An oxindole efflux inhibitor potentiates azoles and impairs virulence in the fungal pathogen *Candida auris*

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*Candida auris* is an emerging fungal pathogen that exhibits resistance to multiple drugs, including the most commonly prescribed antifungal, fluconazole. Here, we use a combinatorial screening approach to identify a bis-benzodioxoylindolinone (azofluxin) that synergizes with fluconazole against *C. auris*. Azofluxin enhances fluconazole activity through the inhibition of efflux pump Cdr1, thus increasing intracellular fluconazole levels. This activity is conserved across most *C. auris* clades, with the exception of clade III. Azofluxin also inhibits efflux in highly azole-resistant strains of *Candida albicans*, another human fungal pathogen, increasing their susceptibility to fluconazole. Furthermore, azofluxin enhances fluconazole activity in mice infected with *C. auris*, reducing fungal burden. Our findings suggest that pharmacologically targeting Cdr1 in combination with azoles may be an effective strategy to control infection caused by azole-resistant isolates of *C. auris*.
The rise in antimicrobial resistance has become a major threat to public health. Although the focus has primarily been on pan-resistant bacteria, there is growing concern over the multidrug-resistant fungal pathogen, *Candida auris*. This emerging pathogen has galvanized researchers, health care workers, and the media due to its high rates of drug resistance and transmissibility. In its most recent report, the U.S. Centers for Disease Control and Prevention classified *C. auris* as one of the five pathogens that are the most urgent threat to public health. Thus, the emergence of *C. auris* highlights the need for more therapeutic options to combat drug-resistant fungal infections.

*C. auris* has an interesting history. Since it was first identified in 2009 in Japan, genomic analyses have revealed the near simultaneous emergence of distinct lineages across six continents, encompassing over 30 countries within the past 40 years. Currently, the majority of *C. auris* isolates fall into four major geographical clades: South Asian (I), East Asian (II), African (III), and South American (IV). This species has a remarkable ability to persist on human skin and other surfaces for extended periods of time, which facilitates hospital transmission amongst patients who are already vulnerable to infection. Additionally, the prevalence of drug resistance amongst *C. auris* isolates is widespread as recent studies show that over 80% of clinical isolates are resistant to the azole antifungal fluconazole. Resistance levels vary significantly between clades, with some isolates exhibiting resistance to all three major antifungal drug classes available to treat systemic infections.

The prevalence of fluconazole resistance amongst *C. auris* isolates is challenging from a clinical perspective as fluconazole is the most widely administered antifungal. This is due to its oral bioavailability, broad spectrum of activity, and favorable safety profile. Fluconazole inhibits the biosynthesis of ergosterol, the major sterol in fungal cell membranes, through inhibition of lanosterol dimethylase, which is encoded by *ERG11*. Inhibition leads to an increase in the Erg11 substrate lanosterol, and the production of aberrant sterol intermediates, including 14α-methyl-3,6-diole. Mechanisms of fluconazole resistance amongst *C. auris* isolates are highly variable and often clade specific, the nuances of which are still being elucidated. One major mechanism of fluconazole resistance involves point mutations in hot spot regions in its target gene *ERG11*, which are known to confer resistance in other fungi. In addition to *ERG11* mutations that are shared across all clades, the most common substitutions found in clade I and IV are *Erj1* and *Erj1* or *Erj1* and *Erj1* whilst clade III isolates commonly have an *Erj1* substitution. Notably, strains from clade II generally have no specific *ERG11* mutations and include the most sensitive isolates. In addition to target alteration, *C. auris* encodes an array of multidrug transporters, several of which are strongly induced under various conditions, including fluconazole treatment. Finally, *C. auris* isolates possess other genetic alterations that could confer fluconazole resistance, such as gene duplication leading to a higher copy number of *ERG11*, or transcriptional upregulation of efflux pumps through mutations in *TAC1B*. Overall, the diversity of *C. auris* resistance mechanisms is extensive, and the prevalence of fluconazole resistance threatens to render this important therapeutic obsolete in treatment of the rising number of *C. auris* infections worldwide.

A well-established strategy to thwart drug resistance and restore antimicrobial efficacy is the use of combination therapy, which has been successfully implemented for many difficult to treat infections, including HIV-AIDS, tuberculosis, and malaria. By identifying agents that re-sensitize pathogens to existing therapeutics, the lifespan of existing antifungals could be extended. In vitro data suggests combining existing antifungals can be effective against *C. auris*. An example of an antifungal potentiator is provided by iKIX1, a novel compound that inhibits interaction of the transcription factor Pdr1 with the Mediator complex in the fungal pathogen *Candida glabrata*, thus preventing upregulation of the multidrug transporter Pdr3. Combination treatment with iKIX1 and fluconazole abrogated intrinsic azole resistance and improved survival in a murine model of *C. glabrata* infection. Clearly, the inclusion of agents capable of impairing the most common, readily anticipated modes of antifungal resistance provides a rational, readily implemented strategy in the development of more efficacious combination treatment regimens.

In this study, we applied a combinatorial approach to screening of a chemically diverse library against an azole-resistant strain of *C. auris* to identify molecules that specifically enhanced the activity of fluconazole. We identified azofloxin as a compound that synergized with fluconazole by increasing intracellular fluconazole accumulation through inhibition of the major multidrug efflux transporter Cdr1. Using azofloxin as a chemical probe, we established that efflux is a major mechanism of resistance in isolates belonging to three of the four major *C. auris* clades. Notably, clade III isolates carrying specific mutations in *ERG11*, in addition to upregulating the multi-drug transporter Mdr1, remained resistant to fluconazole in the presence of azofloxin, despite the compound blocking efflux of Nile red and fluconazole in these isolates. Azofloxin showed cross-species activity by potentiating fluconazole activity against a resistant isolate of *Candida albicans*, the most common human fungal pathogen. In culture, azofloxin transformed fluconazole from ineffective to highly active in rescuing mammalian cells infected with drug-resistant *C. auris*. In mice infected with drug-resistant *C. auris*, azofloxin not only enhanced fluconazole activity but also reduced fungal burden by ~1000-fold as a single agent. Collectively, our findings demonstrate pharmacological inhibition of Cdr1 function may be an effective way to control infection with azole-resistant isolates of *C. auris*.

