Targeting fungal membrane homeostasis with imidazopyrazoindoles impairs azole resistance and biofilm formation

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Fungal infections cause more than 1.5 million deaths annually. With an increase in immune-deficient susceptible populations and the emergence of antifungal drug resistance, there is an urgent need for novel strategies to combat these life-threatening infections. Here, we use a combinatorial screening approach to identify an imidazopyrazoindole, NPD827, that synergizes with fluconazole against azole-sensitive and -resistant isolates of Candida albicans. NPD827 interacts with sterols, resulting in profound effects on fungal membrane homeostasis and induction of membrane-associated stress responses. The compound impairs virulence in a Caenorhabditis elegans model of candidiasis, blocks C. albicans filamentation in vitro, and prevents biofilm formation in a rat model of catheter infection by C. albicans. Collectively, this work identifies an imidazopyrazoindole scaffold with a non-protein-targeted mode of action that re-sensitizes the leading human fungal pathogen, C. albicans, to azole antifungals.
In recent decades, modern medicine has revolutionized the treatment of numerous human diseases through the use of immunomodulatory and invasive medical interventions. As a consequence, opportunistic fungal pathogens have become a leading cause of human morbidity and mortality in vulnerable, immune-compromised populations. Invasive fungal infections are responsible for global mortality rates that exceed that of malaria or breast cancer, with over 1.5 million deaths per year worldwide. Candida species are a major contributor to these infections, with Candida albicans reigning as the fourth most common cause of hospital-acquired bloodstream infections, with mortality remaining at ~40%. This underappreciated public health threat has been met with the overuse of a limited arsenal of antifungals for prophylactic purposes, leading to the widespread emergence of multidrug-resistant pathogenic fungi.

In contrast to approximately two dozen classes of antibacterials in current clinical use, only three major classes of antifungals are approved for the treatment of invasive fungal infections. Fun- gustatic azoles have been the most widely deployed treatment for systemic candidiasis for decades and target the ergosterol biosynthetic enzyme lanosterol 14α-demethylase, encoded by ERG11. Analogous to cholesterol in mammalian cells, ergosterol is critical for maintaining fungal cell membrane integrity and fluidity. Azole-induced depletion of ergosterol from the cell is accompanied by accumulation of alternative sterol intermediates that perturb membrane stability and arrest fungal growth. The pleiotropic effects on membrane homeostasis induced by azoles include severe defects in vacuolar integrity and impairment of vacuole–vacuole fusion. Widespread overuse of the fungistatic azoles has led to the emergence of various mechanisms of resistance, including modification and/or overexpression of the drug target, upregulation of drug-efflux pumps, and compensatory alterations within the ergosterol biosynthesis pathway. Moreover, lipidomic analysis of azole-resistant isolates has revealed alterations in both sterols and sphingolipids, key membrane components that regulate membrane fluidity and stabilize membrane-resident proteins. Given the increasing frequency of resistance, an urgent need exists for the development of novel therapeutic strategies that can be used in combination with current treatments and restore their efficacy.

Despite decades of effort, progress in the development of novel antifungal agents has been modest. Evidence from the model yeast Saccharomyces cerevisiae suggests that antifungal therapeutic strategies need to take into account the highly interconnected and functionally redundant genetic networks that underpin fungal biology. This genetic buffering partly explains the inherent difficulty of identifying single compounds that possess fungal-selective cytotoxicity and advocates for the development of combination therapies. Combination therapy has the potential to confer enhanced efficacy, more selective fungal-toxicity, and reduced vulnerability to the evolution of drug resistance. While combination therapy has been underutilized in the treatment of most invasive fungal diseases, the combination of the polyene antifungal amphotericin B with the anti-metabolite 5-flucytosine is the current gold standard for cryptococcal meningitis, a fungal infection that afflicts roughly 220,000 annually. The benefit of the combination in preventing the rapid emergence of resistance to 5-flucytosine serves as proof-of-principle and highlights the promising, but relatively untapped potential of combination strategies.

In this work, we screened ~20,000 compounds from the RIKEN Natural Product Depository (NPDepo), a collection of natural products and their derivatives, in combination with a minimally inhibitory concentration of the conventional antifungal fluconazole to identify combinations with enhanced activity against C. albicans. This screen identified several compounds sharing an imidazopyrazoindole scaffold that strongly synergize with fluconazole against diverse fungal species, especially azole-resistant isolates of C. albicans. Using chemical-genetic, biochemical, and biophysical approaches, we establish a relatively unusual mode of action for the most active hit, NPD827, which involves disruption of membrane homeostasis rather than inhibition of a specific protein target. With promising efficacy in diverse preclinical models of C. albicans infection, NPD827 provides a promising lead compound for further development of a combination treatment approach to block key fungal virulence traits and re-sensitize resistant fungal isolates to azoles.

Results
Identification of imidazopyrazoindoles with azole-potentiating activity. To explore combinatorial antifungal chemical space, we screened the RIKEN NPDepo chemical library, a collection of natural products and their derivatives, in the absence and presence of fluconazole (1 µg/mL) against a wild-type strain of C. albicans. Compounds that reduced growth after 24 h by 20–median absolute deviations (MADs) from the median alone were classified as single-agent hits. Compounds that reduced growth after 24 h by 20-MADs in the presence of fluconazole compared to no-fluconazole controls (% fluconazole potentiation) were classified as potentiation agents. Of 19,840 compounds screened at 10 µg/mL, we identified 56 single agents (hit rate: 0.28%) and 151 fluconazole potentiators (hit rate: 0.76%) (Fig. 1a), highlighting the potential of screening compounds in combination to dramatically expand the antifungal space. The bioactivity of compounds that could be independently sourced was verified through standard dose–response assays, yielding 59 compounds with confirmed azole-potentiating activity or below their screening concentration. Hits were further filtered based on the magnitude of their synergistic interaction with fluconazole, with 24 molecules displaying synergy against C. albicans (Fractional Inhibitory Concentration Index [FICI] < 0.5; Table 1). Finally, hits were prioritized by comparing their EC50 values against the human liver cancer-derived cell line, HepG2, to their EC50 values against C. albicans. A total of 14 compounds were at least two-fold more potent against C. albicans in the presence of 0.25 µg/mL of fluconazole compared to their potency against HepG2 cells in culture (Fig. 1b). Interestingly, this analysis identified a shared imidazopyrazoindole core in four prioritized molecules with a maximum common substructure (MCS) Tanimoto score of >0.5 (Fig. 1c). The compound NPD827 was chosen for follow-up given its potent activity against C. albicans in the presence of fluconazole (EC50 < 0.1 µg/mL; Fig. 1b) and minimal cytotoxicity against HepG2 cells (EC50 > 80 µg/mL; Fig. 1b).

To further assess the antifungal activity of NPD827, we performed checkerboard assays in combination with fluconazole against two clinical isolates of C. albicans. These isolates included an azole-tolerant strain from an HIV-infected patient recovered early in the course of a persistent Candida infection (CaCi-2) and an azole-resistant isolate recovered from the same patient after multiple treatments with fluconazole (CaCi-17). NPD827 was synergistic with fluconazole against both CaCi-2 and CaCi-17 (Fig. 1d), highlighting the ability of this compound to augment azole activity despite overexpression of efflux transporters and the presence of resistance-conferring mutations in the azole target gene ERG11. This observation was further supported by performing checkerboard assays against diverse laboratory-derived azole-resistant isolates as well as additional clinical isolates. In all cases NPD827 enhanced azole efficacy (Supplementary Fig. 1a). To assess the antifungal spectrum of combination treatment, checkerboard assays were performed...
against isolates of other non-albicans Candida species (C. glabrata and C. auris), the basidiomycete Cryptococcus neoformans, and the filamentous mold Aspergillus fumigatus. NPD827 enhanced azole activity against select C. auris isolates, C. neoformans, and A. fumigatus, but displayed no activity against either of the two C. glabrata strains examined (Fig. 1d, Supplementary Fig. 1b, c).

To learn whether NPD827 might enhance the activity of other ergosterol biosynthesis inhibitors, we performed checkerboard assays with NPD827 and terbinafine, which targets Erg1, and fenpropimorph, which targets Erg2 and Erg4. NPD827 enhanced the activity of both these ergosterol biosynthesis inhibitors against a wild-type strain of C. albicans (Fig. 2a). This activity appeared restricted to compounds which impair sterol biosynthesis because NPD827 had an antagonistic interaction with amphotericin B, a polyene that extracts ergosterol from the fungal cell membrane (Supplementary Fig. 1d). Further, NPD827 showed no chemical–chemical interaction with the cell

Fig. 1 RIKEN Natural Product Depository screen against C. albicans identifies compounds that potentiate azole activity. a Screening data are reported as percent growth inhibition (%) in the absence and presence of fluconazole (FLC, 1 μg/mL). Compounds are colored based on classification as single agent-active (blue, ≥20-MAD above median growth inhibition), or azole potentiator (red, ≥20-MAD above median growth inhibition with fluconazole). b Fungal versus human growth inhibition for the top 24 compounds identified in the primary screen. EC50 values were calculated by nonlinear regression curve fitting of triplicate determinations from dose-response assays against human cells (HepG2) and C. albicans (CaSS1). All assays were performed in the presence of 0.25 μg/mL of FLC. Pink data points indicate compounds belonging to the imidazopyrazoindole class. Red data point highlights NPD827. c Checkerboard assays with four imidazopyrazoindoles in combination with FLC. Checkerboards were performed as described in (c). After 24 h (for C. albicans and C. auris) or 48 h (for C. neoformans, A. fumigatus), growth was measured and plotted as in (c). Source data are provided as a Source Data file.
Table 1 Top 24 azole potentiators displaying a synergistic interaction with fluconazole.

