of action for one class of hits in that they do not compete at the conserved ATP-binding pocket targeted by classical inhibitors. Genetic analysis confirms, however, that they work by modulating function of the Hsp90 chaperone system (L.W., C. McLellan, and S.L., unpublished data).

Hsp90 inhibitors may provide an even broader therapeutic paradigm for the treatment of life-threatening infectious diseases caused by eukaryotes other than fungi. One key mediator of Hsp90-dependent drug resistance is the client protein calcineurin (14, 15). Inhibitors of Hsp90 and calcineurin both possess potent antimalarial activity, thus extending their impact to the protozoan parasite *Plasmodium falciparum* (35, 36). High-throughput genomic and proteomic studies reveal that Hsp90 interacts with up to 10% of the yeast proteome (37), while a systems analysis in *P. falciparum* indicates that Hsp90 interacts with a complex network of client proteins (36). Targeting a highly conserved protein that has an essential role in governing diverse cellular signaling pathways may minimize the probability of the emergence of resistance to Hsp90 inhibitors. Notably, Hsp90 inhibitors also have antiviral activity and evolutionary constraints on chaperone-mediated protein folding may provide a strategy refractory to the development of drug resistance (38). Clearly, the challenge ahead for successfully harnessing Hsp90 function in the treatment of infectious disease now lies in developing potent and specific inhibitors capable of discriminating between the chaperone machineries of pathogen and host.

Materials and Methods

Strains and Culture Conditions. The *C. albicans* clinical isolate (CaCi-2) used in this study was recovered from an HIV-infected patient at an early time point during FL treatment (15, 39). Construction of the tetracycline-repressible *HSP90* system is described in the *SI Text*. The *A. fumigatus* clinical isolate was from a patient at Children's Hospital in Boston, MA (CH 03–197 0861). Species identification was performed as described in the *SI Text*. *C. albicans* strains were grown in either rich YPD medium or a defined medium, RPMI, prepared according to standard protocols (15). *A. fumigatus* was grown on potato dextrose agar (PDA) at 35 °C to induce conidiation.

Antifungal Susceptibility Testing. The effects of Hsp90 inhibitors on antifungal drug resistance were determined with E-test strips (AB Biodisk) on a rich medium (0.5X YPD) and a defined medium (RPMI) essentially as described (15). For *C. albicans*, cell densities of overnight cultures were determined by optical density at 595 nm and by hemacytometer counts; $\approx 10^5$ cells were plated before application of an antifungal test strip. For *A. fumigatus*, conidia were prepared by suspension in PBS, the concentration was determined by hemacytometer count, and $\approx 10^5$ conidia were plated before application of a test strip. GdA, 17-AAG, and 17-DMAG were obtained from Invivogen; drug stocks were prepared in DMSO (Sigma Aldrich Co.). Plates were photographed after 48 h at 35 °C in the dark.

Viability Assay. Duplicate cultures for each condition were grown in liquid YPD at 30 °C with constant agitation. The starting inoculum was normalized to 1×10^6 cells/ml; samples were removed at the indicated times and CFU counts assessed by serial dilution and plating.

***G. mellonella* Killing Assay.** Injection of fungal pathogens and antifungal drugs was performed essentially as described (21). In brief, larvae in the final instar were obtained from Vanderhorst, Inc.. Sixteen larvae (330 ± 25 mg) were used per group. Each injection of 10 μ l of fungus, drug, or control into the hemocoel was performed via a distinct proleg. Drugs were injected individually after inoculum delivery. Larvae were incubated at 37 °C and the number of dead larvae scored daily. *C. albicans* inoculum was prepared by growing 50-ml YPD cultures overnight at 30 °C. Cells were pelleted at $1,308 \times g$ for 10 min followed by 3 washes in PBS. Cell densities were determined by hemacytometer count and dilutions prepared in PBS. *A. fumigatus* inoculum was prepared in PBS by suspending conidia from PDA. Drug stocks were prepared

as indicated in the *SI Text*. Kill curves were plotted and estimation of differences in survival (Log-rank test) analyzed by the Kaplan-Meier method (GraphPad Prism). Histology of infected larvae is described in the *SI Text*.