**Results**

**Chemical screen identifies azole-synergizing compound.** To identify novel compounds that enhance the activity of fluconazole against *C. auris*, we screened a diversity-oriented, synthetic library created by Boston University’s Center for Molecular Discovery (BU-CMD). This library of 2454 molecules, many natural product-inspired, has been curated to encompass greater structural complexity than conventional chemical libraries, which is a feature that increases the likelihood of identifying compounds with bioactivity against microorganisms. The BU-CMD library was screened at 50 μM in the absence or presence of a concentration of fluconazole that inhibited growth of the fluconazole-resistant clade I *C. auris* strain VPCI 673/P/12 by ~20%. Through Sanger sequencing we confirmed this strain harbored an *Erj1* or *Erj1* and *Tac1b* substitution. Compounds that reduced growth after 48 h compared to the control by 7-median absolute deviations from the median alone were classified as single agent antifungals; their mechanism of action has been described elsewhere. Compounds for which antifungal activity was only observed in combination with fluconazole were classified as fluconazole potentiators (Fig. 1a). Of the three fluconazole potentiators identified, we prioritized the bis-benzodioxoarylondolinone CMLD012336, a 3,3-diarylated oxindole hereafter referred to as azofloxin, due to its strong synergistic interaction with fluconazole against a resistant strain of *C. auris*, C66684 (Fig. 1b, c). C66684 harbors an *Erj1* substitution but not known activating substitutions in *Tac1b*
synergistic effect, a common mechanism involves one compound enhancing the biological effect of another agent by targeting parallel pathways or improving bioavailability. To investigate these possibilities for azofloxin, we profiled the sterol composition of C. auris with or without prior exposure to compounds. Our hypothesis was that if azofloxin heightens the effects of fluconazole-mediated Erg11 inhibition, a low concentration of fluconazole combined with azofloxin would have an equally profound impact on sterol composition as a high concentration of fluconazole alone. Using LC-MS, we evaluated how exposure of C. auris to a combination of azofloxin and fluconazole for 18 h affected the abundance of three membrane sterols: ergosterol, lanosterol, and the azole-induced aberrant sterol intermediate 14α-methyl-3,6-diol (Fig. 2a). Minimal changes in abundance of these sterols were detected between untreated and azofloxin-treated cells. Compared to untreated cells, fluconazole treatment resulted in significant 1.4-fold and 3.9-fold increases in ergosterol and lanosterol, respectively, and a larger >250-fold increase in 14α-methyl-3,6-diol (p < 0.05; Fig. 2a). This suggested that while exerting a minimal effect on growth, the low fluconazole concentration partially inhibited Erg11, causing a compensatory upregulation in ergosterol biosynthesis. However, azofloxin dramatically amplified the impact of the low fluconazole concentration on sterol composition. Most notable was a >1000-fold increase in 14α-methyl-3,6-diol compared to untreated cells (Fig. 2a). The relative abundance of all three sterols upon treatment with both azofloxin and a low concentration of fluconazole was similar to that seen in the sterol profile upon treatment of

Azofloxin enhances azole efficacy in a Cdr1-dependent manner. Of the many ways in which drug combinations can exert a synergistic effect, a common mechanism involves one compound enhancing the biological effect of another agent by targeting parallel pathways or improving bioavailability.
cells with a higher concentration of fluconazole alone, treatments that both resulted in ~50% growth inhibition (Fig. 2a).

To determine whether azofluxin enhances the effect of fluconazole treatment by increasing intracellular fluconazole abundance, we measured intracellular levels of fluconazole using LC-MS after 1 h of treatment. We detected significantly more intracellular fluconazole in the combination treatment group compared to treatment with either a high or low fluconazole concentration alone (p < 0.001; Fig. 2b). This amounted to a ~2.5-fold increase in intracellular fluconazole in the combination treatment compared to high fluconazole, despite the fact that these treatments result in similar changes to the sterol profile of the cell (Fig. 2a). This discrepancy is likely due to the variant time points at which these assays were performed. Furthermore, we were able to detect intracellular levels of azofluxin, which were increased in the combination treatment relative to azofluxin alone. These results indicate that this compound acts intracellularly and fluconazole enhances its accumulation, perhaps due to the disruption of membrane homeostasis by azoles (Fig. 2b). Overall, our LC-MS profiles suggest that azofluxin synergizes
Fig. 2 Azofluoxin increases intracellular accumulation of fluconazole (FLC) by inhibiting Cdr1-mediated efflux in C. auris. a Abundance of ergosterol (blue), lanosterol (red), and 14-α-methyl-3,6-diol (yellow) was determined in C. auris strain in competition treatment (● indicates concentrations used in combination treatment) relative to internal cholesterol standard. Growth inhibition (%) caused by each treatment is presented in table. Data are presented as mean ± SD of technical triplicates. Significance was determined by two-sided unpaired Student’s t test of condition compared to untreated; “p-value < 0.05”, “*p-value < 0.1”. Fold-change for each treatment is indicated above the respective bar. b Intracellular concentrations of FLC (green) and azofluoxin (gray) were measured after treatment for 1 h. Data are presented as mean ± SD of technical triplicates. Significance was determined by two-sided unpaired Student’s t test; “p-value < 0.05”, “*p-value < 0.01”, “**p-value < 0.001”. c Transcript levels of C. auris CDR1 (teal) and CDR4-1 (red) were measured. Cells were treated with indicated concentrations of compound (● indicates concentrations used in combination treatment). Transcript levels were normalized to ACT1 and GAPDH and are relative to the untreated control. Data are presented as mean ± SEM of technical triplicates. Significance of differences between untreated control and treatment was determined by two-sided unpaired Student’s t test; “p-value < 0.05”, “*p-value < 0.01”, “**p-value < 0.001”. Fold-change is indicated above each bar. d C. auris treated with azofluoxin, followed by addition of Nile red. Scale bar represents 5 μm. e Cells from Fig. 2d and Fig. S2 were analyzed by flow cytometry. Histograms depict relative fluorescence intensity (PE-A) of events, values depict median fluorescence intensity (MFI). Table displays mean fold-change in MFI of azofluoxin-treated, Nile red stained cells ± SD for biological triplicates. Significance of difference determined by a two-sided unpaired Student’s t test; “p-value < 0.05 compared to parental average MFI.” f Dose-response assays were conducted as in Fig. 1e. FLC was applied as a 2-fold dilution series in the absence or presence of azofluoxin (50 μM). Growth was monitored and normalized to no-drug control (see color bar). Source data are provided as a Source Data file.

with fluconazole by increasing the intracellular accumulation of fluconazole through an undetermined mechanism that we sought to define.

In pursuing mechanistic studies, we reasoned that an increase in intracellular azole accumulation could be caused either by enhancing permeability or by impeding drug efflux. To discriminate between these two models, we first tested the hypothesis that the increase in intracellular fluconazole caused by azofluoxin treatment resulted from impairment of multidrug efflux transporter activity. Impairment could be achieved by either a transcriptional mechanism that reduces the expression of genes encoding transporters or through a post-transcriptional mechanism. In order to evaluate potential transcriptional effects, we profiled the relative expression of six putative C. auris efflux genes, identified by Muñoz and coworkers, following treatment with azofluoxin, fluconazole, or a combination of the two compounds (Fig. S1b). Of the six transporter genes assessed, those encoding the putative ABC transporters Cdr1 (B9J08_000164) and Cdr4-1 (B9J08_000479) demonstrated similar expression profiles. At exposures to azofluoxin alone, which had no effect on growth, we saw a concentration-dependent increase in both CDR1 and CDR4-1 transcript levels, which was greater than the induction observed upon treatment with fluconazole (Figs. 2c and S1b). Furthermore, we observed a greater increase in CDR1 and CDR4-1 expression upon combination treatment than the increase seen with any individual compound treatment (Fig. 2c). The observation that azofluoxin causes an increase in transcript level of two efflux genes but increases intracellular accumulation of fluconazole, suggested a model in which azofluoxin directly inhibits efflux transporter function post-transcriptionally, resulting in the compensatory upregulation of efflux gene expression.

