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Structural basis for species-selective targeting of Hsp90 in a pathogenic fungus

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New strategies are needed to counter the escalating threat posed by drug-resistant fungi. The molecular chaperone Hsp90 affords a promising target because it supports survival, virulence and drug-resistance across diverse pathogens. Inhibitors of human Hsp90 under development as anticancer therapeutics, however, exert host toxicities that preclude their use as antifungals. Seeking a route to species-selectivity, we investigate the nucleotide-binding domain (NBD) of Hsp90 from the most common human fungal pathogen, *Candida albicans*. Here we report structures for this NBD alone, in complex with ADP or in complex with known Hsp90 inhibitors. Encouraged by the conformational flexibility revealed by these structures, we synthesize an inhibitor with >25-fold binding-selectivity for fungal Hsp90 NBD. Comparing co-crystals occupied by this probe vs. anticancer Hsp90 inhibitors revealed major, previously unreported conformational rearrangements. These insights and our probe’s species-selectivity in culture support the feasibility of targeting Hsp90 as a promising antifungal strategy.
Fungal pathogens are major contributors to human morbidity and mortality, infecting billions of people worldwide and killing in excess of 1.5 million per year. The most vulnerable are individuals with compromised immune systems, including those undergoing chemotherapy, recipients of solid organ or hematopoietic stem cell transplants, and those infected with HIV. Candida albicans is a common commensal member of the human mucosal microbiome, but it is also the most frequent cause of invasive fungal infections in hospitalized patients, with mortality rates for disseminated disease approaching 40%, even with current treatments. The development of drugs with selective activity against fungi has been hampered by the close evolutionary relationship between fungi and their human hosts. An armamentarium of only three broadly deployed drug classes currently exists, and the frequent emergence of resistance makes cure of invasive fungal infections often unachievable. New agents with non-cross-reactive modes of action and non-overlapping toxicities are urgently needed.

A promising strategy to enhance the efficacy of established antifungals and improve clinical outcome is the co-targeting of regulators of fungal stress responses. We previously established that the evolutionarily ancient, highly conserved molecular chaperone Hsp90 promotes antifungal drug tolerance and the evolution of drug resistance in species of Candida and Aspergillus. Hsp90 is essential in all eukaryotes where it regulates the form and function of diverse client proteins, many of which are key signal transducers. Hsp90 enables drug resistance via multiple molecular mechanisms, most notably through the stabilization of the stress-activated protein phosphatase calcineurin and multiple components of Pkc1 mitogen-activated protein kinase (MAPK) cell wall integrity pathway. Hsp90 also serves as the previously enigmatic thermal sensor that controls temperature-dependent morphogenetic switching in C. albicans, a key factor contributing to the organism’s virulence. In animal models, limiting Hsp90 function restores sensitivity in drug-resistant fungal pathogens, impedes the evolution of drug resistance, and leads to clearance of otherwise lethal infections. Unfortunately, compromise of host Hsp90 function with human homolog-optimized drugs (currently being tested in patients with cancer) comes with prohibitive side effects in the context of systemic fungal infections. Thus, while fungal Hsp90 serves as a promising therapeutic Achilles heel, the utility of targeting it depends on whether sufficient inhibitor selectivity can be achieved to avoid compromise of its human homolog.

The ability to inhibit nucleotide binding within the Bergerat fold present in Hsp90’s N-terminal domain was initially established by two structurally unrelated macrocyclic natural products, the resorcylic acid lactone radicicol and the benzoquinone ansamycin geldanamycin. With the goal of disrupting Hsp90’s unique role in enabling oncogenesis, multiple structure-guided anticancer drug development programs have yielded a diverse array of inhibitor chemotypes. Utilizing a variety of high affinity binding modes, these compounds all mimic the unusual conformation adopted by ATP upon binding within the chaperone’s nucleotide-binding domain (NBD) and inhibit ATP binding and hydrolysis, thereby blocking chaperone function.

Consistent with its evolutionarily ancient and essential role in maintaining eukaryotic protein homeostasis, Hsp90 is highly conserved across phyla. Bacterial, yeast and human Hsp90, an ordered three-state cycle of conformational changes associated with the hydrolysis of ATP is fundamental to the protein’s chaperoning activity. However, the equilibria between these states and the intrinsic ATPase activity of the isoforms are highly species-specific. The disparity has been hypothesized to reflect “evolutionary tuning to meet the client protein and metabolic environment of an organism.” An additional layer of conformational regulation is provided by a suite of co-chaperones and accessory proteins, which also varies in composition across species. These proteins physically associate with Hsp90 to modulate its client-protein binding and the conformational changes associated with its chaperoning cycle. Thus, despite a high degree of primary sequence conservation, functionally important conformational differences have been demonstrated between the Hsp90 isoforms expressed in humans and the model fungus Saccharomyces cerevisiae. To date, however, no Hsp90 structural studies have been performed in pathogenic fungi such as C. albicans.

Seeking to target potential species-dependent differences in conformation or flexibility within the ATP-binding pocket of Hsp90, we pursued an iterative chemo-structural approach to achieve fungal-selective inhibition. We first solve the structure of the C. albicans Hsp90 N-terminal NBD, alone and in complex with several known Hsp90 inhibitors. The insights gained helped guide synthesis of a fungal-selective probe molecule, CMLD013075 (1). This probe is then co-crystallized with the NBD to reveal a surprising, previously unreported binding-site rearrangement permitting accommodation of the ligand. The unique binding mode observed for our probe and the structural flexibility of the fungal NBD suggest a promising path to selectivity despite high conservation at the primary sequence level. Our findings provide proof-of-principle for the feasibility of selectively targeting fungal Hsp90, while the biological activities of our probe support the therapeutic potential of targeting this key molecular hub to counter the escalating problem of drug-resistant fungal infections.

**Results**

**Unique features of C. albicans Hsp90.** To identify potentially exploitable differences in the Hsp90 expressed by a fungal pathogen vs humans, we determined the crystal structure of the C. albicans Hsp90 N-terminal domain, which includes the nucleotide-binding pocket targeted by all Hsp90 inhibitors in clinical development. We obtained structures for the domain under three conditions: apo (unliganded), in complex with ADP, and in complex with the prototypical inhibitor radicicol (Fig. 1a, Supplementary Table 1). Despite the presence of different ligands, overlay of the three crystal structures showed an almost identical conformation for this domain with a root mean square deviation (r.m.s.d.) of 1.32 Å or less over all atoms (Fig. 1a and Supplementary Table 2). Nonetheless, localized structural differences between the apo and liganded complexes were observed in the loop between α-helices 4 and 5 (residues Gly97 to Thr104), a region on the edge of the ATP binding site (Supplementary Figure 1). A portion of this region also differed between the fungal protein and its human homolog, amid otherwise very similar structures (Fig. 1b, c, upper panels). In previously reported human apo structures, residues 104–111 adopt either an open (PDB: 1YES) or closed (PDB: 1YER) loop conformation; in the C. albicans apo structure, the corresponding region (94–101) adopts an open loop conformation (Fig. 1c, upper panel). Overall comparison between both human apo structures (open and closed) and the fungal N-terminal domain showed no major differences, with main-chain atom r.m.s.d. of 1.02 and 1.15 Å, respectively. Global structural similarity between fungal and human Hsp90 was also observed when comparing the chaperone-ADP and -radicicol complexes (Fig. 1b, middle and lower panels). Additionally, the N-terminal domain of C. albicans Hsp90 shares the same architecture at the nucleotide-binding site, which interacts with both ADP and radicicol through hydrogen-bond interactions at the same amino acids (Asp92 and Thr174, Fig. 1c, middle and lower panels).
Given the structural similarities between human and C. albicans Hsp90 NBD, we asked whether the core ATPase activity of purified full-length homodimers from each species would also be conserved. The dissociation constant ($K_d$) for ATP of C. albicans Hsp90 was similar to that previously reported for S. cerevisiae Hsp90 (125 vs 132 µM), but somewhat lower than that reported for human Hsp90 (240 µM; Supplementary Figure 2a, 2d). The ATPase activity of C. albicans Hsp90 obeyed Michaelis–Menten kinetics (Supplementary Figure 2b and 2c), yielding values for catalytic efficiency similar to that reported for S. cerevisiae ($11.6 \times 10^{-5}$ vs $15.6 \times 10^{-5}$) but more than twofold higher than that reported for human Hsp90 ($4.6 \times 10^{-5}$; Supplementary Figure 2d). At the level of biochemical activity, therefore, a difference is apparent between C. albicans Hsp90 and its mammalian counterpart despite extensive sequence conservation within the NBD.