Spotting. Strains were grown to saturation in YPD and cell concentrations were standardized based on optical density. Fivefold dilutions (from $\approx 1 \times 10^6$ cells/ml) were spotted on media using a spotter (Frogger, V&P Scientific, Inc). The plates were photographed after 2 days in the dark at 30 °C.

Murine Model of *C. albicans* Infection. *C. albicans* was cultured from frozen stocks on Sabouraud agar and incubated at 35 °C for 24 h (48 h for *tetO-HSP90/hsp90Δ* because of slower growth). Multiple colonies were suspended in PBS to a concentration of 1×10^6 cells/ml and 150 μ l was inoculated into Sabouraud broth and incubated for 15 h at 37 °C at 250 rpm (Thermo Fischer Scientific Inc., Forma Model 490 Incubator Shaker). Cultures were centrifuged at $234 \times g$ for 15 min, washed with PBS 3 times and diluted to the desired concentration (1×10^6 cells/ml) by hemacytometer counting. Concentration was verified by serial dilution and culture. We observed discordance between cell counts and CFU measurements for the *tetO-HSP90/hsp90Δ* strain, such that CFU values were $\approx 60\%$ lower than expected. A 10-fold increase in inoculum concentration for *tetO-HSP90/hsp90Δ* (1×10^7 cells/ml) produced ≈ 4 -fold higher CFU/ml than the inoculum for the wild type, and thus was chosen as the dose of *tetO-HSP90/hsp90Δ* for these experiments (see *SI Text*).

For murine exposure, male CD1 mice (Charles River Laboratories) aged 8 weeks (30–34 g) were infected via the tail vein with 200 μ l of a 1×10^6 CFU/ml PBS suspension for the wild type ($n = 13$ mice for untreated and 7 for FL treated), an inoculum previously determined to produce morbidity but not

mortality when using strain SC5314 at 4 days following injection. The inoculum for *tetO-HSP90/hsp90Δ* was 200 μ l of a 1×10^7 cells/ml PBS suspension which corresponds to an ≈ 4 -fold increase in CFU/ml relative to the wild type ($n = 22$ mice for untreated and 10 for FL treated). For groups receiving FL (Pfizer, Inc.), 2 mg/kg was administered i.p. at 1 h after infection and then daily. Mice were observed 3 times daily for signs of illness and weighed daily. At day 4 following injection, mice were killed by CO₂ asphyxiation and the left kidney was removed aseptically, placed in PBS, homogenized via bead beating using a FastPrep 120 (QBiogene) and serial dilutions plated for determination of kidney fungal burden. CFU values in kidneys were expressed as CFU/g of tissue, log-transformed and compared using a Kruskal-Wallis test with posthoc testing of significance between groups by Dunn's Multiple Comparison Test (GraphPad Prism). All murine work was performed under a protocol approved by the Institutional Animal Use and Care Committee at Duke University Medical Center.

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Supporting Information

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SI Text

Strain Construction. *C. albicans* transformations were performed according to standard protocols. Details on strain construction are provided below.

CaLC367 (HSP90/hsp90Δ). The plasmid pLC53 was digested with *Bss*HII to liberate the cassette to target the tetracycline-repressible transactivator *tet-R* to the *HIS1* locus and was transformed into CaLC239 [SN95, (1)]. Proper integration was verified by PCR with primers oLC239 (CaHIS1-587-F, GTAATTTGGTAGTGAAAACCC) and oLC218 (CartTA + 300-R, CCGTGTACCTAAATGTAC). The *SAP2* promoter was induced to drive expression of FLP recombinase (2, 3) to excise the NAT marker cassette and the clone was verified for presence of the transactivator with the same primers as above. This strain was transformed with the *C. dubliniensis* *HIS1* marker and ~350 bases of homology upstream and downstream of *HSP90*, amplified from plasmid pLC64 with primers oLC268 (CaHsp90-FP-1, cggagatgtattgactgtgg) and oLC271 (CaHsp90-FP-6, CTCTATGTTATGTTACTGGG). Proper deletion of *HSP90* was confirmed by PCR with primers oLC255 (CdHIS1 + 1032-F, GTCCATTAACCTCAAACC) and oLC277 (CaHsp90 + 2627-R, CCACCCTGAACCTAATTTGG) as well as oLC276 (CaHsp90-427-F, GAGATGGCAAATCGTTAGG) and oLC254 (CdHIS1-710-R, CGTCTCTTGATGTATATGG). Genotype: *arg4Δ/arg4Δ his1Δ/his1Δ URA3/ura3::imm⁴³⁴ IRO1/iro1::imm⁴³⁴ HIS1/his1::tetR-FRT HSP90/hsp90::CdHIS1*.