To test our model experimentally, we determined whether azofluoxin directly inhibited transporter function by monitoring accumulation within C. auris of the relatively promiscuous efflux pump substrate Nile red. Flow cytometry revealed an 18.7-fold increase in relative cell-associated Nile red signal caused by treatment with azofluoxin (50 μM; Fig. 2d, e). To determine if Cdr1 and/or Cdr4-1 were relevant targets of azofluoxin, we utilized a C. auris strain in which CDR1 had been deleted and also generated a CDR4-1 deletion strain. If azofluoxin acts by inhibiting the activity of either transporter, then deletion of that transporter should reduce or eliminate the increase in Nile red accumulation caused by azofluoxin treatment. Although deletion of these efflux genes did not completely block the increase in Nile red accumulation caused by azofluoxin treatment, the magnitude of the increase was significantly diminished (p < 0.05) in the cdr1Δ strain (Figs. 2e, S2, and S3a), implicating Cdr1 as a likely target of azofluoxin in C. auris. It is possible that other C. auris transporters are also targets of azofluoxin, as Nile red signal was still enhanced upon azofluoxin treatment in the absence of CDR1 or CDR4-1. However, Cdr4-1 is unlikely to be a relevant target given that loss of this transporter had no significant effect on the increase in Nile Red staining caused by azofluoxin.

Encouraged by the effects seen on Nile red as a model efflux substrate, we next assessed the functional relevance of Cdr1 for potentiating of fluconazole activity by azofluoxin. As would be expected if azofluoxin enhances fluconazole activity via inhibition of Cdr1, we found that deletion of CDR1 abolished the ability of azofluoxin to potentiate the antifungal activity of fluconazole (Fig. 2f). In dose-response assays, deletion of CDR1 reduced fluconazole MIC to that observed upon combination with azofluoxin in a wild-type background (Fig. 2f). In contrast, deletion of CDR4-1 did not alter fluconazole sensitivity nor the ability of azofluoxin to potentiate fluconazole (Fig. 2f). This finding fits with previous reports in C. albicans that implicate Cdr1 in azole efflux but not Cdr4-1, despite both being transcriptionally upregulated in response to fluconazole.

Given the extensive range of substrates that Cdr1 is reported to act on, we investigated whether azofluoxin potentiated the effects of other intracellularly acting compounds to the same extent as deletion of CDR1. Consistent with our model for its mode of action, azofluoxin sensitized C. auris to the compounds gecpinacin, cerulenin, and cycloheximide to the same extent as CDR1 deletion, with no further sensitization to these compounds observed by azofluoxin in the CDR1 null (Fig. 3). Although mechanistically diverse, these compounds all act intracellularly and are known to be Cdr1 efflux substrates. In contrast, azofluoxin had no impact on sensitivity to the extracellularly acting compounds caspofungin and amphotericin B (Fig. 3). Considering all these findings, we conclude that azofluoxin enhances the antifungal activity of intracellularly acting compounds, such as fluconazole, by inhibiting drug transporters, most notably Cdr1, in C. auris.

Azofluoxin is active against diverse C. auris strains. Given the extensive genetic diversity identified amongst different clades of C. auris, we investigated whether azofluoxin synergized with fluconazole against representative isolates from all four major clades. Intriguingly, when synergistic activity was assessed by checkerboard assay, azofluoxin potentiated fluconazole in multiple isolates from three of the four major clades. The clade III isolates from South Africa were the exception (Fig. 4). Clade III is generally distinguishable from the others by both a V125A and
F126L substitution in Erg117 and the absence of drug-resistance mutations in TAC1B, the transcriptional regulator of Cdr1 which are commonly found in clades I and IV21 (e.g. the A640V substitution in our screening strain VPCI 673/P/12). Examining whole genome sequences of 304 isolates representing each of the four major clades 7, identified a unique non-synonymous substitution, N647T, in the transcription factor domain of Mrr1 (B9J08_004061) in 49 of 51 clade III isolates. In C. albicans, Mrr1 is a transcription factor that controls the expression of the major facilitator superfamily (MFS) transporter Mdr1, which is involved in fluconazole efflux40,41. Indeed, when expression of MDR1 (B9J08_003981) was assessed in the clade III isolates B11221 and

Fig. 3 Azofluxin potentiates intracellular acting compounds against C. auris, to a similar degree as deletion of CDR1. Dose-response assays were conducted with a C. auris Ci6684 parental strain in the absence and presence of 25 µM azofluxin where indicated, as well as with a strain with the efflux pump gene CDR1 deleted. Indicated compounds were titrated in a 2-fold serial dilution. Growth was measured after 24 h in YPD as described in Fig. 1e (see color bar). Source data are provided as a Source Data file.

Fig. 4 Synergistic activity of azofluxin is clade specific. Checkerboard assays were performed fluconazole (FLC) and azofluxin as described in Fig. 1b with isolates from each major clade of C. auris. CDC identification number is followed by the clade number to which the isolate belongs. Relative growth was measured in YPD medium after 24 h using OD600 and normalized to a no-drug control well (see color bar). The FICI calculated for each checkerboard is shown in the top right of each plot, with values <0.5 indicating synergy, values >0.5 indicating no interaction, and N/A indicating an FICI that could not be calculated due to a lack of growth inhibition. Source data are provided as a Source Data file.

F126L substitution in Erg117 and the absence of drug-resistance mutations in TAC1B, the transcriptional regulator of Cdr1 which are commonly found in clades I and IV21 (e.g. the A640V substitution in our screening strain VPCI 673/P/12). Examining whole genome sequences of 304 isolates representing each of the four major clades7, identified a unique non-synonymous substitution, N647T, in the transcription factor domain of Mrr1 (B9J08_004061) in 49 of 51 clade III isolates. In C. albicans, Mrr1 is a transcription factor that controls the expression of the major facilitator superfamily (MFS) transporter Mdr1, which is involved in fluconazole efflux40,41. Indeed, when expression of MDR1 (B9J08_003981) was assessed in the clade III isolates B11221 and
Fig. 5 Azofluxin does not potentiate fluconazole (FLC) against most clade III isolates despite intracellular FLC accumulation and increased CDR1 expression.

a Relative transcript levels of MDR1 (B9J08_003981) and b relative transcript levels of CDR1 (B9J08_000164) were measured in clade I isolate Ci6684 (gray) and clade III isolates B11221 (red) and B11222 (blue) (∗ indicates concentrations of FLC and azofluxin used in combination (combo) treatment). Data are presented as mean ± SEM of technical triplicates. A two-sided unpaired Student’s t test was performed to evaluate significance of differences between Ci6684 and each clade III isolate ∗∗ p-value < 0.05, ∗∗∗ p-value < 0.01, and ∗∗∗∗ p-value < 0.001.

c Flow cytometry was used to measure the Nile red accumulation in C. auris clade III strains as described in Fig. 2e. Values in histogram plots depict median fluorescence intensity (MFI) and table shows the mean fold-change in MFI ± SD for biological triplicates.

d The relative intracellular azofluxin abundance and e the relative intracellular FLC abundance was quantified by LC-MS as described in Fig. 2b in Ci6684 (gray), B11221 (red), and B11222 (blue). Data are presented as mean ± SD of technical triplicates. Significance of differences between azofluxin and the combination treatment for each strain was determined by two-sided unpaired Student’s t test, ∗ p-value < 0.05, and ∗∗∗ p-value < 0.001 comparing.