**Increased conformational flexibility in C. albicans Hsp90.** Seeking to reconcile apparent structural conservation with disparity in ATPase activity, we probed the conformational flexibility of the C. albicans NBD by solving structures of the domain

![Figure 1](image-url)
in complex with two clinically relevant Hsp90 inhibitors, SNX-2112 (Fig. 2a, Supplementary Table 1) and AUY922 (Fig. 2b, Supplementary Table 1). These compounds were chosen because previously reported human Hsp90 structures had shown distinct conformational changes in the region defined by Hsp90 residues 104–111 in co-crystals with these ligands36,37. While the human Hsp90-AUY922 complex shows an apo-like, closed-loop conformation, the human NBD in complex with SNX-2112 shows an extended α-helical conformation. This conformation exposes a hydrophobic pocket that has been previously characterized in human Hsp9051. Interestingly, compounds that induce a helical conformation of the 104–111 region show orthologue selectivity within the human Hsp90 family, binding poorly to Grp94 and Trap-1.

To correlate structural insights with the relative binding affinity for compounds, we used a fluorescence polarization (FP)-based competition approach that allows quantitation of ligand binding in assays utilizing purified NBD proteins or whole-cell lysates. Use of purified proteins allows determination of actual ligand dissociation constants for specific NBD32. Use of lysates interrogates overall ligand binding by Hsp90 in its native complexes with co-chaperones and in the case of human cells, the mixture of Hsp90 isoforms present in the lysate. Lysate experiments can only provide assay-dependent relative EC50 values for inhibitors, but may be more relevant to selectivity at the whole-cell level. To avoid problems with auto-fluorescence typical of lysate, we synthesized a previously reported, red-shifted Cy3B-geldanamycin conjugate to use as the FP probe in both configurations of the assay33. Importantly, using this probe with purified human NBD protein yielded an inhibition Ki for geldanamycin consistent with previously reported values for full-length recombinant Hsp90a34. In our FP assay, SNX-2112 showed threefold higher affinity for the fungal than human Hsp90-binding domain (Fig. 2a, right panel and Supplementary Figure 3a, 3b). In lysates, the compound displayed even greater fungal selectivity, but the improvement may have resulted from the relatively poor affinity of SNX-2112 for Grp94 which is reported to be present in almost equimolar concentration to Hsp90 αβ in HepG2 cells from which the human lysate was prepared (Supplementary Figure 3b and 3c)55.

The structure of the C. albicans NBD–SNX-2112 co-crystal showed that the aminobenzamide and cyclohexanol portions of SNX-2112 occupy the adenosine region of the ATP-binding pocket, making similar polar contacts with the fungal Hsp90 as those seen in the human structure; overall both complexes showed considerable structural similarity (r.m.s.d. 1.0 Å, main-chain atoms) including almost identical poses for SNX-2112 (Fig. 2a). However, in the fungal NBD, the dihydroindazolone moiety of SNX-2112 induced a localized structural rearrangement of the Val93–Ser102 (103–112 in human Hsp90) region distinct from the one observed in the human complex structure. Unlike the extended α-helix observed for the human NBD in complex with SNX-2112, the C. albicans NBD adopted a bulged-out loop conformation with a shortening of α-helix 4 (Fig. 2a, right panel). A similar conformation was previously described for a complex of T. brucei Hsp90 NBD with SNX-2112 complex (PDB: 3OPD). Interestingly, the Hsp90 in this organism showed increased affinity for SNX-211246. An overlay between C. albicans NBD apo and SNX-2112 structures further demonstrates the significance of the structural rearrangement within the Val93–Ser102 region upon binding to SNX-2112, as this region shows the largest differences between both structures (Fig. 2c and Supplementary Figure 1). The plasticity of the C. albicans Hsp90 ATP-binding site appears to be an important element in understanding differences in binding affinity for ortho-benzamide-type Hsp90 inhibitors like SNX-2112, due to the highly localized nature of the structural differences between the liganded C. albicans and human chaperones, and between the C. albicans liganded and apo structures.

In contrast to SNX-2112, the structure of C. albicans Hsp90 NBD in complex with AUY922 was different from all previously reported structures for an Hsp90 of any species, revealing a large structural rearrangement that extended beyond the nucleotide-binding site (Fig. 2b–d). This fungal co-crystal structure contrasts with a previously reported human NBD-AUY922 complex structure, which adopts a closed-loop apo conformation (PDB: 2VCI)57. Remarkably, the large structural rearrangements observed in the C. albicans AUY922 complex were accompanied by very high binding affinity for the compound, although less than that observed for the human NBD (Fig. 2b, right panel and Supplementary Figure 3b). As with SNX-2112, the high affinity of AUY922 for human Hsp90 NBD also appears to have been obscured somewhat in HepG2 lysate by the lower affinity of Grp94 for this compound (Supplementary Figure 3b and 3c). An overlay of the fungal and human complexes highlights the extent of the structural changes associated with AUY922 binding to C. albicans NBD (Fig. 2b and Supplementary Figure 1, r.m.s.d. 3.8 Å, main-chain atoms). The observed conformational change extends beyond the nucleotide-binding site to include the N-terminal β-strand (β1) and helix (a1), and the lid subdomain comprising residues 94–126 (including helices a5 and a6) (Figure 4a). The loss of β1 also leads to a shortening of the adjacent strand (β6) and repositioning of the loop connecting β5-β6 (Supplementary Figure 4a).

Despite major structural differences between the C. albicans and human Hsp90 complexes with AUY922, the ligand maintains the majority of polar and non-polar contacts in each, adopting nearly identical orientations with the exception of the morpholine substituent (Fig. 2b, Supplementary Figure 4a and 4b). In complex with human Hsp90a, the morpholine points away from the chaperone into the solvent, engaging in limited interactions with the host protein at residues Thr109 and Gly135. In the C. albicans complex, the heterocycle is flipped ~180° towards the protein core, engaging in contacts with residues Asn95, Gly124 and Phe123 (Supplementary Figure 4b). The conformational changes induced by AUY922 binding in C. albicans Hsp90 suggest a far greater degree of conformational flexibility for the fungal chaperone compared to its human homolog.

To better understand the roles of the NBD’s structural elements in ligand-binding, we overlaid the C. albicans Hsp90 apo and AUY922 structures (Fig. 2c, right). As previously noted, the C. albicans NBD-AUY922 complex showed a large conformational change leading to major overall differences with respect to the apo structure (r.m.s.d. 3.47 Å, all atoms). Upon AUY922 binding, residues 94–101 adopt a helical conformation that fuses helices 4 and 5 (Fig. 2d). This fused helical element peels the lid region away from the ATP-binding site to accommodate positioning of AUY922’s morpholine ring. This repositioning is accompanied by disappearance of the N-terminal β-strand (β1), affecting β6 and its preceding loop. Despite this clear change, the rest of the protein’s structure in both the apo and AUY922 complexes is almost identical. The portion of the C. albicans NBD which interacts with the base moiety of ATP, defined by residues from the β-sheet (β2 to β5) and helices a2, a3, a8, and a9, is extremely rigid. Furthermore, this rigid region showed no significant differences across all of the co-crystal structures we solved for the fungal NBD (Supplementary Figure 1). Considered together, our structural observations define the conformationally mobile portions of C. albicans NBD that participate in the binding of specific ligands. Most importantly, the magnitude of structural reorganization observed upon binding of AUY922 indicated a much higher degree of conformational flexibility in the fungal than human Hsp90 NBD.