CaLC436 (tetO-HSP90/hsp90Δ). The plasmid pLC330 was digested with *Bss*HII to liberate the cassette to replace the native *HSP90* promoter with the *tetO* promoter and was transformed into CaLC367. Proper integration was verified by PCR using primers oLC308 (CaHSP90-698-F, GATTATTTGCTCACGGAACC) and oLC275 (pJK863up-R, AAAGTCAAAGTTC-CAAGGGG) as well as oLC309 (CaHsp90 + 415-R, CTTG-GACGTGATCAGCAACC) and oLC274 (pJK863down-F, CTGCAAGGGTATTCTGG). The *SAP2* promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. Genotype: *arg4Δ/arg4Δ his1Δ/his1Δ URA3/ura3::imm⁴³⁴ IRO1/iro1::imm⁴³⁴ HIS1/his1::tetR-FRT FRT-tetO-HSP90/hsp90::CdHIS1*.

CaLC946 (tetO-HSP90/hsp90Δ + HSP90). To complement the deleted *HSP90* allele in CaLC436, pLC455 was sequentially digested with *Sac*II and *Apa*I to liberate a cassette to replace the deleted allele with a wild-type allele. NAT-resistant transformants were PCR tested for proper integration of the cassette at the *HSP90* locus with primers oLC274 (pJK863down-F, CTGCAAGGGG-TATTCTGG) and oLC335 (CaHsp90 + 3116-R, gttggtgtgattctcc). To determine whether the cassette integrated at the deleted allele or the *tetO-HSP90* allele, presence of *CdHIS1* at the *HSP90* locus was tested using primers oLC255 (CdHIS1 + 1032-F, GTCCATTAACCTCAAACC) and oLC277 (CaHsp90 + 2627-R, CCACCCTGAACCTAATTTGG). Lack of a band suggested that the deleted allele was replaced and the *tetO-HSP90* allele remained intact. To confirm this, presence of *tetO* within the *HSP90* promoter was tested by PCR using primers oLC267 (CaHsp90upR-BamHI, CGGGATCCCATAAT-GAATATTGATTTG) and oLC609 (CaHsp90 + 308R-SacI, CGAGCTCCAGATTTAGCAATAGTACC). If *tetO* was still present (i.e., the deleted allele was replaced), then the expected band would be ~1.2 kb; if *tetO* was absent then the expected band

would be 785bp. This complemented strain has the deleted allele replaced with a wild-type *HSP90* allele and the *tetO-HSP90* allele remains intact. The NAT marker was excised. Genotype: *arg4Δ/arg4Δ his1Δ/his1Δ URA3/ura3::imm⁴³⁴ IRO1/iro1::imm⁴³⁴ HIS1/his1::TAR-FRT FRT-tetO-HSP90/HSP90-FRT*.

Plasmid Construction. Recombinant DNA procedures were performed according to standard protocols. Details on plasmids construction are described below. Plasmids were sequenced to verify the absence of any nonsense mutations.