f Checkerboard assay as described in Fig. 1b using parental clade III isolate B12037 and the strain with CDR1 deleted, in YPD medium. Relative growth was measured after 24 h as described in Fig. 1b (see color bar). The FICI for each checkerboard is shown as described in Fig. 4. Source data are provided as a Source Data file.
B11222, we observed a >6-fold increase in expression under nearly all conditions tested compared to the clade I screening strain C66684, suggesting that MDRI is constitutively upregulated in clade III isolates (Fig. 5a). In addition, despite the absence of known TAC1 activating mutations in clade III isolates, we observed increased expression of CDR1 in both clade III isolates relative to C66684 under all treatment conditions except for the drug combination (Figs. 5b and S3b). The lack of fluconazole potentiation by azofloxin in clade III isolates coupled with the observation that efflux pump expression is high in these strains suggested an efflux-independent fluconazole-resistance mechanism in strains B11221 and B11222. To probe whether azofloxin is able to inhibit drug efflux activity in clade III strains, cellular accumulation of Nile red, which is a substrate for both ABC and MFS efflux pumps, was measured in the absence and presence of compound. Treatment with azofloxin led to a 14.2-15-fold increase in Nile red accumulation in B11221 and B11222 (Fig. 5c), which was not significantly different in magnitude from the increase observed with the clade I screening isolate C66684 (Fig. 2e). Furthermore, LC-MS confirmed that azofloxin accumulated intracellularly in B11221 and B11222 (Fig. 5d), and levels of intracellular fluconazole were significantly increased by combination treatment compared to fluconazole alone (Fig. 5e). These results suggest the lack of fluconazole potentiation by azofloxin in clade III isolates is not due to an inability of the compound to inhibit efflux pumps, but rather that azole resistance in these isolates is not due solely to efflux.

To investigate whether the Erg11V125A/F126L and/or Mrr1N647T substitutions were likely responsible for resistance to the fluconazole-enhancing effects of azofloxin in clade III isolates, we assessed the activity of azofloxin against a clade III isolate (B12037) that does not contain the Erg11 substitutions or the Mrr1 substitution shared by most members of this clade. While more sensitive to fluconazole than other clade members at baseline, this strain showed increased Nile red accumulation upon treatment with azofloxin (Fig. 5c) and showed potent synergistic interaction between azofloxin and fluconazole (Fig. 5f). Furthermore, when CDR1 was deleted in this background, it abolished the synergistic activity (Fig. 5f), highlighting the importance of Cdr1 for azole tolerance of this clade III isolate. Together, our results suggest that substitutions in Erg11 and/or Mrr1 in B11222 and B11221 enable fluconazole resistance which is recalcitrant to the effects of azofloxin.

Azofloxin displays activity against azole-resistant C. albicans. Our studies so far supported a model in which azofloxin inhibits Cdr1-dependent fluconazole resistance in C. auris. Notably, our initial findings suggested this compound combination was ineffective against C. albicans, C. glabrata, or S. cerevisiae (Fig. 1e), indicating either species-specific differences in the manner by which azofloxin inhibits efflux pumps, or that efflux does not play a role in the azole sensitivity of the strains we tested. To learn whether azofloxin had activity against strains of C. albicans in which fluconazole resistance is mediated through enhanced efflux, we assessed activity of the azofloxin–fluconazole combination treatment against isolates from a patient who had received intermittent therapy with fluconazole over the course of 2 years. We observed no potentiation in the early clinical isolate, CaCi-2 (Fig. 6a), which is reported to have no bona fide resistance mutations, consistent with our finding of no potentiation in an azole-sensitive laboratory strain, SN95 (Fig. 1e).

Interestingly, azofloxin did potentiate fluconazole against the late clinical isolate, CaCi-17, which possesses the substitutions A736V in Tac1 and G947S in Mrr1 that lead to upregulation of multiple efflux genes, in addition to gain-of-function mutations in the transcription factor UPC2 that lead to overexpression of Erg11. The ability of azofloxin to potentiate fluconazole in CaCi-17 was abolished upon deletion of CDR1 (Fig. 6a), similar to what we observed in C. auris (Fig. 2f). Additionally, azofloxin was able to mildly potentiate fluconazole in three laboratory-generated C. albicans strains with verified gain-of-function mutations in TAC1, but not the sensitive parental strain (Fig. 6b).

To confirm that azofloxin inhibited efflux in C. albicans, we assessed Nile red accumulation in CaCi-17 by flow cytometry. We observed a similar increase in Nile red accumulation upon azofloxin treatment in both the parental CaCi-17 and the CaCi-17 cdr1Δ/cdr1Δ strain (Fig. 6c). This suggests that while azofloxin is able to inhibit efflux pumps in C. albicans, Cdr1 is either not the major Nile red transporter or other transporters are able to compensate upon its deletion. Finally, to confirm that combination treatment was blocking efflux and resulting in increased fluconazole accumulation, we used LC-MS to measure intracellular compound concentrations in SN95, which was replete with the potentiation effects of azofloxin (Fig. 1e), and compared combination treatment to that with each compound alone, we only detected a significant increase in fluconazole in CaCi-17 (Fig. 6d), while in both strains there was a significant increase in azofloxin (Fig. 6e). These data indicate that azofloxin blocks fluconazole efflux in a resistant clinical isolate of C. albicans, increasing its sensitivity to fluconazole, establishing bioactivity for azofloxin beyond C. auris.

Combination treatment reduces fungal burden in vivo. Encouraged by the intriguing mode of action we had uncovered, we next assessed the therapeutic potential of combining azofloxin with fluconazole. We first examined the ability of this combination to rescue human kidney-derived (293T) cells when infected with fungus. 293T cells constitutively expressing firefly luciferase as a reporter were either grown alone or in co-culture with C. auris C66684 under various treatment conditions. Luminescence was used as an indicator of relative viable human cell number. In the cases of solvent control, azofloxin alone, or low fluconazole, C. auris growth was unhindered, which resulted in near complete human cell loss and an absence of luminescent signal (Fig. 7a). However, with combination treatment (azofloxin and low fluconazole) we saw dramatic rescue of the human cells, comparable to that achieved with a 16-fold higher concentration of fluconazole alone (Fig. 7a). Notably, for 293T cells grown in the absence of C. auris we saw no significant change in luciferase signal under any treatment condition, indicating minimal cytotoxicity in vitro (Fig. 7a).