| Compound ID   | FICI | Compound ID   | FICI |
|---------------|------|---------------|------|
| NPD13393      | 0.268| NPD2864       | 0.258|
| NPD827        | 0.188| NPD3363       | 0.258|
| NPD3675       | 0.258| NPD3155       | 0.375|
| NPD13211      | 0.258| NPD7295       | 0.313|
| NPD3774       | 0.258| NPD4066       | 0.281|
| NPD1752       | 0.281| NPD2887       | 0.258|
| NPD2470       | 0.258| NPD2955       | 0.258|
| NPD2889       | 0.313| NPD4138       | 0.258|
| NPD3126       | 0.455| NPD2756       | 0.281|
| NPD3540       | 0.266| NPD3858       | 0.258|
| NP771         | 0.250| NPD4407       | 0.258|
| NPD2396       | 0.258| NPD2600       | 0.258|

Synthetic interactions were determined by performing checkerboard assays with each NPD compound and fluconazole (FLC) against wild type C. albicans (SN95). Growth in checkerboard assays were measured using absorbance at 600 nm after 24 h at 30 °C. Synergistic interactions were quantified by calculating the Fractional Inhibitory Concentration Index (FICI) for each compound pair. FICI is calculated as: FICI = (MIC_{drug} in combination/MIC_{drug} alone) + (MIC_{FLC} in combination/MIC_{FLC} alone), where MIC_{drug} represents the minimal inhibitory concentration at 90% growth inhibition.

Perturbation of lipid homeostasis by NPD827 activates membrane-associated stress responses. Lipid droplets are dynamic storage organelles that support homeostasis by sequestering excess free-lipid species, providing immediate protection from lipotoxicity, and serving as critical mediators of cellular stress responses. Thus, we next investigated whether NPD827-induced changes to lipid composition altered the lipid-droplet content of cells. Treatment of C. albicans with NPD827 caused a concentration-dependent increase in both lipid-droplet number and size, as monitored by BODIPY 493/503 staining, compared to a vehicle-only control (Fig. 4a). Interestingly, treatment of C. albicans with either low (0.25 µg/mL) or high concentration (16 µg/mL) fluconazole did not increase lipid droplet content of cells; however, the combination of low-NPD827 with low-fluconazole greatly enhanced accumulation of lipid droplets (Fig. 4a).

The endoplasmic reticulum (ER) is the major site for synthesis of sterols and early sphingolipid intermediates. This organelle relies on intrinsic stress responses, especially the unfolded protein response (UPR), to respond to the accumulation of misfolded proteins as well as disruptive changes in lipid composition. Activation of the UPR occurs via auto-phosphorylation of the serine-threonine kinase and endoribonuclease, Ire1, resulting in alternative splicing of mRNA encoding the transcription factor HAC1. Interestingly, lipid droplets have been implicated in the alleviation of ER stress, sequestering excess free fatty acids and misfolded proteins. To determine if NPD827 activates the UPR in C. albicans, a PCR-based assay was performed to monitor intron presence or absence in HAC1 mRNA using cDNA prepared from NPD827-treated cells. As observed with the ER stress-inducing positive control, DTT (5 mM), NPD827 drove splicing of HAC1 message in a concentration-dependent manner, indicating that NPD827 activity induces ER stress (Fig. 4b). Notably, treatment with even a high concentration of fluconazole had no impact on HAC1 splicing (Fig. 4b).
In addition to the formation of lipid droplets, ER stress triggers calcium influx through the Cch1-Mid1 calcium channel, leading to activation of stress responses regulated by the protein phosphatase calcineurin. This calcium-calcineurin signaling pathway acts together with the UPR to promote long-term survival of cells undergoing ER stress. Therefore, we examined whether NPD827 activates a calcineurin-dependent stress response using a well-established reporter system that exploits the downstream calcineurin effector, Crz1. The transcription factor Crz1 is activated by calcineurin in response to calcium, translocating to the nucleus, and up-regulating the expression of genes containing calcineurin-dependent response elements (CDREs) in their promoters. We used a strain harboring a construct with the CDRE-containing promoter UTR2p fused to lacZ to assess calcineurin activation in response to NPD827. The canonical calcineurin activator calcium chloride, as well as another ER stress-inducing compound DTT robustly activated this reporter as expected. NPD827 also activated reporter
expression and the calcineurin inhibitor FK506 blocked this activation, confirming that transcriptional activation was indeed calcineurin-dependent (Fig. 4c).

Following up on these results, we examined whether modulating the activity of calcineurin in *C. albicans* would affect sensitivity to NPD827. Addition of the calcineurin inhibitor FK506, or deletion of either the catalytic (CNA1) or regulatory (CNR1) subunit of calcineurin, increased sensitivity to NPD827 (Fig. 4d). Additionally, a mutant lacking the auto-inhibitory domain of the phosphatase that causes constitutive activation of calcineurin (CNAtr), decreased susceptibility to NPD827 (Fig. 4d). To complement these calcineurin-related studies, we also investigated the effects of NPD827 on calcium signaling. In response to disturbances of Ca^{2+} homeostasis in the ER, Ca^{2+} influx through the plasma membrane is stimulated via a voltage-gated channel composed of Cch1 and Mid1.34,35 Deletion of either *CCH1* or *MIDI* resulted in a level of hypersensitivity to NPD827 similar to that seen in calcineurin-deletion mutants (Fig. 4d). Taken together, these results indicate that NPD827 disrupts lipid homeostasis in a far-reaching manner, which activates key stress-response pathways to cope with the imbalance.

NPD827 rapidly disrupts membrane dynamics and impairs drug efflux. To begin investigating the molecular mechanism(s) by which NPD827 disrupts lipid homeostasis, we leveraged a chemical-genetic dataset in the model yeast *S. cerevisiae*.36 The RIKEN NPDepo collection, including NPD827, has been profiled using a collection of mutants that encompass a diagnostic set of essential genes and NE denotes non-essential genes. Genes coloured in blue were significantly upregulated compared to NPD827 5 µg/mL, <0.0001 (all samples); additionally, a mutant lacking the auto-inhibitory domain of the phosphatase that causes constitutive activation of transmembrane transport (p-value: *<0.05, **<0.005, ***<0.0005.

Overview of ergosterol biosynthesis pathway components were profiled at a fixed concentration of NPD827 (5 µg/mL) in the absence and presence of doxycyline (DOX, 0.05 µg/mL). Data are shown for those strains with significant differences, presented as mean ± SD of technical triplicates. P-value: 0.00279 (tetO-ERG10/erg10Δ), 0.00042 (tetO-HMG1/hmg1Δ), 0.00024 (tetO-ERG12/erg12Δ), 0.00345 (tetO-ERG8/erg8Δ), 0.00088 (tetO-ERG17/erg17Δ), 0.00644 (tetO-ERG17/erg17Δ), 0.00045 (tetO-ERG11/erg11Δ), 0.00019 (tetO-ERG24/erg24Δ), 0.02180 (tetO-ERG6/erg6Δ), 0.00603 (tetO-ERG2/erg2Δ), 0.00022 (tetO-ERG3/erg3Δ). Significance was determined by performing multiple unpaired t-tests comparing plus DOX conditions for each strain with no DOX; p-value: <0.05, **<0.005, ***<0.0005.

To complement these calcineurin-related studies, we also investigated the effects of NPD827 with respect to ergosterol homeostasis, which NPD827 disrupts lipid homeostasis in a far-reaching manner, which causes the accumulation of endosome-like vesicles, suggesting impairment of trafficking by the compound, which was further exacerbated by a sub-inhibitory concentration of fluconazole (Fig. 5a). Previous studies in *S. cerevisiae* have implicated endocytosis and vacuole-mediated degradation in proper recycling of the ABC-transporter Pdr5, a homolog of *C. albicans* Cdr1.37 Therefore, we examined the impact of NPD827-induced perturbation of the endomembrane system on localization of the multi-drug efflux pump, Cdr1. Using a GFP-tagged strain of Cdr1, we observed a dramatic increase in fluorescence at the plasma membrane upon treatment with NPD827, as qualitatively observed by fluorescence microscopy and quantified by flow cytometry (Fig. 5b, c and Supplementary Fig. 2a). This increase was not observed with fluconazole treatment alone, nor was the signal enhanced by drug combination (Fig. 5b, c). Importantly, despite increased Cdr1-GFP at the plasma membrane, NPD827 increased intracellular accumulation of a fluorescent efflux substrate, rhodamine-6G, indicating that drug efflux was actually impaired rather than enhanced (Fig. 5d). These NPD827-induced changes occurred very rapidly with Cdr1 accumulating at the membrane and efflux being impaired within 30 min, suggesting immediate rather than secondary effects of compound exposure. To confirm the hypothesis that NPD827-mediated impairment of Cdr1 function would lead to increased intracellular fluconazole accumulation, we measured fluconazole levels within the cell using LC–MS after 1 h of treatment. We detected significantly more intracellular fluconazole upon combination treatment (5 µg/mL NPD827, 0.25 µg/mL fluconazole) compared to the equivalent fluconazole concentration alone (0.25 µg/mL) (Fig. 5e). Notably, when measuring intracellular NPD827 levels, the addition of fluconazole did not lead to significantly increased NPD827 intracellular accumulation (Supplementary Fig. 2b), indicating that NPD827 treatment increases the bioavailability of fluconazole within the cell but not vice versa.