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Fig. 2 Comparison of inhibitor-bound structures for C. albicans vs human Hsp90 NBD. 

a Detailed view of the binding mode of SNX-2112 to C. albicans Hsp90 nucleotide-binding domain (NBD) (dark blue), overlaid with a human Hsp90α NBD structure in complex with SNX-2112 (light blue; PDB id 4NH7). Several of the C. albicans Hsp90 residues interacting with the compound are identified, and the conformation of the 93–102 region is indicated by a dotted rectangle. Binding affinities for the compound were determined by equilibrium competition FP assay using purified human and fungal NBDs. \( K_i \) values are indicated below the structures. The mean ± SEM of three independent experiments is presented.

b Detailed view of the binding mode of AUY922 to C. albicans Hsp90 NBD (orange), overlaid with a human Hsp90α NBD structure in complex with the same compound (light orange; PDB id 2VCI). Several C. albicans Hsp90 residues interacting with the compound are identified. The helical conformation of the 93–102 region is indicated by a dotted rectangle. Hsp90 NBD binding affinities were determined as described for panel a. The mean ± SEM of three independent experiments is presented. 

c Inhibitor-induced conformational changes in C. albicans NBD. Ribbon representation of C. albicans Hsp90 NBD bound to Hsp90 inhibitors SNX-2112 (left image) and AUY922 (right image). Apo structure (green) is overlaid. Compounds are represented as sticks and color-coded according to heteroatom composition. A detailed view of the conformational differences in the 93–102 region between apo and SNX-2112 bound crystal structures is provided; SNX-2112 is shown as sticks and a semitransparent yellow surface.

d Views from different angles of the C. albicans Hsp90 NBD in its apo and AUY922-bound states. Overlays of the crystal structures are shown in cartoon/ribbon representation. Secondary structure elements are color-coded to highlight the conformational changes associated with AUY922 binding; dotted arrows indicate the direction of the movement of secondary structure elements. The outline of the apo surface is indicated by a black line, and the AUY922 position is indicated as a semitransparent yellow sphere representation. Source data for the determination of all inhibitory constants are provided as a source data file.
Semi-synthesis of fungal-selective Hsp90 inhibitors. Disparity between the ligand-induced conformational changes we identified in fungal vs human NBDs encouraged further work to synthesize fungal-selective inhibitors. To identify the most promising scaffold from which to begin, we tested a panel of Hsp90 inhibitor chemotypes for their ability to inhibit fungal growth. We used a strain of *S. cerevisiae* in which the genes encoding both isoforms of its endogenous Hsp90 (HSP82 and HSC82) have been disrupted and growth is dependent upon *C. albicans* Hsp90 expressed from a plasmid. The natural product radicicol was the only Hsp90 inhibitor found to significantly inhibit growth of our engineered fungal strain (Supplementary Figure 5a, 5b). Our structural studies had demonstrated considerable ligand-induced flexibility within the NBD of fungal Hsp90, including an ability of the fungal, but not human, NBD to significantly rearrange to accommodate AUY922, an inhibitor which conserves the resorcylic substructure of radicicol. As an approach to exploit this apparent flexibility, we pursued the introduction of sterically bulkier rigidifying moieties to radicicol via oxime derivatization (a strategy previously employed for metabolic stabilization of the compound)\(^{38,39}\). We began by investigating the binding to *C. albicans* vs human Hsp90 of KF58333, a previously described radicicol oxime (Fig. 3a).

To monitor relative Hsp90-binding affinities, we again used an FP-based competition approach in whole-cell lysates. We observed potent binding for KF58333, but nearly equivalent EC\(_{50}\) values for both human and *Candida* Hsp90 (7–10 nM) (Fig. 3b and Supplementary Table 3). Presumably the oxime substituent in KF58333, which had been optimized to preserve activity in humans, is not sufficiently bulky to hinder binding of the compound. To address this, we next generated a small set of known (2)\(^{40,41}\), (8) (USPTO Patent Application 20100292218) and novel oxime analogs (3–7) through the direct chemical modification of radicicol\(^{42}\). The compounds were prepared using standard conditions with commercially available alkoxyamines varying in size and substitution (Fig. 3c and Supplementary Methods). Also included in the set was an analog, CMLD013075 (1) derived from the structurally related des-chlorinated natural product monocillin I (Fig. 3c), which is roughly equipotent to radicicol as an Hsp90 inhibitor in plants\(^{43}\). By FP assay, we found a moderate (1.4- to 4.6-fold) gain in fungal selectivity for several of our new oxime analogs in binding Hsp90 in whole-cell extracts prepared from *C. albicans* (SC5314) compared to HEPG2, a human cancer cell line (Fig. 3b). Although our design efforts were inspired primarily by structural and biochemical data pertaining to radicicol, we serendipitously discovered that the most selective inhibitor was monocillin-derived CMLD013075. This compound exhibited superior fungal selectivity over all other analogs tested by FP lysate assay, with an EC\(_{50}\) of ~500 nM in *C. albicans* lysate translating to a normalized 4.6-fold selectivity factor (Fig. 3b and Supplementary Table 3). Overall, our compound series supports a model in which the sterically bulkier oxime oximes can limit binding to the less flexible human Hsp90 NBD, yet still maintain, if not improve, fungal Hsp90-binding activity.

A critical consideration in the development of an effective antifungal is not just target binding, but also access to the target across microbial cell membranes and cell walls. Therefore, we evaluated the potency and selectivity of oxime analogs using drug susceptibility assays. We used strains of *S. cerevisiae* where both endogenous genes encoding isoforms of Hsp90 have been disrupted and growth is solely dependent upon *C. albicans* or human Hsp90 isoforms. All our oxime derivatives, including CMLD013075, displayed greater potency in limiting fungal proliferation than geldanamycin and KF58333 (Fig. 3d). Consistent with selectivity findings by FP, CMLD013075 was more active against a yeast strain engineered to express *C. albicans* Hsp90 than isogenic strains expressing either human Hsp90α or Hsp90β proteins. The converse was true of radicicol (Fig. 3e).

Hsp90 residues key to the fungal selectivity of CMLD013075. To identify molecular contacts contributing to the relative selectivity of CMLD013075 for *C. albicans* Hsp90, we solved a co-crystal structure of the compound bound within the protein’s N-terminal domain (Fig. 4a). Structural analysis revealed a substantial conformational rearrangement of the NBD associated with binding of the derivative, with an r.m.s.d. of 1.86 Å upon comparison to a radicicol co-crystal structure (Supplementary Table 2). Conformational rearrangement was associated with an inhibitory constant (K\(_i\)) by FP assay of 7.7 nM for binding of CMLD013075 to the fungal NBD and selectivity over binding to the NBDs of human Hsp90 and Grp94 of ~28- and ~5-fold respectively (Fig. 4c). The binding affinity of radicicol could not be assessed by FP due to its Michael reactivity and inactivation of the compound by thiol-reducing agent in the assay buffer\(^{38,39}\). The compound-induced conformation involved major movements of the N-terminal β-strand and repositioning of the lid subdomain that included α-helices 1, 5–7 and their linking loops (Supplementary Figure 1 and 6). These structural changes are accompanied by a partial remodeling of the ATP-binding site to accommodate the bulkier p-methoxybenzyl moiety of this compound (Fig. 4b and Supplementary Figure 8a, S8b). The macrocyclic portion of CMLD013075 occupies a position identical to that observed in the radicicol complex structure, with the oxime-linked p-methoxybenzyl group participating in several hydrophobic interactions as well as an apparent N-H⋯π bond to Asn40 (Fig. 4d).