The same experimental design was performed in 24-well plate format followed by Periodic-acid Schiff (PAS) staining of polysaccharides to visualize effects of the various treatments on both the fungal and human elements within the co-cultures. Results supported findings obtained using the quantitative assay. We observed extensive damage to the human cell monolayer (stained pale purple) and sloughing in conjunction with the presence of abundant C. auris (stained pink) in wells exposed to azofloxin alone or low fluconazole alone, comparable to the untreated co-culture. With combination treatment, we observed an intact human cell monolayer which was similar to the no fungus control, and scant fungal burden. In the high fluconazole condition, some disruption of the human cell monolayer was evident and fungal cells were readily apparent, suggesting that high fluconazole was effective at reducing fungal toxicity to the monolayer, but less effective than our combination treatment in arresting fungal proliferation (Fig. 7b).
Justified by promising results in culture, the therapeutic potential of azofluxin was investigated in mice. First, we assessed the stability of azofluxin in mouse plasma by bioassay. Incubation of the compound in 100% mouse plasma for 1 h caused no decrease in azofluxin potentiating activity. Technical controls consisted of the experimental antifungal gepcinacin, which is readily inactivated by plasma and caspofungin, a stable clinical antifungal (Fig. 7c). Next, a single dose pharmacokinetic study was performed in mice to inform design of appropriate regimens to assess tolerability and efficacy. Following a 10-mg/kg bolus dose, the peak plasma concentrations of azofluxin achieved were well above those required forazole-potentiating activity in vitro (>1 µg/mL) and a half-life of ~ 2.6 h was defined (Fig. 7d). These findings encouraged us to proceed with a repeated-dose tolerability study which confirmed the absence of any physical signs of
acute systemic toxicity after 4 days of treatment and 100% survival after 21 days (Fig. 7e).

Finally, we evaluated the efficacy of azofluxin in a well-characterized mouse model of systemic C. auris infection. Immunocompromised mice were infected intravenously with theazole-resistant clade IV isolate B11801, which we had confirmed as susceptible to the fluconazole-potentiating effects of azofluxin (Fig. 8a). After four days of well-tolerated treatment, fluconazole alone reduced kidney colony forming units (CFU) compared to untreated mice, however, the addition of azofluxin significantly enhanced this activity (p-value < 0.001; Fig. 8b) compared to either treatment alone. Unexpectedly, azofluxin alone reduced fungal burden by \(-3\log_{10}\) CFU despite having shown no effect on C. auris growth in vitro (Fig. 8). This surprising result suggests that disruption of Cdr1 function by azofluxin impairs C. auris virulence in immunocompromised mice, as has been previously
Fig. 8 Azoffuxin increases the antifungal activity of fluconazole (FLC) in mice. a Checkerboard assays were performed as described in Fig. 1b with C. auris clade IV isolate B11801. Relative growth was measured after 24 h using OD₆₀₀ and normalized to no-drug control wells (see color bar). The FICI is shown in the top right of each plot, with values <0.5 indicating synergy. b Kidney fungal burden (CFU) in mice from each treatment group that had been infected with C. auris B11801. Input is the CFU recovered in an aliquot of the fungal suspension used to inoculate mice. All other values are the CFU recovered from kidney homogenates after 4 days of treatment. Fluconazole was administered at 32 mg/kg intraperitoneally twice daily and azoffuxin at 10 mg/kg subcutaneously four-times daily. Data are presented as mean ± SD of n = 3 mice per treatment group. Experiment was performed in two independent replicates (purple and blue). The significance of differences between combination treatment and treatment with each compound alone was determined for each replicate by two-sided unpaired Student’s t test, **-p-value < 0.01 and ***-p-value < 0.001. Source data are provided as a Source Data file.

Demonstrated using genetic approaches in isolates of C. glabrata⁵⁰. Overall, azoffuxin displays antifungal activity in vivo, further validating the concept of targeting resistance mechanisms in combination with current antifungals to combat C. auris infections.

Discussion
In this study, we leveraged a diversity-oriented chemical library to discover CMLD012336 (azoffuxin) as a new compound that enhances the susceptibility of resistant fungal pathogens to diverse intracellularly acting antifungals. This compound inhibits the activity of multidrug efflux transporters, most notably the ABC transporter Cdr1. Enhanced multidrug efflux is a frequently encountered and problematic mechanism of antimicrobial resistance⁴⁶,⁴⁷,⁴⁸,⁴⁹. With the escalating problem of antifungal resistance to public health, the ability of azoffuxin to inhibit drug efflux in a non-toxic manner could have potential therapeutic implications.

The 3,3-diarylated oxindole we named azoffuxin was identified as an unexpected side product in a Lewis-acid mediated Friedel-Crafts/Prins reaction process intended to generate spirocyclic oxindoles.⁵⁴. 3,3-diarylated oxindoles are a subset of the medicinally “privileged” 3,3-disubstituted oxindole class, which have a rich history of reported biological activities. For example, the diphenolic oxindole oxyphenisatin and other 3,3-diarylated oxindoles have been widely reported to inhibit the growth of diverse cancer cell lines, in many cases the activity being ascribed to inhibition of eIF2α-mediated translation initiation⁵⁵,⁵⁶. Another 3,3-diarylated oxindole BHPI, has been reported to act as a non-classical agonist of human estrogen receptor α and was recently shown to deplete intracellular ATP in estrogen receptor-positive cancer cells, thereby disrupting ATP-dependent ABC transporter-mediated drug efflux.⁵⁷. Whether BHPI might directly inhibit human or fungal ABC transporters is unknown. Other 3,3-bisaryl oxindoles have been reported as mineralocorticoid receptor antagonists⁵⁸, as well as antioxidants⁵⁹. Although azoffuxin falls into the general class of 3,3-disubstituted oxindoles, its specific substituents impart structurally distinct features to the compound. In addition to differences at the level of chemical structure, divergence in the processes reported to be impacted by other class members between mammalian and fungal cells, and azoffuxin’s lack of cytotoxicity to human cells and good tolerability in mice make it unlikely that azoffuxin operates through the mechanisms previously described for other 3,3-disubstituted oxindoles.

Using azoffuxin as a chemical probe, we found that C. auris strains belonging to clades I, II, and IV were sensitized to fluconazole, implicating efflux as a major factor contributing to their high azole-resistance. Indeed, inhibition of efflux in these strains markedly reduced their fluconazole resistance, despite the presence of Erg11 alterations, most notably Erg11Y132F and Erg11K143R that have been reported to cause a >4-fold increase in azole MIC.⁶⁰. Furthermore, azoffuxin reduced azole resistance of a C. albicans strain harboring numerous resistance-conferring mutations in genes such as ERG11, TAC1, MDR1, and UPC2.⁶¹.⁶². Deletion of CDR1 in specific strains of C. auris and C. albicans abolished azoffuxin-mediated potentiation. In contrast, we did not observe azoffuxin-mediated fluconazole potentiation in the more divergent fungal species C. glabrata and S. cerevisiae. One explanation would be that Pdr5, the homologous efflux pump in these species, is not inhibited by azoffuxin, which is plausible given the relatively low 56% sequence identity between Cdr1 and Pdr5.⁶³. Alternatively, efflux may not be a driving factor in determining the fluconazole sensitivity of the strains used as representatives of these species.