Given the rapid timescale on which NPD827 increased Cdr1 at the membrane and impaired efflux, we reasoned that NPD827 might directly impair membrane dynamics by altering the biophysical state of the plasma membrane, rather than inhibiting a specific protein target. To explore this possibility, we performed fluorescence recovery after photobleaching (FRAP) lateral mobility analysis using a *C. albicans* strain in which the GTPase...
Ras1 was GFP-tagged. We specifically used Ras1 as it is plasma membrane-associated, highly mobile, and lipid modifications are known to influence its dynamics within the membrane. Upon treatment of this strain with NPD827 (5 µg/mL), the time to recover after post-photobleaching (i.e., half-time) was increased and a concordant decrease in the mobile fraction within the membrane was observed, both within minutes of compound addition (Fig. 5f). These observations suggest that NPD827 treatment causes measurable changes to the physical state of the plasma membrane, restricting mobility of at least some membrane-associated proteins.

To determine if the altered membrane dynamics seen by FRAP in whole cells could be explained by direct interaction of NPD827 with membranes, we moved to a cell-free, artificial system and used isothermal titration calorimetry (ITC) to monitor NPD827 interactions with sterol-containing membranes. As previously shown, amphotericin B bound to both ergosterol- and cholesterol- but not lanosterol-containing LUVs. NPD827, on the other hand, bound to lanosterol-, ergosterol-, and cholesterol-containing LUVs. Interestingly, titration of NPD827 with lanosterol-containing LUVs yielded a significantly higher increase in total net exotherm compared to ergosterol-containing LUVs, suggesting that NPD827 may preferentially partition into membranous compartments where less mature intermediates within the sterol biosynthetic pathway reside. Consideration of both the whole-cell and biophysical findings acquired so far suggests that NPD827 disrupts lipid homeostasis by intercalating into sterol-containing membranes, thereby altering their

**Fig. 3** NPD827 treatment results in accumulation of long chain sphingoid bases and the depletion of modified ceramides. a) Conditional expression mutants of sphingolipid biosynthesis pathway components were profiled at a fixed concentration of NPD827 (5 µg/mL) in the absence and presence of doxycycline (DOX, 0.05 µg/mL). Data are shown for those mutants with significant differences, presented as mean ± SD of technical triplicates. p-value: 0.00053 (tetO-LCB1/ichb1Δ), 0.00002 (tetO-LCB2/ichb2Δ). Significance was determined by performing multiple unpaired t tests comparing plus DOX conditions for each strain with no DOX. p-value: **<0.005, ***<0.0005. b) Overview of sphingolipid biosynthesis pathway labeled with gene names of conditional expression mutants that were tested for sensitivity to NPD827. E denotes essential genes and NE denotes non-essential genes. Genes coloured in red were identified as significantly hypersensitive to NPD827 upon transcriptional repression with doxycycline (DOX, 0.05 µg/mL). c) Checkerboard assays were performed with NPD827 and the SPT inhibitor myriocin against a wild-type strain of *C. albicans* (SN95). Assays were performed and are presented as previously described in Fig. 1c. FICI values are displayed in the top right corner of each checkerboard. d) Abundance of key sphingolipid species with significant differences in compound-treated samples (star indicates concentrations used in combination treatment). Data are expressed as mean ± SD of *n* = 3, biologically independent replicates. p-value, compared to untreated: Dihydrosphingosine, 0.0086 (NPD827 10 µg/mL); 0.0023 (NPD827 20 µg/mL); 0.0008 (FLC 16 µg/mL); Phytophosphingosine, 0.0009 (NPD827 20 µg/mL); 0.0313 (Combination [FLC 0.25 µg/mL + NPD827 5 µg/mL]); OH-Ceramide, <0.0001 (NPD827 5 µg/mL, 20 µg/mL, FLC 0.25 µg/mL); 0.0001 (NPD827 10 µg/mL); 0.0007 (FLC 16 µg/mL); 0.0102 (Combination [FLC + NPD827]); Glucosyl Ceramide, <0.0001 (all samples). Significance determined by one-way ANOVA with Bonferroni’s multiple comparisons test, where all treatment conditions were compared to the wild-type untreated control. p-value: *<0.05, **<0.005, ***<0.0005. Source data are provided as a Source Data file.
biophysical properties, perturbing vesicular trafficking, impairing the function of membrane-associated proteins, and ultimately increasing intracellular accumulation of fluconazole.

Mutations within the ESCRT-III complex confer resistance to NPD827 in a C. albicans erg3Δ/erg3Δ background. As an orthologous genetic approach to investigate how the sterol-interacting properties of NPD827 might confer azole-potentiating activity, we selected for NPD827-resistant isolates in a NPD827-sensitized C. albicans background that harbors a homozygous deletion of the ergosterol biosynthetic gene, ERG3. We selected for NPD827-resistant mutants by plating erg3Δ/erg3Δ cells (1 × 10^8) on solid YPD medium containing NPD827 (40 µg/mL) and performing whole-genome sequencing of six independent resistant-isolates (Fig. 6a). Three of six resistant-mutants had non-synonymous heterozygous mutations in VPS4, a gene encoding an AAA-ATPase involved in transport from multi-vesicular bodies (MVBs) to the vacuole and in disassembly of the Endosomal Sorting Complex Required for Transport (ESCRT)-III complex. Two of six resistant-mutants had non-synonymous heterozygous mutations in genes encoding members of the ESCRT-III complex itself: VPS20, encoding a protein critical for initiation of ESCRT-III assembly, and SNF7, encoding the most abundant ESCRT-III subunit responsible for forming membrane-sculpting protein filaments. The remaining resistant isolate had a
heterozygous non-synonymous mutation in UBI4, a gene encoding the ubiquitin precursor peptide required for tagging of proteins for degradation by the vacuole via the MVB pathway.

To verify the mutations identified by our sequencing analysis were necessary and sufficient for the resistant phenotype, we performed allele-swap experiments. The parental allele was introduced into the resistant mutant (RM) and reciprocally, the mutant allele was introduced into the parental strain. Expression of one allele of VPS4<sup>D234Y</sup>, VPS4<sup>G178E</sup>, or VPS20<sup>E194*</sup> in an erg3<sup>Δ/Δ</sup> background conferred a level of resistance to NPD827 equivalent to erg3<sup>Δ/Δ</sup>-RM1, -RM4, and -RM6, respectively, demonstrating that each of these mutant alleles is sufficient to confer NPD827 resistance (Fig. 6b). Reciprocally, replacement of the mutant allele with the ancestral VPS4 or VPS20 allele in the respective selected lineage abrogated resistance to NPD827, demonstrating that these mutations are necessary for resistance in these strains (Fig. 6b). Homozygous deletion of either VPS4 or VPS20 in the erg3<sup>Δ/Δ</sup> background resulted in resistance to NPD827, suggesting that the identified mutations are not only loss-of-function but also dominant mutations (Fig. 6c).
Interestingly, these mutations in VPS4 or genes encoding members of the ESCRT-III complex were not necessary or sufficient for resistance in an alternative sensitized-background, cna1Δ/cna1Δ, and did not alter the potentiation of fluconazole activity by NPD827 in a wild-type background (Supplementary Fig. 3).

The NPD827-resistance conferring mutations identified were reminiscent of a resistance profile reported for the alkyllysophospholipid analog, edelfosine that is thought to bind to ergosterol in the plasma membrane resulting in disorganization of lipid microdomains and inducing internalization and vacuole-dependent degradation of membrane-bound proteins40. Loss-of-function of ESCRT-III has been proposed to provide an alternative route for membrane-bound enzymes and associated lipids to be recycled, which avoids their toxic accumulation within the vacuole. As with NPD827, we confirmed the heterozygous non-synonymous mutations identified in the NPD827-resistant lineages or homozygous deletion of VPS4 or VPS20 conferred edelfosine resistance in an erg3Δ/erg3Δ background (Fig. 6c). Loss-of-function of Vps4 or ESCRT-III restored the capacity of the cell to respond to lipotoxic stress. Increases in lipid-droplet size and vacuolar fragmentation caused by NPD827 were reduced compared to the erg3Δ/erg3Δ control (Fig. 6d and Supplementary Fig. 4a). However, these mutations did not reverse impairment of multi-drug efflux, as Cdr1-GFP rapidly accumulated at the plasma membrane and rhodamine-6G efflux was still impaired (Supplementary Fig. 4b). Taken together, the results support a mode of action for NPD827 that involves sterol-dependent targeting of membrane biophysics rather than inhibition of a specific protein target.