Given the significant structural rearrangements associated with binding of CMLD013075 and AUY922 to *C. albicans* Hsp90, we next sought to directly compare these changes with respect to each other and to the apo structure (Supplementary Figure 6). Both the CMLD013075 and AUY922 complexes show similar changes in several secondary structure elements, such as the disappearance of the N-terminal β-strand (β1), the extension and tilting of helix a1, and the displacement of the lid subdomain (Supplementary Figure 6). However, in the CMLD013075 complex residues 94–101 adopt a bulged-in loop conformation, associated with a movement of the lid subdomain that appears essential to accommodate the p-methoxybenzyl group within the ATP-binding site (Supplementary Figure 8b). This conformational change differs considerably from the AUY922-induced structural rearrangement that involved the fusion of helices 4 and 5, and repositioning of the lid subdomain to accommodate AUY922’s morpholine ring (Supplementary Figure 6).

To rationalize the enhanced selectivity of CMLD013075 for fungal Hsp90, we evaluated each amino acid residue that differed between the *C. albicans* and human NBD and assessed the individual contributions of these residues to local conformation by comparing their H-bond networks. In this way, we identified several residues within the fungal NBD that might affect the protein’s conformation (Fig. 4e). We tested their importance by generating three *C. albicans* Hsp90 variants with the native fungal residues changed to their corresponding human residues (Fig. 5a, b). The first variant was a single substitution, replacing fungal threonine with human glutamine at position 12 (Thr12Gln) located between the N-terminal β-strand and α-helix. This substitution was predicted to affect the flexibility of the Gly94–Gly100 loop linking helices 4 and 5, thus potentially limiting movement of the lid region and N-terminus seen in the CMLD013075 complex structure. The second variant involved two amino acid changes within α-helix 7 (Leu130Ala and Phe131Tyr) which were predicted to impact contact with the
The experiment was repeated once with quantitatively similar results. Source data for the determination of EC50 values are provided as a source data file.

Relative growth inhibition by radicicol (top) or oxime derivative CMLD013075 is displayed in the same manner and indicated as normalized EC50 values. A red box highlights the oxime derivative CMLD013075 (1) in our panel of analogs which demonstrated the greatest combination of potency and selectivity for fungal Hsp90. EC50 values were determined by variable slope 4-parameter curve fitting of triplicate determinations at each concentration tested ($R^2 > 0.95$, all curves). All fungal values were normalized by the ratio of geldanamycin EC50s measured in human vs fungal lysate. The experiment was repeated once with quantitatively similar results. Source data for the determination of EC50 values are provided as a source data file.

The experiment was repeated once to confirm results. 

**Fig. 3** Synthesis and characterization of fungal-selective Hsp90 inhibitors. **a** Structure of previously reported anticancer radicicol oxime KF58333. **b** Plot of relative Hsp90-binding affinity for compounds determined in cytosolic extracts prepared from human (HEPG2) vs fungal (SC5314) cells. The relative binding affinities for compounds were determined in cytosolic extracts prepared from human vs fungal cells by fluorescence polarization (FP) assay and are indicated as normalized EC50 values. A red box highlights the oxime derivative CMLD013075 (1) in our panel of analogs which demonstrated the greatest combination of potency and selectivity for fungal Hsp90. EC50 values were determined by variable slope 4-parameter curve fitting of triplicate determinations at each concentration tested ($R^2 > 0.95$, all curves). All fungal values were normalized by the ratio of geldanamycin EC50s measured in human vs fungal lysate. The experiment was repeated once with quantitatively similar results. Source data for the determination of EC50 values are provided as a source data file. 

**c** Summary of R-group substitutions and scheme for the synthesis of monocillin-derived and radicicol-derived oxime analogs 1-8. **d** Susceptibility testing of Hsp90 inhibitor panel using a strain of *S. cerevisiae* engineered to express *C. albicans* Hsp90 as its sole source of Hsp90. Relative growth inhibition by each compound over a twofold dilution series of concentrations is displayed in heat-map format. Each colored box represents the mean of duplicate determinations. Color scale for relative growth is provided in lower right of panel, green: no inhibition to black: complete inhibition. The experiment was repeated once to confirm results. 

**e** Inhibitor sensitivity of isogenic *S. cerevisiae* strains engineered to express the indicated HSP90 genes as their sole source of Hsp90. Relative growth inhibition by radicicol (top) or oxime derivative CMLD013075 is displayed in the same manner and using the same color scale as in **d**. The experiment was repeated as an independent biological replicate to confirm results.

**para**-methoxybenzyl substituent. The final variant also involved two amino acid substitutions, Lys158Ser and Thr162Arg, located within β-strand 6. This strand is adjacent to β-strand 1 in the Hsp90 N-terminal β-sheet. To experimentally test the importance of these residues in providing fungal selectivity, we constructed strains of *S. cerevisiae* in which each of these variants constituted the sole source of Hsp90, and then compared the antifungal activity of radicicol and CMLD013075 against these strains. The *C. albicans* Hsp90 Thr12Gln or composite Lys158Ser/Thr162Arg substitutions did not alter the potency of either compound; radicicol remained less potent and CMLD013075 remained more potent against strains expressing these variants than against strains carrying the human isofoms (Fig. 5c). In contrast, the composite Leu130Ala/Phe131Tyr mutation increased potency of
radicicol while decreasing potency of CMLD013075 relative to wild-type C. albicans Hsp90 (Fig. 5c). In fact, these substitutions in C. albicans Hsp90 resulted in even less sensitivity to CMLD013075 than observed upon expression of human Hsp90 isoforms, highlighting the importance of these amino acids in conferring fungal selectivity to the oxime.

**Species-selective activity of CMLD013075 in culture.** We focused on CMLD013075 for further biological characterization, because it possessed the most potent and selective activity in whole-organism assays (Fig. 3d, e). We began by measuring inhibition of C. albicans growth in culture. Both radicicol and CMLD013075 showed comparable single-agent activity against a laboratory strain of C. albicans (SN95) as well as a more drug-resistant clinical isolate (CaCi-2) from an HIV-infected patient recovered early in the course of persistent Candida infection (Fig. 6a).

Next, we investigated the fungal selectivity of our Hsp90 inhibitors using standard microplate-based dye-reduction assays...
to measure concentration-dependent mammalian cytotoxicity. CMLD013075 was >10-fold less toxic to human HEPG2 liver cancer cells than the non-selective parent compound radicicol, and >50-fold less toxic than ganetespib, a resorcylic clinical inhibitor (Fig. 6b). Cytotoxicity was also tested against Raw264.7, mouse cancer cells of macrophage lineage, where again CMLD013075 was much less toxic than radicicol or ganetespib (Fig. 6b). We also examined the toxicity of CMLD013075 and several known Hsp90 inhibitors to non-cancer cells, i.e., the non-tumorigenic mouse fibroblast line 3T3 (Supplementary Figure 7a). Both radicicol and CMLD013075 were less cytotoxic to 3T3 cells than HEPG2 cells by at least 10-fold and both showed a plateau in their dose–response curves consistent with the presence of a non-proliferating, relatively inhibitor-insensitive population in the contact-inhibited 3T3 line.