Within C. auris, we found that isolates from clade III were distinct in their lack of susceptibility to the fluconazole-sensitizing effect of azoffuxin. Clade III isolates often express an Erg11 variant with V125A/F126L substitutions and harbor a unique substitution in Mrr1 (N647T), which likely leads to upregulation of the MFS pump Mdr1. Unlike clade I and IV, this clade does not possess candidate drug-resistance substitutions in Tac1b. While we confirmed upregulation of MDR1 and CDR1 in clade III isolates compared to clade I, azoffuxin treatment of these strains still increased accumulation of fluconazole, and Nile red which is a substrate of both Mdr1 and Cdr1. The mechanism(s) underlying this observation is unclear but include the possibility that azoffuxin inhibits Mdr1 as well as Cdr1. Alternatively, inhibition of Cdr1 in these strains might be sufficient to increase compound accumulation despite Mdr1 upregulation, or azoffuxin is able to inhibit the function of yet other efflux pumps for which these compounds are also a substrate. Irrespective of their mechanism, our findings and the observation that azoffuxin synergized with fluconazole against an atypical clade III isolate that did not
carry the Erg11V125AF126L or Mrr1N647T substitutions suggest that one or more of these resistance-conferring mutations is responsible for the inability of azoloflouxin to potentiate fluconazole activity against most clade III isolates. In light of a previous report that Mdr1 does not play a role in fluconazole resistance of clade I isolates and our finding that azoloflouxin enhanced fluconazole accumulation in clade III isolates, it is likely that the impact of Mdr1 upregulation on azole resistance in clade III strains is negligible. Rather, the specific Erg11V125AF126L mutant target protein in most clade III strains is likely the dominant determinant of their fluconazole resistance and would explain the inability of an efflux pump inhibitor such as azoloflouxin to restore azole sensitivity. Efflux is regulated by complex and highly interconnected genetic circuitry. Recent analyses of S. cerevisiae genetic interaction networks show that perturbation through deletion of specific ABC transporter genes can paradoxically lead to an increase in azole resistance. This response was mediated, at least in part by ABC transporter genes which have previously been shown to enhance development and growth of S. cerevisiae. In an additional distinct challenge to the efficacy of azoloflouxin in mediating antifungal drug resistance across diverse fungal pathogens, From a therapeutic perspective, utilizing a chemical combination of which one compound targets an essential process and the other disables a major resistance mechanism provides an attractive strategy that has been explored for both antimicrobial and cancer treatments. In the case of efflux inhibitors, not only does this strategy enhance the efficacy of the other compound, but if applied early in the course of intervention, it can also reduce the rate at which resistance emerges. Despite the conceptual appeal, no efflux inhibitor combination therapies have proven effective in patients. This failure in clinical translation has largely been due to host toxicity, off target effects, and/or the poor pharmacokinetics that have plagued current efflux inhibitors such as verapamil, cyclosporin A, and valspodar. In one study, we found azoloflouxin to be very well tolerated both in culture and in mice. As well, azoloflouxin significantly enhanced fluconazole activity in an immunocompromised mouse model of invasive C. auris infection, and reduced fungal burden by ~1000-fold even as a single agent, consistent with the role of Cdr1 in virulence of C. glabrata. An additional distinct challenge to the efficacy of efflux inhibitors is the emergence of target-based and other resistance mechanisms that can render efflux a less important factor contributing to the overall resistance level of a fungal pathogen. Encouragingly, azoloflouxin remains able to sensitize azole-resistant Candida strains despite the presence of target-based resistance mechanisms. While overall results are encouraging, they are clearly discovery-phase in nature and will require further development for translation to clinical application. As the number of drug-resistant infections continues to rise, there remains a need to understand the relative contribution of different resistance mechanisms to the diminishing efficacy of our limited antifungal armamentarium and to design new resistance-evasive treatment strategies.

Methods

Strain construction. All strains, plasmids, and oligonucleotides used in this study are listed in Supplementary Information.

CauC6410: C. auris Cie6684 cdr4-Δcdn4-Δnat. The C. auris strain with CDR4-1 (B9198_000479) deletion was constructed using homologous recombination and an electroporation transformation approach. Approximately 1 kb of sequence homology upstream of CDR4-1 was amplified using primers OLCS164/OLCS165 and ~1 kb of homology downstream of CDR4-1 was amplified with OLCS166/OLCS167. The interior primer of each set contained 40 bp homology to a nourseothricin (NAT) resistance cassette (pLC1098), which was amplified with primers OLCS296/OLCS304. Using fusion PCR with nested primers OLCS168/OLCS169, the NAT cassette and CDR4-1 homology regions were combined into a single DNA fragment. This PCR product was ethanol precipitated. C. auris CauLC5083 cells were prepared by growing in 50 mL YPD medium to an OD600 of 1.6–2.2. Cells were pelleted for 5 min at 3000×g, then subcultured for 1 h in 10 mL 10 mM Tris-HCl (Biohop), and 1 mM EDTA (Biohop) in 1x TE buffer and 0.1 M Lithium Acetate (Sigma). In all, 250 µL of 1 M DTT was added to the culture for 30 min. Cells were washed twice in cold ddH2O followed by a wash in cold 1 M sorbitol (Biohop). In all, 3 µg of DNA was electroporated into 40 µL CauLC5083 cell suspension in a 2-mm electroporation cuvette (VWR) with the following settings on a BTX ECM830 electroporator: 1.8 kV, 200 μF, 25 μF and outgrown in YPD medium for 4 h at 30 °C. Transformants were plated on YPD plates containing 150 µg/mL NAT. Colonies were patched and genotyped for integration of the deletion construct (OLCS164/OLCS306 and OLCS274/OLCS167) and for the absence of the wild-type allele (OLCS052/OLCS053).

CauC5447: C. albicans CaGi-17 cdr1Δcdr1Δ. Both alleles of CDR1 (C3_05220W) were deleted by a transient CRISPR method. The guide construct was made of two components: The SN522 promoter amplified with the universal primer OLCS929 and guide specific primer OLCS966, and the guide scaffold and terminator amplified with the guide specific primer OLCS967 and universal primer OLCS927. The fusion construct was PCR amplified with the universal nested primers OLCS928/OLCS929. Repair template was digested from pLC1083 by Apal (NEB) and SacI (NEB). Gene deletion was verified by the absence of the CDR1 specific amplicon with OLCS968/OLCS969.

CauC5589: C. albicans SN152 TAC1/TAC1. Stepwise insertion of wild-type TAC1 back into the TAC1ΔTAC1Δ deletion mutant CalC4525 to match gain-of-function strains below. OLCS1096 was digested by BamHI (NEB) and SacI (NEB) and used to transform CalC4525. NAT resistant transformants was further triaged by a histidinase auxotrophy. Re-introduction of the TAC1 ORF was verified by the presence of the OLCS7041 and OLCS7042 ampicillin. Subsequently, pLC1092 was digested by BamHI and SacII and used in a second transformation. Transformants protrophic for histidine were further selected for NAT resistance.

CauC5591: C. albicans SN152 Tac1A2029G/Tac1A2031C (Tac1A2029G/Tac1A2031C) insertion. Stepwise insertion of a Tac1A2029G (Tac1A2031C) resistance marker from pLC1049, which was amplified with primers OLCS296/OLCS304. Repair template was digested from pLC1083 by Apal (NEB) and SacI (NEB). Gene deletion was verified by the absence of the Tac1A2029G/Tac1A2031C specific amplicon with OLCS968/OLCS969.

CauC5599: C. albicans SN152 Tac1A2502G/Tac1A2503A (Tac1A2502G/Tac1A2503A) deletion. Stepwise insertion of a WT Tac1A2502G/Tac1A2503A into the Tac1A2502G/Tac1A2503A deletion mutant CalC4525. OLCS1097 was digested by BamHI and SacII and used to transform CalC4525. NAT resistant transformants was further triaged by a histidinase auxotrophy. Re-introduction of the Tac1A2502G/Tac1A2503A ORF was verified by the presence of the OLCS7041 and OLCS7042 ampicillin. Subsequently, pLC1093 was digested by BamHI and SacII and used in a second transformation. Transformants protrophic for histidine were further selected for NAT resistance.