pLC53. [tet-R integration at HIS1 vector, NAT, ampR (4)]. The *C. albicans* tetracycline-repressible transactivator (*tet-R*) was PCR amplified using primers oLC221 (CaTA-ApaI-F, TTGCGGGCCCTT-GAGATGGAGCCGTCAAATATCC) and oLC222 (CaTA-ApaI-R, ACCGGGGCCCGACATTTTATGATGGAAT-GAATGG) and cloned into plasmid pLC49 [pJK863, FLP-CaTAT, ampR (3)] at *Apa*I. Clones were PCR tested for the orientation of the insert using primers oLC221 (CaTA-ApaI-F TTGCGGGCCCTT-GAGATGGAGCCGTCAAATATCC) and oLC203 (CaSAP-65R, CCAAGAGGAATCAACTATCC). Approximately 300 base pairs upstream of *HIS1* were amplified from SC5314 genomic DNA using primers oLC231 (CaHIS1UPKpnI-F, CGGGGTACCCAATTTGGGGACA-GAAGAGG) and oLC232 (CaHIS1UPKpnI-R, CGGGGTAC-CCATTATCGGTAGTTGGTGG) and was cloned into pLC49 at *Kpn*I. The proper orientation of the homology was PCR verified with primers oLC231 (CaHIS1UPKpnI-F, CGGGGTACCCAATTTGGGGACAGAAGAGG) and oLC203 (CaSAP-65R, CCAAGAGGAATCAACTATCC). Approximately 300 base pairs downstream of *HIS1* were amplified with primers oLC233 (CaHIS1DOWNSacII-F, TCCCCGCGGTA-AAAGAAGTGATAGTTTCTC) and oLC234 (CaHIS1DOWNSacII-R, TCCCCGCGGGAATTGGATAGATTATTTGGG) from SC5314 genomic DNA and was cloned into pLC49 containing the upstream homology at *Sac*II. The proper orientation of the homology was PCR verified with primers oLC202 (CaNAT1 + 544F, GCTTTGTATATGTCTATGCC) and oLC234 (CaHIS1DOWNSacII-R, TCCCCGCGGGAATTGGATAGATTATTTGGG). The cassette to target *tet-R* to the *HIS1* locus can be excised with *Bss*HII, which leaves an additional ~40 bases of plasmid sequence.

pLC64 (CaHSP90 knock-out vector, CdHIS1, ampR). Approximately 350 base pairs of homology upstream of *HSP90* were amplified from SC5314 genomic DNA with primers oLC268 (CaHsp90-FP-1, CGGAGATGTATTGACTGTGG) and oLC269 (CaHsp90-FP-3, TGCTGTGATTTATACCAGGGAGAGAC-CTCGTGACATCCATAATGAOACTATTGATTTG). Approximately 350 base pairs of homology downstream of *HSP90* were amplified from SC5314 genomic DNA with primers oLC272 (CaHsp90-FP-4, TATCTCAGCACACGGGACAGC-TACGAGACCGACACCGTAAACACCAGAAGGGCTAC) and oLC271 (CaHsp90-FP-6, CTCTATGTTATGTTACTGGG). The *Candida dubliniensis* *HIS1* selectable marker was amplified from pSN52 (1) with primers oLC270 (CaHsp90-FP-2, CCCTGGTATAAATCACAGCACCAGTGTGCTGGAATTCG) and oLC273 (CaHsp90-FP-5, CTGTCCTGTGCTGAGATAGGATATCTGCAGAATTTCG). The 3 components were assembled by fusion PCR using primers oLC268 (CaHsp90-FP-1, CGGAGATGTATTGACTGTGG) and oLC271 (CaHsp90-FP-6, CTCTATGTTATGTTACTGGG).

This PCR product was TA cloned into pCRII-TOPO vector (Invitrogen Co.).

pLC330 (CaHSP90 promoter replacement vector, tetO-CaHSP90, NAT, ampR). The tetracycline-responsive promoter was amplified from pNIM1 (5) with oLC300 (Tetp-F-NotI, ATAAGAATGCGGC-CGCGTTTGGTTCAGCACCTTGTCG) and oLC301 (Tetp-R-SacII, GGCACCGCGGCGACTATTTATATTTG-TATGTGTGTAGG) and cloned into pLC49 at NotI and SacII. Approximately 350 base pairs of homology upstream of the promoter were amplified from SC5314 genomic DNA with primers oLC294 (CaHsp90-597-F-KpnI, CGGGGTAC-CGATTTTCAGGTTGAAGAATTTGC) and oLC295 (CaHsp90-250-R-ApaI, TTGCGGGCCCCTGTTATAGG-TAGTAATATGG) and was cloned into pLC49 containing the *tetO* promoter at KpnI and ApaI. Approximately 350 base pairs of homology downstream of the promoter were amplified from SC5314 genomic DNA with primers oLC296 (CaHsp90-1-F-SacII, TCCCCGCGGTATGGCTGACGCAAAAGTTG) and oLC297 (CaHsp90 + 348-R-SacI, CCCGAGCTCTCAGCAC-CAGCACTTAAAGC) and cloned into pLC49 containing the *tetO* promoter and the upstream homology at SacII and SacI. The cassette to replace the native *HSP90* promoter with the *tetO* promoter can be excised with *Bss*HII.