As an explanation for the greater activity of N-terminal Hsp90 inhibitors against cancers compared to normal tissues, it has been reported that Hsp90 in cancer cells resides predominantly in an activated hetero-complex form with higher inhibitor affinity44,45. To investigate whether the binding of CMLD013075 might differ in lysate from non-cancer cells and HEPG2 cells, we performed FP assays with CMLD013075 and geldanamycin in lysate prepared from quiescent 3T3 cells. Affinity for both CMLD013075 and geldanamycin was indeed lower in 3T3 lysate
than HEPG2 lysate suggesting a relative improvement in selectivity for fungal Hsp90 (Supplementary Figure 7b). In light of the effects on geldanamycin affinity, however, care must be taken in the interpretation of these results because the probe used for these FP assays is itself geldanamycin-based. Although we cannot resolve the potential contributions of higher Hsp90 affinity state, higher Hsp90-dependence for proliferation/survival or even increased Grp94 content to the increased sensitivity of HEPG2 cells to CMLD013075, it is likely that using cancer cells as a comparator underestimates the compound’s fungal selectivity.

To determine whether CMLD013075 retained selective activity against C. albicans in the presence of human cells, we optimized conditions for growth of both cell types together in a co-culture model. Established monolayers of adherent human HEPG2 cells were infected with CaCi-2 at a multiplicity of infection (MOI) of ~0.2 and test compounds added. After 24 h of growth at 37 °C, cultures were formalin-fixed and stained to visualize fungal burden and underlying human cells (light pink signal). Representative fields from conventional light photomicrographs are presented at the same magnification in all panels. The experiment was repeated in two independent biological replicates to confirm results. Scale bar, 100 μm

**Selectivity reduces target-related mammalian toxicity.** Compromise of host immune function and other target-related toxicities preclude the use of non-species-selective Hsp90 inhibitors...
in the treatment of fungal infections. Host defense against invasive infection relies heavily on the ingestion and destruction of *C. albicans* by innate immune cells, such as macrophages. Thus, we compared the effect of CMLD013075 to that of radicicol on phagocytosis of *C. albicans* by macrophages. We pre-treated RAW264.7 mouse cells for two hours with radicicol or CMLD013075 then infected cultures with log-phase *C. albicans*. After an additional two hours, phagocytosis of the fluorescently labeled fungi was measured by flow cytometry. Neither compound impaired engulfment of *C. albicans* by these macrophages (Fig. 7a). Likewise, both compounds had similar effects in driving the yeast-to-filament transition reported for classical Hsp90 inhibitors (Fig. 7b). This Hsp90-dependent morphogenetic program has an important role in the organism’s virulence and interactions with host immune effectors such as macrophages.

In contrast to phagocytosis, Hsp90 inhibitor treatment did impair macrophage activation as measured by secretion of the pro-inflammatory cytokines TNF-α and IL-1β. Consistent with its selectivity for fungal Hsp90, however, CMLD013075 caused far less suppression than radicicol of cytokine production (Fig. 7c), a critical requirement for induction of robust immune responses that aid in the clearing of fungal infections. In addition to effects
similar to that previously reported for other Hsp90 inhibitors, CMLD013075 could potentiate antifungal activity in a manner similar to that previously reported for other Hsp90 inhibitors. Reversal of antifungal drug resistance induced the reporter to a measurable extent (Fig. 7d).

The opposite pattern of potency held true in C. albicans; CMLD013075 induced a peak threefold activation of the heat-shock reporter at 1 μM whereas neither ganetespib nor radicicol induced the reporter to a measurable extent (Fig. 7d).

Reversal of antifungal drug resistance. To determine whether CMLD013075 could potentiate antifungal activity in a manner similar to that previously reported for other Hsp90 inhibitors, we used a standard dose-response matrix (checkerboard) approach involving gradients of Hsp90 inhibitor and FL, the most commonly used clinical antifungal. By Bliss independence modeling analysis, we found strong synergy for both radicicol (δ = 22.6) and CMLD013075 (δ = 13.0) in combination with FL against the C. albicans clinical isolate CaCi-2 (Fig. 8a). Given that azoles such as FL are fungistatic against C. albicans alone but fungicidal in combination with an Hsp90 inhibitor, using a reference strain of C. albicans, we again performed clonogenic survival assays with CMLD013075 to determine whether it conserved this important property of Hsp90 inhibition. Using a reference strain of C. albicans, we again performed checkerboard assays titrating FL and CMLD013075. After 48 h in liquid culture (Supplementary Figure 9a), cells were transferred onto drug-free solid medium to assess viability (Supplementary Figure 9b). With similar potency, both radicicol and CMLD013075 transformed the fungistatic azole into a fungicidal combination.

To learn whether CMLD013075 would eliminate the FL-resistant growth of strain CaCi-2 in a manner similar to that previously reported for other Hsp90 inhibitors, the wells in plates that had been previously seeded with luciferized human cells were infected with GFP-marked CaCi-2. Hsp90 inhibitors were then added over a range of concentrations in addition to a fixed concentration of FL. After 48 h at 37 °C, relative fungal burden was measured by GFP fluorescence and survival/proliferation of the human cells was evaluated by luminescence. While all Hsp90 inhibitors were widely used as a sensitive pharmacodynamic endpoint for target engagement in the clinical development of Hsp90 inhibitors as anticancer agents, we also examined activation of the evolutionarily conserved heat-shock response by CMLD013075. Such activation is well-defined toxicity of impairing Hsp90’s essential role in maintaining protein homeostasis in cells and has been widely used as a sensitive pharmacodynamic endpoint for target engagement in the clinical development of Hsp90 inhibitors as anticancer agents. To compare the degree to which CMLD013075 vs classical Hsp90 inhibitors activated the heat-shock response, we used cells stably expressing reporter proteins (β-galactosidase in C. albicans and firefly luciferase in human 293T kidney-derived cells) under transcriptional control of heat-shock promoter elements from an HSP70-family gene of the relevant species. Treatment of human cells with the anticancer Hsp90 inhibitor ganetespib or radicicol itself drove concentration-dependent reporter activation as expected. Although CMLD013075 also activated the reporter, activation required much higher concentrations of this compound (Fig. 7d).
inhibitors displayed comparable activity in sensitizing CaCa-2 to FL, CMLD013075 was much less toxic than radicicol or ganetespib to HEPG2 cells in the co-culture environment (Fig. 2b).

Encouraged by the selective activity of CMLD013075 in co-culture, we made preliminary investigations into the compound’s suitability for administration to mice. To do so, we first developed a sensitive and reliable analytical method to measure levels of the compound in biological media (Supplementary Figure 10a). We then measured the stability of the compound in mouse plasma. As expected, by dampening its Michael reactivity, the oxime modification in CMLD013075 increased plasma stability compared to radicicol (Supplementary Figure 10b). Unfortunately, the pendant 4-methoxybenzyl group also increased susceptibility to P450 metabolism, as reflected by ~80% loss of parent radicicol oxime after 60-min incubation in mouse liver microsomes. Given this pharmacological liability, relatively rapid clearance from the systemic circulation in a single dose pharmacokinetic study of CMLD013075 in mice was observed (Supplementary Figure 10c). Further work will be required to improve the whole-animal pharmacology of our tool compound CMLD013075 before testing in mice can be pursued. Nevertheless, results so far provide strong structural evidence and biological proof-of-principle for the feasibility of targeting Hsp90 in a species-selective manner to combat invasive fungal disease.

Discussion

Heat-shock proteins, especially Hsp90, are notable for their exceptional degree of sequence conservation across species. Despite such conservation, the crystal structures of the C. albicans Hsp90 NBD presented here revealed sufficient conformational flexibility to enable synthesis of first a fungal-selective Hsp90 inhibitor. Our proof-of-concept probe molecule CMLD013075 inhibited the growth of C. albicans, enforced filamentous growth in the absence of an inducing cue, displayed reduced toxicity against mammalian cell lines and potentiated azole activity against a drug-resistant clinical isolate of C. albicans.