NPD827 demonstrates therapeutic promise in preclinical models. To begin evaluating the therapeutic potential of NPD827, we used in vitro co-culture models consisting of human 293T kidney-derived cells stably expressing firefly luciferase and clinical isolates of C. albicans (CaCi-2 and CaCi-17) expressing GFP (Fig. 7a). Assay plates previously seeded with 293 T cells were infected with C. albicans followed by addition of NPD827 in the absence or presence of a fixed concentration of fluconazole. After 48 h, relative fungal burden was measured by quantifying GFP fluorescence and relative rescue of human cell viability was evaluated by luminescence. NPD827 had minimal impact on fungal burden in the absence of fluconazole, but burden of both CaCi-2 and CaCi-17 was significantly reduced in the presence of sub-inhibitory concentrations of fluconazole (0.25 and 16 µg/mL, respectively), resulting in a reciprocal rescue of human cell survival, as measured by luciferase expression (Fig. 7a). While NPD827, alone or in the presence of fluconazole displayed minimal cytotoxicity to human cells in vitro (Figs. 1b and 7a), fluconazole does not inhibit biosynthesis of cholesterol, the analogous sterol in human cells to ergosterol in fungi. To ask whether NPD827 would increase the cytotoxicity of inhibiting cholesterol biosynthesis, we used lovastatin, an inhibitor of HMG-CoA reductase. This enzyme catalyzes an early, rate-limiting step in cholesterol biosynthesis. 293 T cells were cultured with a two-fold gradient of lovastatin in the absence and presence of NPD827. Combination with NPD827 has no impact on lovastatin cytotoxicity, highlighting the tolerability of NPD827 for human cells even when cholesterol synthesis is impaired (Supplementary Fig. 5).

As a non-optimized screen hit, NPD827 did not have the necessary pharmacological properties, especially adequate solubility, to permit testing in mouse models of systemic fungal infection. Therefore, we leveraged an alternative infection model involving the nematode Caenorhabditis elegans41. This simple invertebrate is frequently used as a model host to study Candida–host interactions due to ease of infection and functional similarity between the intestinal tract of C. elegans and mammals, a site from which C. albicans can invade to cause systemic infection. Worms were infected with CaCi-2 or CaCi-17 by allowing them to feed on plates previously inoculated with the fungal strains. After infection, they were transferred to multi-well plates containing solutions of the indicated compounds and their subsequent survival monitored for up to 48 h. Only combination treatment significantly extended the lifespan of worms infected with CaCi-2 (Fig. 7b). In worms infected with CaCi-17, a high concentration of fluconazole alone did significantly extend lifespan, but combination with NPD827 provided significant additional benefit (Fig. 7b).

To further explore the therapeutic potential of NPD827, we took an alternative approach to the worm model and determined whether NPD827-induced perturbation of membrane homeostasis would block a key virulence trait of C. albicans, the ability to transition between yeast and filamentous forms42. Disruption of lipid homeostasis, such as inhibition of ergosterol biosynthesis
with fluconazole, has previously been reported to impair C. albicans filamentation in response to diverse cues. We found that NPD827 alone blocked C. albicans filamentation induced by the host-relevant cues of serum and high temperature in rich YPD medium but was not sufficient to block filamentation induced by culture in RPMI medium at 37 °C (Fig. 7c).

Effects seen on filamentation encouraged us to evaluate activity against biofilms because the capacity to transition between yeast and filamentous forms is critical for robust biofilm development in C. albicans. We performed in vitro assays in which C. albicans biofilms were established in static 96-well plates primed with calf serum under biofilm-inducing conditions (Fig. 7d).

Fig. 6 Selection experiments identify mutations in the ESCRT-III complex that confer resistance to NPD827 in an erg3Δ/erg3Δ background. a Single colonies from six independent lineages were tested for resistance to NPD827 and whole genome sequencing was used to identify candidate resistance mutations. Dose–response assays were performed in YPD medium, and growth was measured by absorbance at 600 nm after 24 h at 30 °C. Optical densities were averaged for duplicate measurements and normalized to no-compound, wild-type strain growth. Results are depicted as in Fig. 2b. Genome sequences were analyzed using Mutect (v1.1.7) and annotated using SnpEff (v4). Non-synonymous substitutions identified in each lineage are listed in the table. ESCRT-III complex members are bolded. b Allele-swap strains were assessed by growth curve analysis in the absence or presence of NPD827 (20 μg/mL) for 48 h in YPD at 30 °C, and growth was monitored every 15 min by absorbance at 600 nm. The areas under the resulting growth curves (AUC) were calculated and the ratio of AUCdrug/AUCsolvent was normalized relative to a wild-type control. Data are expressed as mean ± SD of n = 3 biologically independent replicates. Significance determined by one-way ANOVA with Bonferroni’s multiple comparisons test, where all conditions were compared to the parent (erg3Δ/Δ), ***<0.0005 p-value, p-value, compared to erg3Δ/erg3Δ parent: <0.0001 (Wildtype, RM1 [Vps4D234/Y234], erg3Δ/erg3Δ [Vps4D234/Y234], RM4 [Vps4G178/E178], erg3Δ/erg3Δ [Vps4G178/E178], RM6 [Vps20G194/*194], erg3Δ/erg3Δ [Vps20G194/*194]). c Dose–response assays were performed against allele-swap strains, vps4Δ/vps4Δ, and vps20Δ/vps20Δ, as previously described in (a). Alleles coloured in blue represent those found in the parent. Alleles coloured in red represent those found in the resistant lineage. Growth was measured using absorbance at 600 nm in YPD medium at 30 °C after 24 h. Measurements were normalized to no-drug controls and data are presented as in Fig. 2b. d Lipid droplets were visualized using fluorescence microscopy (GFP filter) of cells stained with BODIPY 493/503 after NPD827 treatment. Parental allele is displayed in blue, resistant-lineage allele is displayed in red. Experiment was repeated in three biologically independent replicates with similar results. Scale bar, 10 μm. Source data are provided as a Source Data file.
Finally, to evaluate the efficacy of NPD827 in a more clinically relevant context, we used a rat model of catheter-associated candidiasis that mimics a common complication of central venous catheters in patients.\(^4\) Infection of implanted catheters with *C. albicans* was performed by intraluminal instillation of a fungal inoculum. Catheters were flushed after 6 h, instilled with either NPD827 (20 µg/mL) or control vehicle and then removed 24 h later. Serial dilutions of the catheter fluid were plated to assess viable colony-forming units (CFU), and catheters were processed for scanning electron microscopy (SEM). Under control conditions, biofilms became well-established in the indwelling catheters, as visualized by scanning electron microscopy (Fig. 7e). In contrast, treatment with NPD827 completely blocked the formation of *C. albicans* biofilms and reduced the recovered CFU by more than 7-fold compared to the untreated control (Fig. 7e). Together, these results provide proof-of-concept for the strategy of targeting lipid homeostasis with NPD827-like compounds to treat fungal infections.

**Discussion**

With the striking rise in antifungal resistance worldwide, an urgent need exists for the development of alternative strategies to control drug-resistant fungal pathogens. Here, we identify a potent azole-potentiating agent with fungal-selective, broad-spectrum activity. We found that the imidazopyrazindo
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Fig. 7 NPD827 reverses azole tolerance in co-culture and inhibits filamentation and biofilm development. a Co-culture of GFP-expressing C. albicans clinical strains with human 293T cells expressing a luciferase reporter. The effect on relative fungal (left axis, fluorescence) and human (right axis, luminescence) cell number by NPD827 in the presence or absence of fluconazole (FLC) after a 48-h treatment is depicted. Data are mean ± SD of technical triplicates. Significance was determined by one-way ANOVA with Bonferroni’s multiple comparisons test comparing each treatment condition to untreated (UN); p-value: *<0.05, **<0.005, ***<0.0005. Data is representative of two independent biological replicates. b C. elegans were infected with C. albicans clinical strains (CaCi-2, left panel; CaCi-17, right panel) and survival monitored in the presence of fluconazole, NPD827, the combination, or without treatment. 20 C. elegans worms were subjected to each condition, and survival was monitored over 48 h in three independent trials. Survival was plotted as a Kaplan–Meier survival curve. Log-rank (Mantel–Cox) test was used to determine p value. c Cultures were grown under filament-inducing conditions with the indicated compound treatment for 4 h. Scale bar, 10 µm. Images are representative of two independent biological replicates. d (Left) Compounds were added immediately to assess effects on biofilm formation. (Right) Compounds were added after 24 h of biofilm growth to assess effects on biofilm maturation. Metabolic activity was measured using an XTT reduction assay. Data are presented as mean ± SD of technical triplicates. Star indicates concentrations used in combination treatment. Significance determined by one-way ANOVA with Bonferroni’s multiple comparisons test, wherever all conditions were compared to wild-type untreated condition. p-value: *<0.05, **<0.005, ***<0.0005. p-value, compared to untreated: Biofilm Prevention, <0.0001 (NPD827 5, 10, 20 µg/mL, Combination); Biofilm Eradication, 0.0055 (NPD827 5 µg/mL); 0.0351 (NPD827 10 µg/mL), <0.0001 (NPD827 20 µg/mL, Combination). e C. albicans biofilms cultured in rat central venous catheters in the presence or absence of NPD827 (20 µg/mL) were examined by scanning electron microscopy at 24 h. Scale bar, 50 µm. Serial dilutions of catheter fluid were plated for fungal colony counts. Data represented as mean colony forming unit (CFU) per catheter ± SD of three technical replicates. Significance determined by a two-tailed unpaired t-test with Welch’s correction, p-value: *<0.05. p-value: 0.0071 (NPD827). Source data are provided as a Source Data file.