pLC455 (CaHSP90 complementation vector, NAT, ampR). The *HSP90* ORF, in addition to ≈ 330 base pairs of promoter region and some terminator region was amplified from SC5314 genomic DNA by PCR with oLC867 (CaHsp90-331F-ApaI, TT-GCGGGCCCCGATTGAGACAGAAGAAGTG) and oLC332 (CaHsp90 + 2491-R-ApaI, TTGCGGGCCCCAACAAAGAT-CAATACCTTACTACC) and cloned into pLC49 at ApaI. Homology downstream of *HSP90* was amplified from SC5314 genomic DNA with primers oLC333 (CaHsp90 + 2492-F-NotI, ATAAGAATGCGGCCGCGATCTATCTTGAAACTCA-GCG) and oLC334 (CaHsp90 + 2884-R-SacII, GGCACCGCG-Gctctgatctacatcc) and cloned into pLC49 containing *HSP90* at NotI and SacII. Presence of the inserts was tested by PCR with oLC274 (pJK863down-F, CTGTCAAGGAGGG-TATTCTGG) and oLC334 (CaHsp90 + 2884-R-SacII, GGCACCGCGGCTCTGATCGTATACCTATCC) as well as oLC275 (pJK863up-R, AAAGTCAAAGTTCCAAGGGG) and oLC200 (CaHsp90 + 1831F, GAAATTTCTCCATCT-TCC). The complementation cassette can be liberated by digestion with ApaI and SacII.

Formulation of Drug Stocks for *Galleria mellonella* Killing Assays.

Fluconazole (Sequoia Research Products Ltd.) was dissolved in water and filter sterilized; caspofungin (courtesy of R. Bagatell, University of Arizona) was provided as a formulation for i.v. delivery; geldanamycin was formulated in DMSO (tissue culture grade, Sigma Aldrich Co.); 17-DMAG was formulated in water

and filter sterilized; 17-AAG was formulated in a complex vehicle: 5% N, N-dimethylacetamide (DMA, Sigma Aldrich Co.); 7.5% Cremophor-EL (Sigma Aldrich Co.); 87.5% saline solution (0.9% saline); specifically, 17-AAG was dissolved in DMA and vortexed vigorously; cremophor was added and vortexed until the 2 layers were completely mixed; the saline solution was added in 3 equal parts with intermittent vortexing until a clear emulsion was achieved; the final solution was filter sterilized.

***Aspergillus fumigatus* Species Identification.** *A. fumigatus* was identified by macroscopic morphology on Sabouraud medium and by microscopic appearance of conidiophores stained with lactophenol cotton blue, showing uniseriate phialides emerging from the upper portion of the vesicle parallel to the conidiophore axis (6).

Histology of *A. fumigatus*-Infected *G. mellonella* Larvae. Larvae were embedded in paraffin, cut at 5 μm , and stained with Grocott's methenamine-silver nitrate fungus stain (7, 8).

Dose-Finding Survival Experiment to Standardize *C. albicans* Inocula for Murine Model of Disseminated Disease. In an attempt to standardize day-4 fungal burden across *Candida* strains, increased inocula of the *tetO-HSP90/hsp90* Δ strain were evaluated in a survival experiment. An inoculum size of 2×10^7 CFU (200 μl of 1×10^8 CFU/ml suspension) produced significant morbidity at 48 h after inoculation, necessitating sacrifice of all mice ($n = 5$). An inoculum size of 2×10^6 CFU (200 μl of 1×10^7 CFU/ml) produced an average log CFU/g kidney of 4.36 ± 1.33 without mortality and was chosen for the dose of this strain for subsequent experiments.