In initial work, the crystal structures of apo, ADP-bound, and radicicol-bound fungal NBD showed minimal structural variation. More problematic, they also showed little variation with respect to the human NBD. Importantly, however, the region spanning residues Asp91 to Gly97 of the C. albicans NBD in its apo state was seen in two conformations. We hypothesized that this observation might result from greater flexibility within the fungal ATP-binding site than was immediately obvious, an increased flexibility that might contribute to observed differences in the intrinsic ATPase activity of the fungal and host homologs. The significance of this observation, however, became clearer when we determined structures for the C. albicans NBD in complex with SNX-2112 and AUY922. The region spanning residues 93–102 adopted very distinct configurations to accommodate each of these compounds. In addition, extensive structural rearrangements, inclusive of regions outside of the nucleotide-binding site were observed in the case of AUY922. In complex with this drug, movement of the NBD lid region was accompanied by disappearance of the N-terminal β-strand. Remarkably, previous work in S. cerevisiae has shown that the first 24 residues of the NBD stabilize it in a rigid state and their removal confers flexibility specifically to the region between amino acids 98 and 120. While no such data are available in Candida, our findings are consistent with N-terminal alterations being capable of affecting the architecture of the overall binding site and vice versa.

Insights gained from the fungal structures reported here inspired synthesis of derivatives of the natural product radicicol and its des-chloro variant monclinol I, all synthesized with the goal of achieving fungal selectivity. We hypothesized that steric challenges would allow for selective accommodation by the more flexible fungal NBD. Amongst the analogs, CMLD013075 showed >25-fold biochemical selectivity for C. albicans Hsp90 NBD compared with the mammalian homolog. Importantly, a co-crystal structure for CMLD013075 in complex with the fungal NBD also showed a major and distinct structural change associated with inhibitor binding. These changes could be due to either stabilization of a pre-existing conformation visited by the NBD during the Hsp90 chaperoning cycle or de novo induced-fit effects, but both mechanisms are dependent on the intrinsic protein flexibility. In the case of CMLD013075, the orientation of the p-methoxybenzyl group towards the protein’s core is proposed to support these conformational changes, with stabilizing hydrophobic and NH···π interactions in a newly formed elongated pocket. This hypothesis is supported by our mutagenesis results demonstrating that the mutations Leu130Ala (affecting ligand-binding) and Phe131Tyr (affecting lid region flexibility) were accompanied by a reduction in the binding affinity of CMLD013075. Notably, these positions are variable in other fungal pathogens, including C. neoformans and A. fumigatus. Such variation could negatively impact CMLD013075 binding affinity in these species and necessitate additional modifications in the pursuit of a pan-selective fungal Hsp90 inhibitor.

Given the remarkable conformational shift associated with the binding of monclinol I-derived CMLD013075, it remains to be determined whether radicicol-derived analogs (3–5), which exhibited more modest fungal selectivity and similar whole-cell bioactivity to CMLD013075, bind in a similar manner. The sole difference between radicicol and monclinol I is a single chlorination of the aryl ring. Among resorcylate Hsp90 inhibitors, replacement of this chlorine with moderately sized aliphatic groups (ethyl, isopropyl) often improves potency, attributed to enhanced hydrophobic interactions in the lipophilic pocket of the ATP-binding region, as seen in the C. albicans and human NBD-AUY922 co-crystals. The top fungal-selective radicicol-derived inhibitor, ortho-chlorobenzyl-substituted 4 (2.9-fold selectivity in lysates, 531 nM EC50, 2.6 μM MIC), would be unable to engage in hydrophobic interactions as observed at the para-position of the CMLD013075 benzyl oxime, and halogenation of this ring should also significantly decrease potential for NH···π binding with Asn40.

Hsp90 ortholog selectivity has been recognized as a promising toxicity-mitigating strategy to improve the clinical efficacy of Hsp90 inhibitors in humans. Several distinct chemotypes have been shown to take advantage of the differential structural plasticity intrinsic to each human Hsp90 orthologue, thus bypassing the limited sequence variation seen in these essential chaperones. The resorcylate analog CMLD013075 achieves fungal specificity in an analogous manner, exploiting the extensicev structural flexibility of the C. albicans NBD. Nonetheless, there are significant variations in the structural repertoire available to the fungal and host chaperones. Both proteins can accommodate compounds such as SNX-2112 with a limited remodeling of the binding site. Only the fungal protein, however, showed remodeling of the N-terminus and lid region to accommodate the binding of CMLD013075 and AUY922. The increased catalytic efficiency of the fungal chaperone compared with the human protein might well be correlated with its enhanced flexibility, but from the perspective of drug discovery, such conformational flexibility also allows access to unique structural features to generate fungal-selective compounds.

The observed conformational change in the fungal NBD showed some similarity to the open conformation described for Grp94 in complex with ADP (r.m.s.d. of 4.337 Å for main-chain
Atoms, and it can be related to an initial conformational change necessary to accomplish a complete catalytic cycle by the full-length chaperone. To investigate orthologous selectivity further, we also overlaid the structure of our C. albicans-CMLD103075 complex with the structure reported for Grp94 in complex with PUWS13, a paralogous-selector of this chaperone (PDB code 3O2F). Consistent with the 5-fold lower affinity of CMLD103075 for Grp94 NBD than fungal Hsp90 NBD, however, drug efflux60, induced overnight with 0.5 mM IPTG. Cells were harvested by centrifugation and subsequently frozen in liquid nitrogen and stored in -80 °C until needed. Fused crystals were obtained in 40 mL of binding buffer per liter of culture, then flash frozen in liquid nitrogen and stored in -80 °C until needed. Frozen cells were thawed in 0.5% CHAPS and 500mL of benzamidine for 45 min at room temperature, for large-scale preparations, an additional mechanical lysis was done using an M-110EH Microfluidizer Processor (Microfluidics Corp., MA, USA). Cells lysates were then centrifuged to eliminate debris and the cleared lysate was loaded onto a DES2 anion exchange resin column (Whatman, MA, USA). This column was then washed with 75 mL of 500mM NaCl, 30mM imidazole, and 5% glycerol. The protein was eluted with 75 mL of a buffer consisting of 50mM HEPES, pH 7.5 with 500mM NaCl, 250mM imidazole and 5% glycerol. Following elution, 1mL EDTA and 1mL TCEP were added to the sample. The protein was further purified by size exclusion chromatography on a Superdex 200 (GE Healthcare, NJ, USA) column equilibrated with buffer consisting of 50mM HEPES, pH 7.5 and 500mM NaCl. The protein was concentrated by Amicon Ultra centrifugal filter device (Millipore, MA, USA) and its identity was confirmed by SDS-PAGE and mass spectrometry.

Protein crystallization and structure determination. Aliquots of purified recombinant C. albicans Hsp90 N-terminal domain (NTD) were crystallized at 23°C in sitting-drop vapour diffusion methods, either alone or in the presence of five different ligands, ADP, radicicol, CMLD103075, SNX-2112 and AUY922. Crystals were obtained by mixing one part of protein solution at 10–15 mg/mL (10mM HEPES, pH 7.5 with 50mM NaCl, 2mM TCEP and 4mM MgCl₂) with or without 2mM ligand with one part of reservoir solution. In the case of apo and ADP co-crystals, the reservoir solution contained 2M ammonium sulphate, 2% PEG400, 100mM HEPES, pH 7.5. For radicicol co-crystals, the reservoir solution contained 8% Tacsimate, pH 5.0 and 20% PEG3350; for CMLD103075 it included, 5mM CoCl₂, 12% PEG3350, 100mM HEPES, pH 7.5; for the SNX-2112 complex it contained, 200mM MgCl₂, 100mM bis-Tris, pH 5.5; and for the AUY922 complex, 25mM MgCl₂, 100mM HEPES, pH 7.5, and 22% polyacrylic acid sodium salt. Crystals appeared within one week and were cryo-protected with ~20–25% ethylene glycol or glycerol supplemented mother liquor before being flash cooled in liquid nitrogen. Crystals were centre of topics.