CauC5693: C. albicans SN152 Tac1 A977D/Tac1 A977D deletion. Stepwise insertion of a WT Tac1A977D/Tac1A977D into the Tac1A977D/Tac1A977D deletion mutant CalC4525. OLCS1098 was digested by BamHI and SacII and used to transform CalC4525. NAT resistant transformants was further triaged by a histidinase auxotrophy. Re-introduction of the Tac1A977D/Tac1A977D ORF was verified by the presence of the OLCS7041 and OLCS7042 ampicillin. Subsequently, pLC1094 was digested by BamHI and SacII and used in a second transformation. Transformants protrophic for histidine were further selected for NAT resistance.

CauC5695: C. albicans SN152 Tac1 A977D/Tac1 A977D deletion. Stepwise insertion of a WT Tac1A977D/Tac1A977D into the Tac1A977D/Tac1A977D deletion mutant CalC4525. OLCS1099 was digested by BamHI and SacII and used to transform CalC4525. NAT resistant transformants was further triaged by a histidinase auxotrophy. Re-introduction of the Tac1A977D/Tac1A977D ORF was verified by the presence of the OLCS7041 and OLCS7042 ampicillin. Subsequently, pLC1095 was digested by BamHI and SacII and used in a second transformation.
Transformants prototrophic for histidine were further selected for NAT resistance. CauLC6750: C. auris B12037 cdf1Δ::NAT. To delete CDF1 (B9J08_00164) in C. auris B12037 a homologous recombination and electroporation transformation approach was used35. Approximately 1 kb of sequence homology upstream of CDF1 was amplified using primers oLC62020/oLC6305 and ~1 kb of homology downstream was amplified with oLC6306/oLC6205. The interior primer of each set contained 40 bp homology to a narrowly specific (NAT) resistance marker from oLC6000, which was amplified with primers oLC6002/oLC6304. Using fusion PCR with nested primers oLC6204/oLC6307, the NAT cassette and CDF1 homology regions were combined into a single DNA fragment. This PCR product was ethanol precipitated, 5 µg of DNA was electroporated into CauLC6554, and transformants were plated on YPD plates containing 150 µg/mL NAT. Colonies were picked and grown for 3 days on NAT containing plates to verify integration of the deletion construct (oLC6221/oLC6308 and oLC274/oLC6023) and for the absence of the wild-type allele (oLC6231/oLC6169).

Statistics and reproducibility. All data presented in this study are derived from two biological experiments in which both results agreed, and data shown in the figures are from technical replicates from a single biological replicate, representative of both, unless otherwise stated. All statistical analysis was performed by a two-sided Student’s t test in Microsoft Excel (version 16.41) unless otherwise stated.

Culture conditions. All fungal strains were stored in 25% glycerol in YPD medium (YPD: 1% yeast extract, 2% peptone, and 2% D-glucose) and maintained at ~80 °C. Strains were grown in either YPD or RPMI medium (10.4 g/L RPMI-1640, 3.5% precipitated, 5°C274/oLC6023) and for the absence of the wild-type allele (oLC6231/oLC6169).

Statistical analysis. All data presented in the study were derived from two biological experiments in which both results agreed, and data shown in the figures are from technical replicates from a single biological replicate, representative of both, unless otherwise stated. All statistical analysis was performed by a two-sided Student’s t test in Microsoft Excel (version 16.41) unless otherwise stated.

Chemical synthesis

General methods. 1H NMR spectra were recorded at 400 or 500 MHz at ambient temperature unless otherwise stated. 13C NMR spectra were recorded at 100 or 125 MHz at ambient temperature unless otherwise stated. Chemical shifts are reported in parts per million (ppm) and referenced to 85% (w/w) D2O (400 MHz) or 99.96 ppm (1H) or 176 ppm (13C) for 500 MHz NMR. Metabolite concentrations were determined by the internal standard deuterium oxide method. 1H and 13C peaks were assigned using 1D and 2D NMR experiments. 1H-1H TOCSY and 1H-13C HSQC spectra were used for structural elucidation. NMR spectra were processed using MestReNova (version 10.4). NMR spectra were processed using MestReNova (version 10.4). The molecular mass of the synthesized compound was verified by MALDI-TOF MS. HRMS was performed by ESI-TOF-MS to determine the elemental composition of the synthesized compounds. The mass spectra were acquired using a Q-Exactive (Thermo Fisher Scientific) equipped with an ESI source.

Analytical methods. All strains were grown in either YPD or RPMI medium (10.4 g/L RPMI-1640, 3.5% precipitated, 5°C274/oLC6023) and for the absence of the wild-type allele (oLC6231/oLC6169). Genotyping for integration of the deletion construct (oLC6221/oLC6308 and oLC274/oLC6023) and for the absence of the wild-type allele (oLC6231/oLC6169).
Extraction and quantification of sterols. To quantify the abundance of steroids in C. auris the targeted metabolomics profiling protocol established by Hoepfner et al.13 was performed. Cells were washed with 0.1% in 1 mL of RPMI supplemented with the indicated compound concentration for 18 h with agitation. After incubation growth (OD_600) was normalized and cell pellets washed and resuspended in 100 μL of PBS. Cell suspension was treated with 1 mL of methanol/CHCl_3 (2.1 v/v) supplemented with 0.01% w/v butylated hydroxyanisole. The mixture was vortexed for 10 min. Samples were centrifuged for 5 min at 16,000 × g. The supernatant was collected and dried. For LC-MS analysis, samples were resuspended in ethanol with cholesterol included as an internal standard. Samples were separated on Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm) using the Acquity UPLC 1-C18 coupled to a Xevo G2-S QTOF equipped with an APCI source (Waters). Chromatographic methods were adopted from Hoepfner et al. as well as the selective reaction monitoring mass transitions specific for each sterol in subsequent quan tion steps. Targetlynx (Waters; version 4.1) was used for peak finding, smoothing and area calculations. All samples were run in biological duplicate and technical triplicate, and a representative replicate was plotted in GraphPad Prism (version 8.4.2).

Intracellular fluoronazole and azofloxin detection. C. auris was subcultured from overnight cultures at a starting OD_600 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 in 1 mL of methanol and pellet washed with PBS and resuspended in 100 μL of RPMI supplemented with 0.1% in 1 mL of RPMI supplemented with the indicated compound concentration for 18 h with agitation. After incubation growth (OD_600) was normalized and cell pellets washed and resuspended in 100 μL of PBS. Cell suspension was treated with 1 mL of methanol/CHCl_3 (2.1 v/v) supplemented with 0.01% w/v butylated hydroxyanisole. The mixture was vortexed for 10 min. Samples were centrifuged for 5 min at 16,000 × g. The supernatant was collected and dried. For LC-MS analysis, samples were resuspended in ethanol with cholesterol included as an internal standard. Samples were separated on Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm) using the Acquity UPLC 1-C18 coupled to a Xevo G2-S QTOF equipped with an APCLI source (Waters). Chromatographic methods were adopted from Hoepfner et al. as well as the selective reaction monitoring mass transitions specific for each sterol in subsequent quan tion steps. Targetlynx (Waters; version 4.1) was used for peak finding, smoothing and area calculations. All samples were run in biological duplicate and technical triplicate, and a representative replicate was plotted in GraphPad Prism (version 8.4.2).