Fig. 8 Model of NPD827 mechanism of action. (Left) Under normal physiological conditions, ergosterol and sphingolipids exist within the plasma membrane as lipid microdomains (yellow shadow). These microdomains harbor membrane-bound proteins, including drug-efflux pumps such as Cdr1. (Middle) NPD827 inserts into the plasma membrane and alters the biophysical properties of the membrane, reducing lateral mobility and impairing the function of membrane-bound proteins (i.e., Cdr1). Internalization of NPD827-associated membrane fragments disrupts the endomembrane system and induces vacuolar fragmentation, thereby perturbing lipid recycling throughout the cell. This disruption results in accumulation of toxic long-chain sphingoid bases, depletion of downstream sphingolipids, such as OH-Ceramide and Glucosylceramide, and induction of membrane-associated stress responses (i.e., lipid droplets, UPR, calcineurin-dependent stress responses). (Right) Due to impairment of drug-efflux by Cdr1, fluconazole accumulates within the cell further perturbing lipid homeostasis. The combination of effects ultimately halts growth of C. albicans in a synergistic manner.

NPD827 acts through a previously undescribed mode of action involving physical association with sterols (Fig. 8). This interaction alters the properties of fungal membranes, resulting in reduced membrane mobility, impaired function of some integral membrane proteins, and disruption of vesicular trafficking. As might be expected, the profound perturbation of membrane homeostasis caused by NPD827 induces a compendium of membrane-associated stress responses, including lipid-droplet accumulation, induction of the unfolded-protein response, and activation of calcineurin-dependent stress responses (Fig. 8). Combining NPD827 with fluconazole increases intracellular accumulation of fluconazole, which enhances disruption of sterol biosynthesis, increases lipid-droplet formation, and exacerbates vacuolar fragmentation (Fig. 8). Selection for NPD827-resistant
mutants in an ergosterol biosynthesis-defective background yielded isolates with loss-of-function mutations in the multivesicular body pathway confirming that fungal membrane composition influences NPD827 activity and revealing that genetically altering vesicular trafficking can mitigate NPD827-induced lipotoxicity in this background.

Major success has been achieved in the development of drugs that target microbial cell membranes, including two out of the three major antifungal classes available for treatment of systemic fungal disease. The azoles, which target ergosterol biosynthesis, have been effective in the clinic and agriculture, however, resistance mechanisms including overexpression of drug efflux pumps, substitutions in Erg11, and loss-of-function of Erg3 are capable of potentiating azoles in strains with well-characterized activity. It is also notable that, in contrast to amphotericin B, which directly target the membrane by extracting ergosterol through a sterol sponge mode of action, have proven to be exceptionally resistance-evasive for more than half a century. Moreover, when resistance does develop, it is associated with profound fitness costs including vulnerability to diverse exogenous stresses. More broadly, the development of resistance to other membrane-targeting antibiotics also appears to occur less frequently than to agents with single protein targets. Here, we show that NPD827 directly binds to sterol-containing lipid bilayers and induces membrane alterations within minutes that affect the function of membrane-bound proteins. Its effects on membrane homeostasis, however, are clearly distinct from that of the polynes because NPD827 displays minimal single-agent activity. It is also notable that, in contrast to amphothericin B, NPD827 interacts strongly with lanosterol-containing lipid bilayers. The ability of NPD827 to interact with not just ergosterol, but also its precursor lanosterol, may explain why NPD827 activity is enhanced in combination withazole-mediated changes in membrane sterol composition. Finally, we show that NPD827 is capable of potentiating azoles in strains with well-characterized resistance mechanisms including overexpression of drug efflux pumps, substitutions in Erg11, and loss-of-function of Erg3. This effect contrasts with other well-characterized molecules that enhance azole activity in C. albicans including inhibitors of the molecular chaperone Hsp90, the protein phosphatase calcineurin, and the protein kinase Pck1. While these stress response inhibitors enhance azole activity againstazole-tolerant isolates and strains with mutations in ERG3, resistant isolates that overexpress efflux pumps remain resistant to the drug combination.

Sterols and sphingolipids are distinct lipid classes that act together to maintain the integrity and function of eukaryotic membranes. Genetic or pharmacological inhibition of the ergosterol biosynthesis pathway results in the depletion of ergosterol and the accumulation of specific sterol intermediates. Pathway inhibition, however, can impact more than just sterol levels. Some disruptions induce compensatory alterations in sphingolipid composition, suggesting that sterol and sphingolipid biosynthesis genes functionally interact. Such interaction may contribute to the synergistic effects of combining NPD827, which affects both early and late sphingolipid intermediates, with fluconazole, which depletes ergosterol and drives accumulation of aberrant, toxic sterols. Beyond purely biochemical effects, the synergistic disruption of lipid homeostasis observed upon combining NPD827 and fluconazole may also occur because genes involved in sterol and sphingolipid biosynthesis are co-regulated by shared trans-acting factors. For example, the transcription factors Ecm22 and Upc2 control ergosterol production, but they also have consensus binding sequences in the promoters of the SPT genes LCB1 and LCB2, and deletion of fatty acid elongases, involved in synthesizing sphingolipid precursors, induces UPC2 expression in C. albicans.

Sterols and sphingolipids are well-known to be connected in human diseases, especially Niemann Pick type C (NPC), where defects in sphingolipid and cholesterol trafficking appear to be interdependent. Reminiscent of NPD827 treatment, mutations in NPC1 or NPC2, which underlie the NPC disorder, cause accumulation of LCBs within the endocytic pathway and the increased production of lipid droplets. Intriguingly, the AAA ATPase SKD1 (mammalian homolog of Vps4) physically interacts with NPC1 and regulates its role in endosomal cholesterol trafficking. In yeast, vps mutants result in entrapment of Ncr1 (yeast homolog of NPC1) in pre-vacuolar compartments and homozygous deletion of NCR1 renders cells resistant to the lipid analog, edelfosine. Edelfosine directly binds to the N-terminal, lipid-binding tunnel of Ncr1 and this association is critical for its integration into the vacuolar membrane. Given the structural similarity of NPD827 to sterols and the similar resistance profile it shared with edelfosine, NPD827 may interact with Ncr1 in a similar way. In this scenario, the NPD827 resistance-conferring loss-of-function mutations we identified within the ESCRT machinery may entrap Ncr1 in pre-vacuolar compartments and prevent NPD827 internalization via Ncr1-mediated shuttling to the vacuole, thus allowing lipids to be recycled back to the plasma membrane and ameliorating toxicity. Conversely, we found that the toxicity of NPD827-induced trafficking defects is exacerbated when alternate sterols are present in the plasma membrane, as with deletion of ERG3. This enhanced effect may be due to increased binding of NPD827 to alternative sterols within the membrane or due to increased cellular stress when trafficking defects occur in the absence of the mature membrane sterol, ergosterol.

Disruptions in lipid homeostasis are tightly linked with induction of key cellular stress responses that help mitigate lipotoxicity and promote cellular survival. Sphingolipids have emerged as important regulators of ER stress. As an example, addition of sphingolipid building blocks including palmitate, or the disruption of sphingolipid biosynthesis by genetic deletion of ORM1/2, results in activation of the UPR. Further, lipid droplets act to buffer lipid uptake, distribution, and storage to finely tune lipid dynamics within the cell. Pharmacological inducers of ER stress, including tunicamycin, brefeldin A, and thapsigargin, promote lipid droplet biogenesis in both yeast and mammalian cells. Under conditions of ER stress, calcium- and calcineurin-dependent stress responses are also essential to sustain the UPR. In particular, disruption of calcium influx via the Cch1-Mid1 Ca2+ channel or blocking downstream signaling by the calcium-dependent phosphatase calcineurin results in rapid cell death in response to ER stress. Here, we demonstrate that these membrane-associated stress responses are activated in response to NPD827-induced lipotoxicity, and that deletion of components involved in the calcineurin-dependent stress responses results in hypersensitivity to NPD827. Of note, calcineurin-dependent stress responses also play an important role in the response of diverse fungal pathogens to azole-induced stress. Further, a downstream effector of the calcineurin pathway, RTA2, is involved in calcineurin-mediatedazole resistance and long chain sphingoid base release in C. albicans. Deletion of RTA2 results in exacerbation of azole-induced stress and the accumulation of dihydrosphingosine, similar to the effects of NPD827 on sphingolipid homeostasis.

From a therapeutic perspective, NPD827 reveals an intriguing way to reverseazole resistance in C. albicans and inhibit key virulence traits, including filamentation and biofilm formation. Although NPD827 does not have the necessary pharmacological properties for systemic use, we demonstrated that NPD827 in combination with fluconazole does act in a fungal-selective manner in a co-culture model with human cells and in a C. elegans model to improve survival in the face of infection with azole-tolerant- and -resistant clinical isolates. Contributing to its
overall antifungal potential, NPD827 also impaired the key C. albicans virulence traits of filamentation and biofilm formation. While these results are encouraging from a therapeutic perspective, more work will be needed at both the basic science and drug-development levels to translate the new biological insights provided by discovery of NPD827 into a useful strategy to counter antifungal drug-resistance.

Methods

Strains and culture conditions. All strains used in this study are listed in Supplemental Table 2. All plasmids used in this study are included in Supplemental Table 2. All strains were cultured in YPD (1% yeast extract, 2% bacto-peptone, and 2% d-glucose) for liquid culture growth, or YPD with 2% agar for solid medium growth, at 30 °C unless otherwise specified. Strains stocks were maintained at −80 °C in 25% glycerol.