Diffraction data were collected using home X-ray sources for the apo and ADP structures. A synchrotron X-ray source (beamline 8OID-1 at the Canadian Light Source (CLS)) was used for radicicol and CMLD103075 complexes. Beamline 23ID-D at APS - Argonne National Laboratory was used for SNX-2112 and AUY922 complexes. Data were processed with HKL300066,67. Structures were determined by molecular replacement using the yeast Hsp90 (PDB code 1A9H)68 as a search model for the first complex structure of the C. albicans Hsp90 NBD. This structure was used as the model for subsequent structures. Phaser66 was used for molecular replacement calculations, while model building was performed with COOT67 and structures were refined with Phenix65. The radicicol and SNX-2112 complexes were twinned, and the structure (as refined in Phenix)58 identified the following merohedral twin h,k,l and a twin fraction of ~0.3 for both crystals. The positions of all compounds in the reported C. albicans Hsp90 (E7-K218) mhhhhhsrghnlylgf<ETHEFTAEBSQMLLSNTVYNSKFIELRNLPSNASADF KIRYQALSDPSQESEFLIPFRIPQDKQVDISGTGMKALDVNLNTIGAT SGKTSFSMEALSAAGDVMISQGQGFGYSLFDVHDYVYKSHHDQYVWES NAGGKFTLYDTRNLGTRMLRLPKDGEDQYELEREKVEKVHKHFSEAY VFFVFF FHHKEX Human Hsp90 beta (M1-K223) mhhhhhsrghnlylgf<ETHEFTAEBSQMLLSNTVYNSKFIELRNLPSNASADF KIRYQALSDPSQESEFLIPFRIPQDKQVDISGTGMKALDVNLNTIGAT SGKTSFSMEALSAAGDVMISQGQGFGYSLFDVHDYVYKSHHDQYVWES NAGGKFTLYDTRNLGTRMLRLPKDGEDQYELEREKVEKVHKHFSEAY VFFVFF FHHKEX Hsp90 NBD expression and purification for crystallographic studies. Sequences of the recombinant NBD used for crystallization and FP binding assays were as follows: C. albicans Hsp90 (E7-K218) mhhhhhsrghnlylgf<ETHEFTAEBSQMLLSNTVYNSKFIELRNLPSNASADF KIRYQALSDPSQESEFLIPFRIPQDKQVDISGTGMKALDVNLNTIGAT SGKTSFSMEALSAAGDVMISQGQGFGYSLFDVHDYVYKSHHDQYVWES NAGGKFTLYDTRNLGTRMLRLPKDGEDQYELEREKVEKVHKHFSEAY VFFVFF FHHKEX
Hsp90-ligand complex crystal structures were unambiguous as indicated by their simulated annealing OMIT maps (Figure S11). CMLD013075 coordinates and geometry restraints were created within the ELBOW. The stereochemistry of both models was checked by MOLPROBITY70. Relevant data collection and refinement statistics are shown in Table S1. Protein structure superpositions were calculated with LSQKAB [1] and implemented in CCP4 [2] to construct protein figures generated using UCSF Chimera package [3]. C. albicans and human Hsp90-AU9Y22 2D interaction diagrams (Figure S4b bottom panels) were generated using Maestro (Schrodinger Release 2016–1: Maestro, Schrodinger, LLC, New York, NY, 2016).

Yeast strains and culture conditions. Archives of all yeast strains were maintained at −80 °C in 25% glycerol. Strains used in experiments were maintained on solid (2% agar) yeast extract peptone (YPD, 1% yeast extract, 2% bactopeptone, 2% glucose) at 4 °C for no more than one month. For experiments, yeast strains were routinely grown in either YPD medium, unless otherwise indicated. Strains used in this study are listed in Supplementary Table 4. Strain construction is described in Supplementary Methods.

Plasmid construction. Recombinant DNA procedures were performed according to standard protocols and construction of vectors is described in Supplementary Methods Supporting Information. Plasmids were sequenced to verify the absence of any nonsynonymous mutations. Plasmids used in this study are listed in Supplementary Table 5. Oligonucleotides used in this study are listed in Supplementary Table 6.

Minimum inhibitory concentration assays. Antifungal tolerance and resistance were determined in flat bottom, 96-well microtiter plates using a modified broth microdilution protocol as described[43]. All Hsp90 inhibitors were formulated in dimethyl sulfoxide (DMSO, Sigma Aldrich Co.); fluconazole was dissolved in sterile ddH2O. Cell suspensions in duplicate on at least two occasions were quantitatively displayed in heat-map format using the program JavaTreeView 1.1.3 (http://jtreeview.sourceforge.net). To test for fungidal activity, cultures from MIC plates were spotted on YPD agar plates using a spotter (Frogger, V&P Scientific, Inc.). Plates were photographed after 48 h of incubations at 30 °C.

Preparation of whole-cell lysates for FP assays. To prepare human cell lysate, approximately 1 × 10^6 HEPG2 or 3T3 cells were collected by centrifugation at 4 °C, washed in cold PBS and resuspended in 250 μL binding buffer (20 mM HEPES pH 7.5, 50 mM KCl, 5 mM MgCl2, 0.01% Triton X-100) supplemented fresh with a protective cocktail tablet (Roche; 1183671001), 20 mM Na2MoO4, and 1 mM DTT. After vortexing, cell lysate was frozen at −80 °C for 2 h, followed by thawing on ice. The vortex-freeze-thaw step was repeated. Complete lysis was confirmed by microscopy followed by centrifugation at 14,000 rpm for 30 min at 4 °C. To prepare yeast lysates, an overnight culture grown in YPD was diluted to an OD600 of 0.15 in 1 L of pre-warmed YPD. This culture was grown for 4 h at 37 °C, then centrifuged at 4000 rpm for 20 min at 4 °C. The pellet was resuspended in 10 mL of the same binding buffer. Yeast lysates were quantitatively divided twice with a French Press and then centrifuged at 14,000 rpm for 30 min at 4 °C. Post centrifugation, yeast and mammalian lysates were filtered (0.22 μm), supplemented with sterile glycerol to a final concentration of 15% (v/v), flash frozen, and stored at −80 °C for up to 5 months. Aliquots were thawed immediately prior to use.

FP assays. Total protein concentration of human and yeast lysates was determined by Bradford assay as described previously8. Titration of Cy3-GdA probe and lysate were evaluated to define conditions that resulted in 75% maximal probe polarization with no competitor present. Serial dilutions of test Hsp90 inhibitors were then assayed under these same conditions to monitor loss of fluorescence polarization as an indicator of probe displacement from Hsp90. All determinations were then assayed under these same conditions to monitor loss of the geldanamycin-based probe35. Signal in millipolarization (mP) units was measured in a SpectraMax i3 microplate reader (Molecular Devices) using Softmax Pro software (version 5.4.1). Each experiment was repeated in at least biological duplicate. Non-linear 4-parameter curve fitting of raw displacement data was performed in GraphPad Prism 5.0 to determine IC50 values as a measure of relative Hsp90-binding affinity. Results were normalized to the value determined for GdA in yeast of each cell type. To determine assay-independent inhibitor Kᵢ values for compounds by FP, the binding affinity of Cy3-GdA probe (0.1 μM) for each NBD (human and yeast Hsp90 10 nM; human Grp94 7.5 nM) was first determined by saturation binding measurements35. Using these values, data from equilibrium competition binding experiments were analyzed by non-linear regression that takes into account the non-ligand saturated conditions inherent to FP assays to calculate the Kᵢ of inhibitors33.