Quantitative real-time-PCR. To determine changes in efflux gene expression, strains were subcultured from a saturated overnight culture at an OD_600 of 0.1 in YPD for 3 h at 30 °C with agitation. Cells were then harvested using 1 mL of methanol containing 0.15% formic acid, 3 mM Ammonium Acetate, and 37.5 ng/μL of the indicated primer pair. The mixture was vortexed, incubated for 10 min at 95 °C, and then incubated at 60 °C for 5 min. The mixture was then added to 90 μL of 293 T cells were seeded at 1 × 10^5 cells/mL in 24-well plates (Corning). In all, 2.5 × 10^5 exponential phase C. auris cells were added to mammalian cells followed by indicated drug or solvent concentrations. Cells were incubated for 48 h at 37 °C. Cultures were fixed with 4% formaldehyde (BioShop) in medium overnight, fixed was removed, and the plate was dried. Fixed cells were then washed with 1 mL ddH_2O and was removed from each well. In total, 1 mL hematoxylin was added and samples were incubated at 37 °C for 10 min. Cells were then thoroughly rinsed for 5 min with ddH_2O. In all, 1 mL hematoxylin was applied for 3 min and cell was rinsed again. Cells were allowed to dry and then were imaged. Experiments were performed in biological duplicate, with one representative image being shown.

Co-culture experiments. To assess the ability of azofloxin to rescue mammalian cell growth in co-culture experiments, 20 μL of 293 T cells were seeded at 1 × 10^5 cells/mL in DMEM media containing 10% FBS and incubated overnight at 37 °C in 5.5% CO_2. The following day 20 μL of DMEM inoculated with 2.5 × 10^5 cells/mL of the indicated compound was added to the wells. The compound was added to be used to DMSO-based compounds to each well at the indicated final concentrations. Co-cultures were incubated for 48 h at 37 °C in 5.5% CO_2. The mammalian cell growth was measured by replacing the media with 20 μL PBS, and adding 20 μL Titer-glow (Promega) to each well, incubating for 10 min, and recording luminescence on a Tecan Infinite M200 Pro. All experiments were performed in technical quadruplicate and biological duplicate and plotted in GraphPad Prism. The cellular glycoproteins were stained in co-culture experiments using a periodic-acid Schiff (PAS) staining kit (Sigma) as per manufacture’s instructions. Mammalian 293 T cells were seeded at 5 × 10^4 cells/well for 24 h in six-well plates (Corning). In all, 2.5 × 10^5 exponential phase C. auris cells were added to mammalian cells followed by indicated drug or solvent concentrations. Plates were incubated for 48 h at 37 °C. Cultures were fixed with 4% formaldehyde (BioShop) in medium overnight, fixative was removed, and the plate was dried. Fixed cells were then washed with 1 mL ddH_2O and was removed from each well. In total, 1 mL hematoxylin was added and samples were incubated at 37 °C for 10 min. Cells were then thoroughly rinsed for 5 min with ddH_2O. In all, 1 mL hematoxylin was applied for 3 min and cell was rinsed again. Cells were allowed to dry and then were imaged. Experiments were performed in biological duplicate, with one representative image being shown.

Mouse studies. The pharmacokinetics of azofloxin in mice were measured by the Preclinical Pharmacology Core at UT Southwestern Medical School. All animal work was approved and conducted under the oversight of the UT Southwestern Institutional Animal Care and Use Committee (IACUC) and the Guide for the Care and Use of Laboratory Animals when establishing animal research standards. Mice were maintained at 20.5 ± 2.2 °C, at 48–52% humidity, with light/dark alternating every 12 h. 21-week-old female CD-1 (Charles River) mice were dosed intraperitoneally (IP) with 10 mg/kg azofloxin. Azofloxin was formulated at 1 mg/mL in 25% PEG-400 (Sigma); 10% DMSO; 4% w/v Tween-90; 0.1% w/v N-benzylbenzamide (Sigma); and 50 μM N-benzylbenzamide. As an internal standard, data were analyzed using Analyst software (AB Sciex; version 1.6.1) A value 3x above the signal obtained in the blank plasma was designated as the limit of detection (LOD). The limit of quantitation (LOQ) was defined as the lowest concentration on the standard curve at which back calculation yielded a concentration within 20% of the theoretical value and above the LOD signal. The LOQ for azofloxin was 0.1 ng/ml. Pharmacokinetic properties were evaluated using the noncompartmental analysis tool in WinNonlin (Certara, Corp; Phoenix WinNonlin version 8.1). Sparse sampling was used for data analysis. Terminal half-life was calculated as the ln (2)/λz where λz is a first order rate constant associated with the terminal (log-linear)
The in vivo tolerability of azofloxin was determined in outbred ICR (CD-1) mice (Envigo). Animal toxicity studies were approved by the Institutional Animal Care and Use Committee of The Lundquist Institute for Biomedical Innovation at Harbor-UCLA Medical Center (#31413), according to the NIH guidelines for animal housing and care. Mice were maintained at 22.2 °C, at 45% humidity, with light/dark alternating every 12 h. Mice were monitored twice daily for signs of distress such as hunching, ruffled fur, weight loss, difficulty moving, or reduced drinking or eating to prevent and minimize unnecessary pain for 21 days.

The antifungal activity of azofloxin was assessed using a well-characterized model49. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin and William S. Middleton VA according to guidelines of the Animal Welfare Act (#DA0081), The Institute of Laboratory Animal Resources Guide for the Care and Use of Laboratory Animals, and Public Health Service Policy. Mice were maintained at 22.2 °C, at 45% humidity, with light/dark alternating every 12 h. Specific-pathogen-free, six-week-old female ICR (CD-1) mice weighing 25–27 g were used. Mice were monitored twice daily for signs of distress such as hunching, ruffled fur, weight loss, difficulty moving, or reduced drinking or eating to prevent and minimize unnecessary pain for 21 days.

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Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Flow cytometry data collected for this study and its analysis is included as Supplementary Data 1. Additional data that supports the findings of this study are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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**Author contributions**

K.R.I conceived the study, performed the majority of experiments, interpreted data, and wrote the initial manuscript. K.C. and R.T. characterized and synthesized the azoflufoxin compound. M.D.I. and S.M.P.E. in the lab of J.R.N. performed LC-MS experiments and interpretation. Z.L. constructed a strain and S.H.K. aided in screening the chemical compounds. S.S., A.S.I., and J.E. performed the murine pharmacokinetic experiment and analysis. N.R., L.W., and L.E.C. helped conceive the study, design the experiments, and interpret data. All authors provided comments in editing the manuscript.

**Competing interests**

L.E.C. and L.W. are co-founders and shareholders in Bright Angel Therapeutics, a platform company for development of novel antifungal therapeutics. L.E.C. is a consultant for small-molecule development company focused on leveraging the unique chemical properties of boron chemistry for crop protection and animal health. The remaining authors declare no competing interests.
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