High-throughput chemical screen. A set of 19,840 compounds from the RIKEN Natural Product Depository (NPDepo) library were used to identify compounds that enhanced fluconazole activity against C. albicans. The NPDepo library is maintained at 1 mg/mL stocks in DMSO (dimethyl sulfoxide) and was screened at a final concentration of 10 µg/mL. A BioTech ADS-384-8 compound dispenser was used to transfer 0.2 µl of 1 mg/mL compound stocks in DMSO from each mother plate into daughter plates. YPD medium alone or containing 1 µg/mL fluconazole was inoculated with ~10^5 cells/mL of C. albicans (CaS11) from a saturated overnight culture. Inoculum was aliquoted into 384-well microtitre plates (Greiner Bio-One #781182) to a final volume of 40 µl/well using a Ranin Liquidator. Dilutions were made to another aliquot at 30 °C for 48 h, at which point 20 µl of 0.03% (w/v) resazurin salt solution was added for 4 h. After incubation, the samples were cooled to room temperature and 1 ml and 3 ml chloroform were added to each sample for extraction. The tubes were vortexed for 10 s 3 times then centrifuged at 1048×g for 5 min. Using Pasteur pipettes, the bottom organic phases were transferred to another borosilicate glass tube (EZ-2 Series SP Scientific) on the high boiling point setting. Samples were dissolved in 600 µl chloroform and 200 µl was transferred into a 1 ml conical thick-walled glass reaction vial for derivatization. Saponified lipid extracts were evaporated to dryness under a stream of N2 gas at an airflow rate of 1 ml/min and the bottom pH 7 phosphate buffered saline. Pellets were weighted and flash frozen with liquid nitrogen and stored at −80 °C.

Intracellular sterols of yeast were extracted as reported previously with slight modification. Cell pellets were mixed with 3 ml extraction solution (25% KOH w/v, 64% methanol v/v) adding 30 µl of 2 mg/ml cholesterol (dissolved in chloroform) to each sample for extraction. The tubes were vortexed for 4 min and were centrifuged at 10,000×g for 10 min before aspiration of the supernatant. The salts were washed with 1 ml methanol and 1 ml chloroform. The organic phases were transferred into 1 ml borosilicate glass vials. Approximately 5 µl of the chloroform phase was dried under a gentle nitrogen stream prior to derivatization. According to the ChromaFID user manual (ChromaFID Systems, Sweden), 3 µl of methanol and 1 µl of 50% methanol and 50% phenol was added to each sample for derivatization. The vials were vortexed for 30 s, then 50 µl of 15% HCl in methanol was added. After 15 min, the vials were cooled to room temperature and then 1 ml water and 3 ml chloroform were added. The LC-MS controls were run under the same conditions. For mass spectrometry analysis, data was plotted in Prism v9.3.0 using the mean relative quantitative –5D.

For sphingolipid profiling, saturated overnight cultures of SN95 were diluted to an OD600 of 0.1 in 10 mL of minimal medium (0.17% yeast nitrogen base without amino acids, 2% D-glucose, 0.1% monosodium glutamate, 1X histidine, 1X arginine) without drug treatment. Cells were grown for 2 h before adding drug and incubating for an additional 4–6 h with shaking at 30 °C. Samples were normalized to ~5 × 10^6 cells/sample and then transferred into reusable glass tubes with phenolic screw caps. Samples were pelleted by centrifugation and washed with 1X PBS before flash freezing with liquid nitrogen. Internal standards (C17-ceramide and C17-sphingosine) were added to the samples, and sphingolipids were extracted as described previously. Briefly, fungal cells were suspended in Mandal buffer (ethanol:distilled water:diethyl ether = 2:1:1, v/v/v) and were sonicated for 10 min. Lipid extracts were then adjusted with a mild alkaline methanolysis of phospholipids. The organic phase was transferred to a new tube, dried, and used for LC–MS analysis, which was performed in a Thermo Finnigan TSQ Quantum Ultra Mass Spectrometer (Thermo Fischer Scientific, USA).

Fluorescence microscopy. Stationary phase C. albicans cultures were diluted to an OD600 of 0.2 and grown at 30 °C for 2 h, shaking, in the absence of drug. After 2 h, NPD827 (or DMSO equivalent) was added to correspond to the IC50 (50% inhibition), which was determined using quantitative color with displaying the use of the program Java TreeView 1.1.3 (http:// jtreeview.sourceforge.net). Experiments were performed in biological duplicate, with one representative shown.
with 1 µg/mL BODIPY (Cayman Chemical, 25892) for 10 min at 30 °C and washed twice in 1X PBS before microscopy. Lipid droplets were visualized using fluorescence microscopy with a GFP filter. To visualize drug efflux, cells were stained with 1 µg/mL Rhodamine-6G (in sterile dH₂O; Sigma-Aldrich) for 30 min at 30 °C and washed twice in 1X PBS before microscopy. To visualize FM4-64-stained cells, cells were stained with 8 µM 1 µg/mL in the dark for 30 min at 30 °C. After 30 min, cells were resuspended in 1 mL YPD medium to remove free FM4-64 (Thermo Fisher Scientific, #13166) and added to 4 mL of fresh YPD medium. Cells were incubated in FM4-64 free medium for 90 min at 30 °C, spun down, and resuspended in 1X PBS. Rhodamine-6G and FM4-64 were visualized using fluorescence microscopy using an RFP filter. To quantify fluorescence, a CytoFlex Flow Cytometer (Beckman Coulter) was used. Cells were flowed through a flow transparent, 96-well plate (Beckman Coulter). Each sample was run using the CytExpert Software (version 2.4) until ~20,000 events had been recorded. Populations were gated to exclude debris and doublets, and the median value was taken for each sample.

For Cdr1-GFP experiments, CaLC7116 was subcultured in YPD medium in 3 mL at an OD₆₀₀ of 0.2 for 3 h with agitation. Indicated compounds were added and cells were incubated for an additional 30 min. Cells were then pelleted by centrifugation at 3000× g for 5 min and washed twice in PBS. Samples were then imaged by microscopy or flow cytometry as indicated in the “Methods” section currently. All assays were done in biological triplicates shown in the figure. Data are plotted in Prism v9.3.0 (GraphPad) showing the mean fluorescence ±SD.

**HAC1 mRNA splicing assay.** To test for the activation and alternative splicing of HAC1, reverse transcription-polymerase chain reaction (RT-PCR) was performed on RNA extracted from C. albicans, as previously described. Stationary phase SN95 cells were used to inoculate a fresh culture at an OD₆₀₀ of 0.4 in 5 mL of YPD in the presence of the indicated compound concentration for 1 h with agitation. Cells were then exposed to 5 mM dithiothreitol (DTT), 10 mM tunicamycin, or varying concentrations of NPD827 for 1 h. Control cultures were treated with the equivalent volumes of DMSO. RNA was isolated using QIAGEN RNeasy kit and further treated with RNase-free DNase (QIAGEN). cDNA was prepared using the AffinityScript cDNA synthesis kit. The reaction products (2 µL) were then mixed with 1 µL each of 10 µM HAC1 primers (olC4345/olC4347), 2 µL of 2.5 mM each dNTP mix, 2.5 µL of the supplemented IBOX 2X buffer, 16.37 µL of dH₂O, and 0.4 µL of TAKARA Taq DNA polymerase (5 U/µL), and subjected to a 30-cycle thermal cycle reaction of 94 °C for 30 s, 54°C for 30 s, and 72°C for 60 s. PCR products were run on 2% agarose gels (1X TAE Buffer) at 100 V for 60 min and the SYBR Safe stained fluorescent images were captured by a ChemiDoc.

**Calcineurin activation assay.** By β-Galactosidase assay with the UTR2p-lacZ reporter, stationary phase C. albicans cultures were diluted to OD₆₀₀ of 0.5 and grown at 30 °C for 2 h, shaking. After 2 h, 100 µL of culture was placed in a 96-well plate (Sarstedt) with test compounds and the plate was grown at 30 °C shaking for another 4 h. Following this, cell cultures were diluted 1:100 in YPD and 30 µL of diluted culture was placed into a 384-well white assay plate (Corning). Pre-diluted Gal-Screen substrate diluted 1.25 with Gal-Screen Buffer was added in each well, and the plate was placed in a Tecan to read luminescence every 15 min for 1 h. Data were normalized to OD₆₀₀ values read with a spectrophotometer after 4 h of compound treatment. All treatments were performed in triplicate and are representative of biological duplicates.

**Intracellular compound quantification.** To quantify intracellular flouconazole levels, C. albicans (SN95) was subcultured from overnight cultures at a starting OD₆₀₀ of 0.4 in 5 mL of YPD in the presence of the indicated compound concentration for 1 h with agitation. Cells were then transferred to falcon tubes and pelleted at 3000xg for 5 min at 4 °C. Cells were washed with 5 mL of cold PBS 3 times at 2000xg for 5 min, flash frozen in 1 mL PBS and stored in --80 °C overnight. Pellets were thawed on ice and 3 µL of 10 µg/mL verapamil was spiked in. 25 µL of 6 N NaOH was added and samples were vortexed for 15 s, 500 µL of 10 mM sodium phosphate pH 6 was added and samples were vortexed for 15 s. 5 mL of CH₃Cl was added to the tubes and samples were vortexed for 5 min and subsequently centrifuged for 10 min at 3000xg at 4 °C. The bottom organic phase was collected and dried by vacuum (EZ-2 Series SP Scientific) on the very low boiling point setting. 200 µL DMSO was added and 10 µL sample was analyzed by HR-ESI-MS (Agilent 1290 Infinity II Series HPLC system coupled to qTOF 6550 ESI/MS) operated in positive ion mode with mobile phase A (0.1% formic acid (H₂O, v/v)) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.191 mL/min using an XDB C8 column (2.1 × 100 mm, 3.5 µm). Peak areas of compounds were normalized to that of internal verapamil standards to control for extraction efficiency and optical density. All assays were done in each biological triplicate and biological duplicates. Data from both biological duplicates are plotted in Prism v9.3.0 (GraphPad) showing the mean relative quantity ±SD.