Microscopy. C. albicans strain SN95 was sub-cultured to an OD600 of 0.1 and grown for 6 h in the absence or presence of Hsp90 inhibitors as indicated. Imaging of cells incubated in liquid was performed by differential interference contrast microscopy with a Zeiss Axioscope.Instrument and Axiovision software (Carl Zeiss, Inc.).

Mammalian cell toxicity. Cancer cells were plated in 96-well format at a density of 5000 per well (HEPG2) or 2000 per well (Rav264.7) in DMEM supplemented with 10% fetal bovine serum (FBS) and 1x Pen-Strep antibiotic. Non-tumorigenic mouse 3T3 fibroblasts were plated in 384-well format at 2500 per well. After overnight incubation, two-fold dilutions of test compounds were added to wells for 72 h followed by measurement of relative viable cell number using standard resazurin dye-reduction assay and microplate fluorometer (Tecan Infinite M1000Pro, 544 nm excitation and 590 nm emission) as previously published34.

Phagocytosis assay. The ingestion of GFP-marked C. albicans CaG-2 (CaLC867, Table S4) by mouse macrophages was monitored using flow cytometry. Rav264.7 macrophage cultures were pre-treated with various concentrations of Hsp90 inhibitors for 2 h at 37 °C and then infected with fungus at a multiplicity of infection of ~2 for an additional 2 h. Wells were harvested by trypsinization and resultant cell suspensions incubated with Guava ViaCount reagent per manufacturer’s recommendation (Millipore Sigma, Cat #4000-0040). Red fluorescent signal was used to identify macrophages while green fluorescent signal identified the fungus. Dual positive (red and green) events with appropriate forward- and side-scatter parameters were quantitated as representative of ingestion of fungus by a macrophage. All determinations were performed in triplicate and the experimental design was repeated twice.

Cytokine production. Rav264.7 cells were plated in 100 μL of media (DMEM supplemented with 10% fetal bovine serum (FBS) and 1x Pen-Strep antibiotic) at a density of 1.5 × 10⁵ cells per well in a 96-well plate. The next day, cells were treated with a dilution series of radicicol or CMLD013075. After 2 h incubation at 37 °C, medium was flipped off and wells washed two times with 200 μL of warm medium. During incubation of macrophages with Hsp90 inhibitor, a culture of C. albicans grown in YPD overnight at 30 °C was spun down and washed twice with PBS. Cell counts were then determined using a hemocytometer and an inoculum of 6 × 10⁵ cells per well was added to the macrophages. Co-culture incubation time was 2 h for measuring secretion of TNF-α and 5 h for measuring IL-1β cytokine levels. To monitor IL-1β production, macrophages were primed with 50 ng/mL of LPS (InvivoGen) for 2 h prior to infection with C. albicans. All cytokines were measured using a Lumitrend reagent kit manufactured by Messung. Experiment 150 mL of media was removed and added to a new 96-well plate that contained a 0.5 mL solution of 7× protease inhibitors in water. The plate was frozen and stored at −80 °C until measurement of cytokine levels using ELISA kits from R&D Systems: TNF-a DuoSet (DY1410) and IL-1β/IL-1f DuoSet (DY1401).

Heat-shock induction assays. Concentration-dependent activation of the heat-shock response in human cell lines by Hsp90 inhibitors was measured in 96-well format as described previously using a single cell stabilizing trypsinization method to construct encoding GFP-luciferase fusion protein under transcriptional control of heat-shock elements from the HSP70B gene74. Activation of the response in C. albicans was also measured in 96-well format using a reporter strain (CalC922) expressing GFP-luciferase elements driven expression of the LacZ locus with the HSP70 promoter element (CalC867). The induction of galactosidase activity was measured using luminescent Gal-Screen reagent per manufacturer’s recommendations (Applied Biosystems; Bedford, MA Cat#T1029).

Co-culture experiments. Monolayers of HEPG2 cells established in 6-well format were infected with log-phase C. albicans (CaG-2) at a concentration of 2.5 × 10⁵ cells/mL in DMEM supplemented with 5% fetal bovine serum and pen-strep antibiotic. After addition of test compounds, culture was continued for 48 h. Wells were fixed in 10% formalin, stained with a periodic acid-Schiff reagent kit (Sigma, 395B-1KT) per manufacturer’s recommendation and photomicrographs obtained under standard white light illumination. To quantify the concentration-dependent toxicity of Hsp90 inhibitors in human-fungal co-cultures, 293T cells stably expressing firefly luciferase were plated in 384-well format (2000 cells in 20 μL per well, black clear-bottom plates) and allowed to adhere overnight. Wells were then infected with a log-phase GFP-serum (CalC867) or GFP-serum (CalC363) in an equal volume of medium supplemented with fluconazole (4 μg/mL). After 48 h, medium was replaced with PBS and relative fluorescence per well measured on a plate reader (Tecan Infinite M1000Pro, 480 nm excitation and 540 nm emission). Stable–Glo luciferase assay reagent (10 μL per well, Promega Cat# E2520) was then added, plates incubated at room temperature for 10 mins and relative luminescence per well measured using an Emission plate reader (Perkin Elmer).

Pharmacological studies. See Supplementary Methods for materials, methods and instrumentation used to evaluate the metabolic stability in vitro and single dose plasma pharmacokinetic profile in mice of CMLD013075. Pharmacokinetic studies
in mice were performed in an ethical manner under animal study protocol (0156-037-19 as reviewed and approved by the Massachusetts Institute of Technology Committee on Animal Care (CAC)).

**Statistical methods.** GraphPad Prism 5 and 7.0 were used to perform curve fitting, calculate EC50 values and generate graphical displays. Technical replicate numbers (n values) for all quantitative data characterizing the biochemical and biological activities of compounds are provided in the relevant figure legends. Informatively biological experiments and compound binding measurements were repeated at least once in an independent experiment to confirm results.

**Data availability**

The coordinates for NBD structures and their structure factors have been deposited with the Protein Data Bank (http://www pdb.org) under the following accession codes: 6CJI (apo), 6CJJ (ADP), 6CJP (radicicol), 6CJP (CMLD013075), 6CJR (SNX-2112), 6CS (AUY292). The source data and calculations underlying Figs. 2a, b, 3b, 4c, Supplementary Figures 3a-c and 7b are provided within the “Source Data” Excel file associated with the manuscript. Other data are available from the corresponding author upon reasonable request.

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Author contributions

L.W., S.L., L.E.B., and L.E.C. conceived and designed the overall study. C.S.N., A.H., R.H., and J.P. designed, performed and analyzed structural studies with J.P. providing overall supervision. D.S.H., R.T., J.A.P., and L.E.B. planned, synthesized, and purified resorcylic oximes with L.E.B. providing overall supervision. L.W., N.R., C.A.M., E.V.I., T.S.-G., C.C., S.C., J.I.X., and U.T. planned, performed, and analyzed microbiological, genetic, cell culture, biochemical, and pharmacokinetic studies with L.E.C. providing overall supervision. L.W., N.R., D.J.K. L.E.B., J.P., and L.E.C. wrote and edited the manuscript.

Additional information

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Competing interests: L.E.C and S.L. are named as inventors on USPTO 8349313. L.W. and L.E.C. are scientific co-founders and stock holders of Bright Angel Therapeutics (BAT), a company pursuing the discovery and development of new antifungals. J.P. is a paid consultant to BAT. The remaining authors declare no competing interests.

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