Quantitative RT-PCR. Cells were grown overnight in YPD at 30 $^{\circ}\text{C}$, diluted to an OD₆₀₀ of 0.2 in 50-ml YPD and grown for an additional 3 h. Then 15 ml of the culture was pelleted and resuspended in either: 50-ml YPD at 30 $^{\circ}\text{C}$, 50-ml YPD at 37 $^{\circ}\text{C}$, or 50-ml YPD with 4- $\mu\text{g}/\text{ml}$ FL. Cultures were sampled by removing 7 ml, placing it on ice for 5 min, pelleting it at $1,308 \times g$ for 5 min at 4 $^{\circ}\text{C}$, and freezing the pellet at -80 $^{\circ}\text{C}$. RNA was isolated using the QIAGEN RNeasy kit and RNase-free DNase (QIAGEN), and cDNA synthesis was performed using the AffinityScript cDNA synthesis kit (Stratagene). PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma Aldrich Co.) with the following cycling conditions: 94 $^{\circ}\text{C}$ for 2 min, 94 $^{\circ}\text{C}$ for 15 s, 60 $^{\circ}\text{C}$ for 1 min, 72 $^{\circ}\text{C}$ for 1 min, for 40 cycles. All reactions were performed in triplicate, using oLC752 (GPD1 + 570-F, AGTATGTGGAGCTTTACTGGGA) and oLC753 (GPD1 + 766-R, CAGAAACACCAGCAACATC-TTC) for *GPD1* and oLC756 (HSP90 + 1051-F, GCTGAA-GAGTTGATTCAGAAT) and oLC757 (HSP90 + 1236-R, GGAGAAAGCAGTGTAGAATTGG) for *HSP90*. Data were analyzed using iQ5 Optical System Software Version 2.0 (Bio-Rad Laboratories, Inc).

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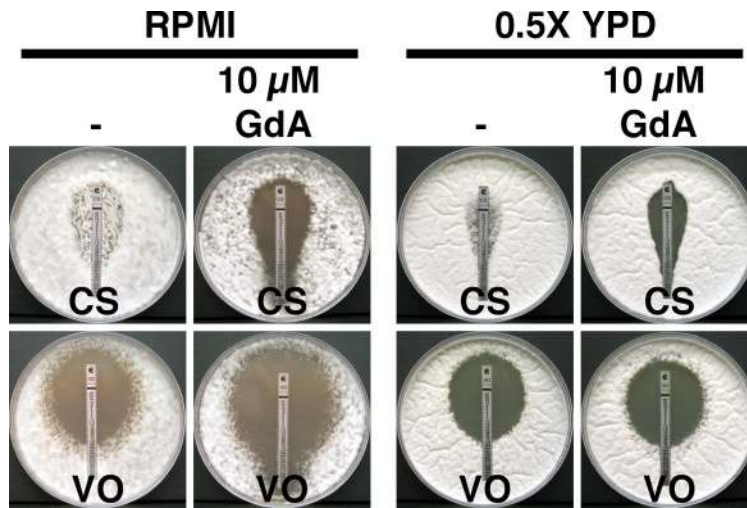


Fig. S1. Pharmacological inhibition of Hsp90 enhances the efficacy of antifungal drugs against *Aspergillus fumigatus*. Resistance of an *A. fumigatus* clinical isolate to the echinocandin caspofungin (CS) or the azole voriconazole (VO) on defined RPMI medium or rich 0.5X YPD medium, as indicated. Antifungal test strips (Etest, AB Biodisk) produce a gradient of drug concentration with the highest at the top. Plates contained vehicle control (DMSO) or geldanamycin (GdA), as indicated.