**FRAP analysis.** FRAP experiments were conducted on an A1R point-scanning confocal system (Nikon). A saturated overnight culture of C. albicans strain (CaLC744), which expresses GFP-Ras1 at the plasma membrane, was subcultured 1:100 into fresh YPD. After 2 h, growth at 30 °C with agitation, 5 x 10⁶ cells were plated on 18 mm glass coverslips that had been previously treated with 2 mg/mL of poly-L-lysine (Sigma-Aldrich). Cells were allowed to settle by gravity over 20 min and were placed in 1X PBS containing 2% glucose for imaging at 30 °C. Prior to imaging, C. albicans was treated with 5 µg/mL NPD827 for 20 min at 30 °C. Images were acquired using a x100/1.4 NA oil objective (Nikon), 1.2-AU pinhole, Galvanic scanning mode, with no line averaging, at 256-pixel resolution and with 1.83 x rectangular zoom. FRAP acquisition was at 3.7 fps, using the 488-nm line at ~1 mW power. For acquisition after a field of up to 5 µL of cultured cells, 3 regions of interest ~1.5 µm in diameter was bleached sequentially for 1 s each using the 405 laser at 100% power, followed by imaging 2 min for fluorescence recovery. Images were exported and analyzed for fluorescence intensity using Velocity software. After background subtraction, fluorescence intensity units were normalized to the non-bleached portion of the membrane and transformed to a 0–1 scale to correct for differences in bleaching depth and allow for comparison of up to 30 individual FRAP curves per condition. Normalizations were done using Microsoft Excel software (v16.60). GraphPad Prism software (v9.2.0) was used to fit the FRAP curves to a single exponential, plotted as fractional recovery over time. Values derived from this non-linear regression were used to calculate mobile fraction, half-time of recovery, and diffusion rate.

**Isotothermal titration calorimetry.** Experiments were performed using a NanoITC isothermal titration calorimeter (TA Instruments). Solutions of the compounds to be tested were prepared by diluting a 15 mM DMSO stock solution to 150 µM with K buffer (5 mM HEPES/HEPES, pH = 7.4). LUV solutions were diluted with buffer and DMSO to give a final phospholipid concentration of 8 mM in a 1% DMSO/K buffer solution. Immediately prior to use, all solutions were degassed under vacuum at 25 °C for 10 min. To prepare LUVs, a 13 × 100 mm test tube was charged with 800 µL of palmitoyl oleoyl phosphatidylcholine (POPC in CHCl₃, from Avanti Polar Lipids) and 230 µL of sterol solution. For each point, the LUV suspension was diluted with 150 µL of K buffer and vortexed vigorously for 3 min to form suspension of multimellar vesicles (MLVs). The resulting lipid suspension was pulled into a Hamilton 1 mL gastight syringe and was placed in an Avanti Polar Lipids Mini-Extruder. The lipid solution was then passed through a 0.20 µm Millipore polycarbonate filter 21 times to prevent carry over of MLVs into the LUV suspension. Titrations were performed by injecting the LUV suspension at ambient temperature into the sample cell (volume = 0.191 mL), which contained the compound of interest. Consistent with standard procedure, the volume of the first injection 0.23 µL (not included in final analysis) and next, nineteen 2.50 µL injections of the LUV suspension was performed with 300 s between each injection. The rate of stirring for each experiment was 350 rpm. Nano/Analyze software (TA Instruments) was used for baseline determination and integration of the injection heats prior to subtraction of dilution heats and calculation of overall heat involved. The overall heat involved during the experiment was calculated using the following formula (1):

\[
\text{cal}_{\text{overall}} = \sum_{i} (\Delta H_{\text{injection}} - \Delta H_{\text{reaction}})
\]

where \( i \) is the injection number, \( n \) is the total number of injections, \( \Delta H_{\text{injection}} \) is the heat of the \( i \)th injection, \( \Delta H_{\text{reaction}} \) is the heat of the final injection of the experiment. All experiments were performed in triplicate and mean ± standard deviation was plotted in GraphPad Prism.

**Selection experiments and whole genome sequencing.** To select for NPD827-resistant mutants, overnight cultures were grown in YPD medium at 30 °C and cell densities were quantified by haemocytometer. Cells (1 x 10⁸) were plated on YPD
containing 40 μg/mL of NPD827. As a control, ~1 × 10⁶ cells were plated on YPD containing DMSO. Selection plates were incubated at 30 °C for 2–3 days, single colonies were picked and subsequently typed for yeast DNA. These selection experiments were performed in six independent biological lineages. Overnight cultures of pure resistant mutant colonies were used to isolate genomic DNA for whole genome sequencing. DNA was prepared using the PureLink Genomic DNA Mini Kit (Invitrogen) according to the manufacturer’s instructions. Sequencing libraries were prepared using the Nextera XT Kit (Illumina) according to the manufacturer’s instructions. Libraries were sequenced on the Illumina NextSeq platform using paired end reads (300 bp). Sequence reads were de-multiplexed and trimmed to remove bases with Phred scores <20. Reads were aligned to the C. albicans genome from CAGL3 using Bowtie2 (v2.4.0)78, and the alignment was visualized using Integrative Genomics Viewer79. MiTeC (v1.1.7) was used to identify unique mutations aligned to the parental strain80.

Filamentation microscopy. The filament-inducing cues tested include YPD medium at the elevated temperature of 39 °C, YPD medium containing 10% (vol/vol) heat-inactivated newborn calf serum at 37 °C, and RPMI medium, pH 7.0 at 37 °C. Overnight cultures of C. albicans (SN95) were diluted to an OD₆₀₀ of 0.2 and grown under the conditions specified while shaking for 4 h. All imaging was performed using DIC microscopy on Zeiss Axio Imager.M1 at ×100 magnification.

In vitro biofilm drug susceptibility. Drug susceptibility assays were performed on biofilms as previously described81. Briefly, strains were grown overnight in YPD at 30 °C. Subsequently, cultures were resuspended in RPMI medium buffered with MOPS to a final concentration of 10⁶ cells/mL. An aliquot of 100 μL was added to each well of a 96-well plate. Triplicates were included for each condition. Wells were gently washed twice with 1X phosphate-buffered saline (PBS) to remove non-adherent cells. To assay for biofilm formation, fresh medium was added with or without NPD827 and/or fluconazole. After 24 h, non-adherent cells were washed away with PBS and biofilm cell metabolic activity was measured using the XTT reduction assay as previously described. Briefly, 90 μL of XTT at 1 μg/mL and 10 μL of phenazine methosulfate at 320 μg/mL were added to each well, followed by incubation at 37 °C for 15 min. Absorbance of the supernatant transferred to a fresh plate and diluted 1:4 in 1X PBS was measured at 490 nm using a spectrophotometer. To assay for biofilm eradication, fresh medium was added to adhered cells without any drug and incubated for 24 h to allow for establishment of mature biofilms. Non-adherent cells were then washed away with 1X PBS and fresh medium containing either NPD827 and/or fluconazole was added. After 24 h, non-adherent cells were washed away with 1X PBS and biofilm cell metabolic activity was measured using the XTT reduction assay, as previously described. Experiments were carried out in a minimum of three technical replicates for each strain/condition.

In vivo biofilm drug susceptibility. In order to evaluate biofilm formation in vivo, a rat central venous catheter infection model was employed82. Rat catheter biofilm and infection assay protocol was used, based on previous studies83–85. Briefly, 48-well plates were prepared with each well containing either: fluconazole (16 μg/mL for CaCi-2 or 256 μg/mL for CaCi-17), NPD827 (40 μg/mL), fluconazole and NPD827 (16 or 256 μg/mL fluconazole and 40 μg/mL NPD827), or no drug treatment. C. elegans worms were allowed to grow from NGM plates using 6 mL of M9 buffer with swarming to dislocate worms from agar and transferred to a 15 mL conical tube. Conical tubes with worms were centrifuged at 1500×g for 30 s, buffer was removed, and worms were washed twice with additional M9 buffer. The worms were counted under a stereomicroscope at ×10 magnification, and the concentration of worms per microliter of solution was calculated, as previously described81. The worm solution was adjusted to 1 worm/μL, and 100 worms were added to BHI + kanamycin plates, and allowed to feed for 3 h. The worms were then rinsed off plates with 500 μL of M9 buffer, transferred to a 15 mL conical tube, 10 mL of additional M9 buffer was added, and the worms were centrifuged at 1500×g for 30 s and washed twice. The worm pellet was resuspended in 300 μL of M9, and worms were allowed to crawl on unseeded BHI plates for 30 min. Worms were rinsed from plate and transferred to a new conical tube with 1 mL of M9 buffer with swarming to dislocate worms from agar and transferred to a 15 mL conical tube. Conical tubes with worms were centrifuged at 1500×g for 30 s, buffer was removed, and worms were washed twice with additional M9 buffer. The worms were counted under a stereomicroscope at ×10 magnification, and the concentration of worms per microliter of solution was calculated, as previously described81. The worm solution was adjusted to 1 worm/μL, and 100 worms were added to BHI + kanamycin plates, and allowed to feed for 3 h. The worms were then rinsed off plates with 500 μL of M9 buffer, transferred to a 15 mL conical tube, 10 mL of additional M9 buffer was added, and the worms were centrifuged at 1500×g for 30 s and washed twice. The worm pellet was resuspended in 300 μL of M9, and worms were allowed to crawl on unseeded BHI plates for 30 min. Worms were rinsed from plate and transferred to a new conical tube with 1 mL of M9 buffer, the number of worms in the sample was determined once again and adjusted to 1 worm/μL. 20 worms (20 μL of sample) was then added to each well of the prepared 48-well plates. Plates were incubated at room temperature overnight, and worm survival was scored at 16, 24, and 48 h under a stereomicroscope at ×10 magnification.

Plasmid construction. Plasmid construction was performed using standard recombinant DNA techniques. Plasmids were sequenced to verify the absence of spurious mutations. Primers are listed in Supplementary Table 3.

pLC1446. From erg3Δ/erg3Δ (CalC7094) genomic DNA, ~300 bp of homology downstream of VSFP was amplified with olC8797/8798 and cloned into pLC49 (pKIp63) at SacI and SacI. Homology from upstream and within VSFP was amplified from erg3Δ/erg3Δ (CalC7094) genomic DNA. PCR fragment was cloned into pLC49 containing the downstream homology at Apal and KpnI. Presence of the insert was confirmed with olC274/olC87800 and olC8799/
VPS4 from RM1 (CaLC7095) genomic DNA using oLC8795/8796 and cloned into pLC49 (pK863) at SacI and SacII. Homology from upstream and within VPS20 was amplified from erg3/3/erg3 (CaLC7094) genomic DNA using oLC8790/3/87840 and cloned into pLC49 containing the downstream homology at Apal and KpnI. Presence of the inserts was confirmed with oLC7470/3/8800 and oLC8799/275. The VPS20 WT allele replacement cassette was liberated by digestion with BssHII.

From erg3/3/erg3 (CaLC7095) genomic DNA, ~300 bp of homology downstream of VPS4 was amplified with oLC7897/8798 and cloned into pLC49 (pK863) at SacI and SacII. Homology from upstream and within VPS4 was amplified from RM1 (CaLC7095) genomic DNA using oLC7895/8796 and cloned into pLC49 containing the downstream homology at Apal and KpnI. Presence of the inserts was confirmed with oLC274/70/2800 and oLC8799/275, respectively. The VPS20 WT allele replacement cassette was liberated by digestion with BssHII.

From erg3/3/erg3 (CaLC7098) genomic DNA, ~300 bp of homology downstream of VPS4 was amplified with oLC8797/8798 and cloned into pLC49 (pK863) at SacI and SacII. Homology from upstream and within VPS4 was amplified from RM4 (CaLC7098) genomic DNA using oLC8795/8796 and cloned into pLC49 containing the downstream homology at Apal and KpnI. Presence of the inserts was confirmed with oLC274/70/2800 and oLC8799/275, respectively. The VPS20 WT allele replacement cassette was liberated by digestion with BssHII.

From erg3/3/erg3 (CaLC7100) genomic DNA, ~600 bp of homology downstream of VPS4 was amplified with oLC8790/3/87840 and cloned into pLC49 (pK863) at SacI and SacII. Homology from upstream and within VPS4 was amplified from RM4 (CaLC7100) genomic DNA using oLC7895/8796 and cloned into pLC49 containing the downstream homology at Apal and KpnI. Presence of the inserts was confirmed with oLC274/70/2800 and oLC8799/275, respectively. The VPS20 WT allele replacement cassette was liberated by digestion with BssHII.

Strain construction. Strains were constructed according to standard protocols. To select for nourseothricin (NAT)-resistant transformants, nourseothricin (Jena Bioscience) was solubilized in water and supplemented into YPD plates at a final concentration of 150 µg/mL.

CalCl7113. The plasmid pClI146 was digested with BssHII to liberate the VPS4 WT allele replacement cassette and was transformed into erg3/3/erg3-RM1 (CaLC7095). For NAT resistant transformants, proper integration was verified by PCR using oLC8799/275 and oLC274/0/2802. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. Replacement of the mutant allele of VPS4 with the wild type was confirmed with oLC8799 by sequencing a PCR product amplified by oLC8787/0/2788.

CalCl7114. The plasmid pClI146 was digested with BssHII to liberate the VPS4 WT allele replacement cassette and was transformed into erg3/3/erg3-RM4 (CaLC7098). For NAT resistant transformants, proper integration was verified by PCR using oLC8799/275 and oLC274/0/2802. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. Replacement of the mutant allele of VPS4 with the wild type was confirmed with oLC8799 by sequencing a PCR product amplified by oLC8787/0/2788.

CalCl7115. The plasmid pClI146 was digested with BssHII to liberate the VPS4 WT allele replacement cassette and was transformed into erg3/3/erg3-RM6 (CaLC7100). For NAT resistant transformants, proper integration was verified by PCR using oLC8799/275 and oLC274/0/2808. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. Replacement of the mutant allele of VPS20 with the wild type was confirmed with oLC8789 by sequencing a PCR product amplified by oLC8780/2/275.

CalCl7116. The plasmid pClI146 was digested with BssHII to liberate the VPS4 WT allele replacement cassette and was transformed into erg3/3/erg3 (CaLC660). For NAT resistant transformants, proper integration was verified by PCR using oLC8799/275 and oLC274/0/2808. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. Replacement of the mutant allele of VPS20 with the wild type was confirmed with oLC8789 by sequencing a PCR product amplified by oLC8780/2/275.

CalCl7117. The plasmid pClI146 was digested with BssHII to liberate the VPS4 WT allele replacement cassette and was transformed into erg3/3/erg3 (CaLC660). For NAT resistant transformants, proper integration was verified by PCR using oLC8799/275 and oLC274/0/2808. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. Replacement of the mutant allele of VPS20 with the wild type was confirmed with oLC8789 by sequencing a PCR product amplified by oLC8780/2/275.

CalCl7118. The plasmid pClI146 was digested with BssHII to liberate the VPS4 WT allele replacement cassette and was transformed into erg3/3/erg3 (CaLC660). For NAT resistant transformants, proper integration was verified by PCR using oLC8799/275 and oLC274/0/2808. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. Replacement of the mutant allele of VPS20 with the wild type was confirmed with oLC8789 by sequencing a PCR product amplified by oLC8780/2/275.

CalCl7119. The plasmid pClI146 was digested with BssHII to liberate the VPS4 WT allele replacement cassette and was transformed into erg3/3/erg3 (CaLC660). For NAT resistant transformants, proper integration was verified by PCR using oLC8799/275 and oLC274/0/2808. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. Replacement of the mutant allele of VPS20 with the wild type was confirmed with oLC8789 by sequencing a PCR product amplified by oLC8780/2/275.

CalCl7120. The plasmid pClI146 was digested with BssHII to liberate the VPS4 WT allele replacement cassette and was transformed into erg3/3/erg3 (CaLC660). For NAT resistant transformants, proper integration was verified by PCR using oLC8799/275 and oLC274/0/2808. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. Replacement of the mutant allele of VPS20 with the wild type was confirmed with oLC8789 by sequencing a PCR product amplified by oLC8780/2/275.

CalCl7121. The plasmid pClI146 was digested with BssHII to liberate the VPS4 WT allele replacement cassette and was transformed into erg3/3/erg3 (CaLC660). For NAT resistant transformants, proper integration was verified by PCR using oLC8799/275 and oLC274/0/2808. The SAP2 promoter was induced to drive expression of FLP recombinase to excise the NAT marker cassette. Replacement of the mutant allele of VPS20 with the wild type was confirmed with oLC8789 by sequencing a PCR product amplified by oLC8780/2/275.
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Author contributions
N.M.R., L.W., N.R. and L.E.C. wrote the paper. N.M.R., K.R.I., M.E.M., J.Z., S.Y., C.M.F., K.J.M., X.C., I.S., M.F., H.S., S.H., S.L., Y.Y., H.H., M.Y. and H.O. performed the experiments. N.M.R., C.B., R.S.S., D.R.A., G.D.W., J.R.N., M.D.P., M.D.B., L.W., N.R. and L.E.C. designed the experiments and interpreted the data. C.B., R.S.S., D.R.A., G.D.W., J.R.N., M.D.P., M.D.B. and L.E.C. provided the materials.

Competing interests
L.E.C. and L.W. are co-founders of and shareholders in Bright Angel Therapeutics, a platform company for development of novel antifungal therapeutics. M.D.P. is a co-founder and chief scientific officer (CSO) of MicroRod Technologies Inc. M.D.B. is a (co-)Founder, consultant, and shareholder for REVOLUTION Medicines, Ambys Medicines, Stungs Therapeutics, Cystic Medicines, and Kinesis Therapeutics. The remaining authors declare no competing interests.

Additional information
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