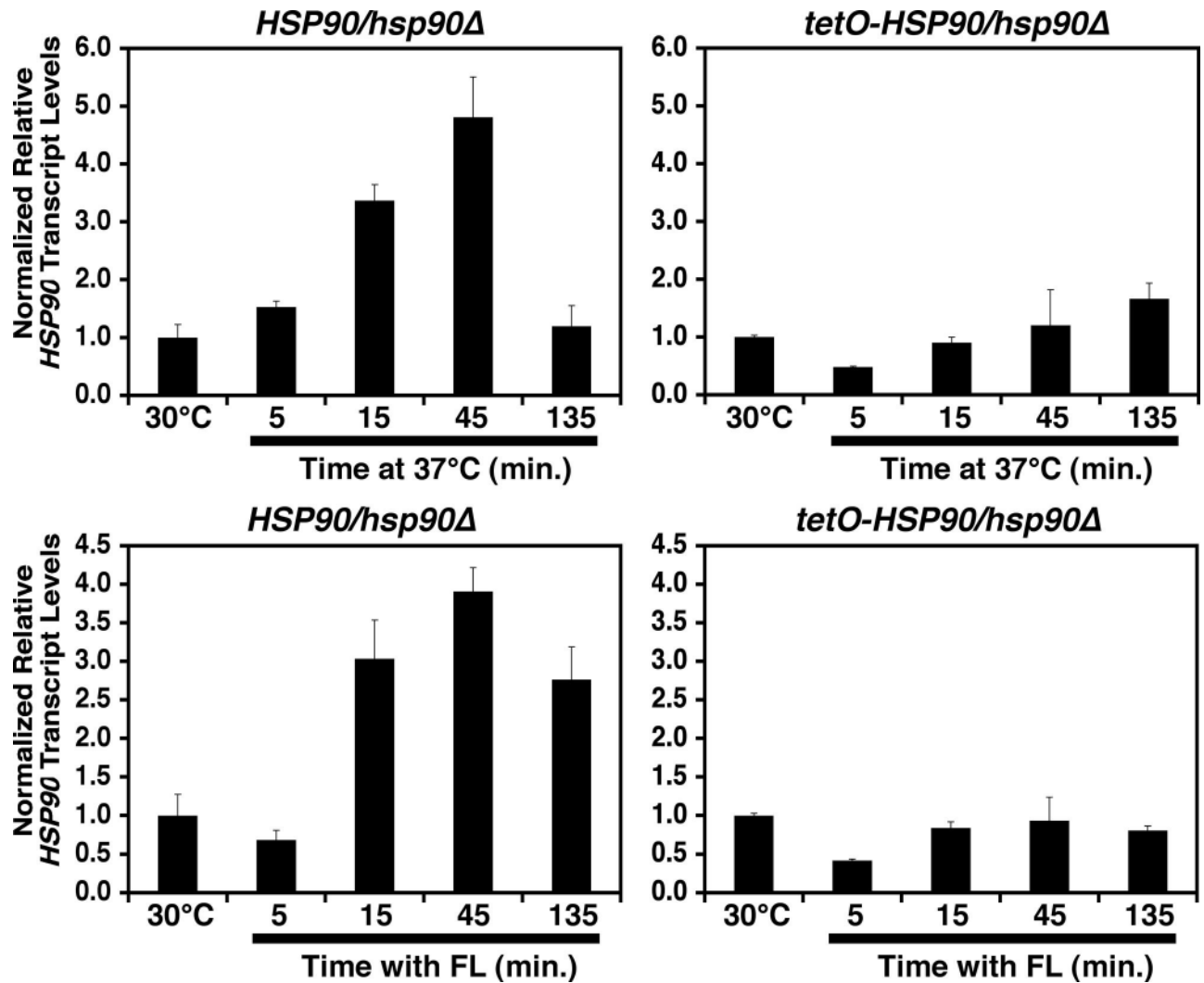


Fig. S2. Replacing the native stress-inducible *HSP90* promoter with the tetracycline-repressible promoter (*tetO*) blocks induction of *HSP90* upon exposure to increased temperature (37 °C) or FL (4 μ g/ml). *HSP90* transcript levels were measured by quantitative RT-PCR and normalized relative to the *GPD1* control. Levels are expressed relative to the control sample at 30 °C with no treatment. Data are means for triplicate samples and standard deviations.