Antifungal tolerance is a subpopulation effect distinct from resistance and is associated with persistent candidemia

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Tolerance to antifungal drug concentrations above the minimal inhibitory concentration (MIC) is rarely quantified, and current clinical recommendations suggest it should be ignored. Here, we quantify antifungal tolerance in Candida albicans isolates as the fraction of growth above the MIC, and find that it is distinct from susceptibility/resistance. Instead, tolerance is due to the slow growth of subpopulations of cells that overcome drug stress more efficiently than the rest of the population, and correlates inversely with intracellular drug accumulation. Many adjuvant drugs used in combination with fluconazole, a widely used fungistatic drug, reduce tolerance without affecting resistance. Accordingly, in an invertebrate infection model, adjuvant combination therapy is more effective than fluconazole in treating infections with highly tolerant isolates and does not affect infections with low tolerance isolates. Furthermore, isolates recovered from immunocompetent patients with persistent candidemia display higher tolerance than isolates readily cleared by fluconazole. Thus, tolerance correlates with, and may help predict, patient responses to fluconazole therapy.
A goal of antimicrobial susceptibility testing is to predict the clinical success or failure of antibiotic therapy. Some infections are recalcitrant to drug treatment due to "resistance", which refers to microbial growth in the presence of drug concentrations that inhibit susceptible isolates. Susceptibility is commonly measured as the minimal inhibitory concentration (MIC) after 24 h of growth in the presence of drug. Fungal infections generally follow the "90/60" rule for predicting therapeutic outcomes based on in vitro susceptibility testing: ~90% of susceptible isolates and ~60% of resistant isolates respond to therapy. This implies that infection outcomes are influenced by host factors as well as features of the pathogen that are not reflected by the MIC. In effect, organisms that cause persistent infections, defined as those that are not cleared by a course of antifungal treatment, have similar susceptibilities to organisms that are readily cleared by a course of antifungal treatment. Accordingly, it is important to identify measurable parameters that can contribute to disease severity.

Only four classes of antifungals are currently in clinical use, and resistance to azoles, including fluconazole (FLC), the most commonly administered antifungal against Candida species, is an increasing problem. Altered drug uptake or efflux and changes in ergosterol biosynthesis (the target for azole drugs) are the major known mechanisms of azole resistance. Stress responses have been proposed as a third mechanism of antifungal resistance. A broad range of small molecules enhance antifungal activity in vitro and in vivo, with inhibitors of Hsp90, calcineurin, and TOR the most prominent among them. Combination therapy using antifungals together with such inhibitors has been proposed as a promising strategy to extend the efficacy of current drugs. In addition, several psychotherapeutic agents such as fluoxetine, flufenazole (FNZ), or sertraline can enhance FLC activity against fungal species. Whether such adjuvants affect therapeutic outcomes remains to be addressed.

Persistent candidemia, defined as the failure to clear a bloodstream infection caused by a susceptible organism, is associated with increased mortality. In one study, the mortality rate was 54% among infections with persistent candidemia and only 3% among those with non-persistent (NP) candidemia. Mechanisms underlying persistent candidemia may include variability in the pharmacology of the drug, suboptimal dosing, presence of fungal biofilms on indwelling catheters, and reduced immunity. We posit that some responses to the drug are not captured by measuring the MIC alone and that additional parameters could be used to predict the likelihood that a clinical isolate might respond poorly to antifungal drugs. Furthermore, understanding the mechanisms that underlie these parameters is critical for the development of new therapeutic approaches against persistent Candida infections.

MIC measurements have been optimized to minimize or ignore residual fungal growth, termed "tolerance" or "trailing growth", which has been discussed in the literature for over 20 years and is detected in 25–60% of clinical isolates. This recommendation is based upon studies of acute infections using the mouse model of bloodstream candidiasis, and the observation that isolates with high trailing growth in mucosal infections respond positively to short-term antifungal treatment, despite later recurrence of infection. Trailing growth is sensitive to environmental conditions, including pH, temperature, and nutrients.Definitions of "tolerance" vary and generally describe survival or growth above inhibitory concentrations, detected as slow growth within the zone of inhibition using E-strips or disk diffusion assays, or in broth microdilution assays. Tolerance can be affected by several adjuvant drugs, iron levels, and genes involved in vacuolar protein sorting, as well as calcium flux. However, the precise relationship between the outcome of fungal infections and tolerance or trailing growth has not been determined. Because tolerant cells continue to divide in the presence of antifungals, we postulated that they contribute to the persistence and/or recurrence of fungal infections.

Here, we measure susceptibility and tolerance in a broad range of clinical isolates spanning the major Candida albicans clades, and ask how these parameters influence fungal infection outcomes. We analyze disk diffusion assays using disk-ImageR, and quantify the radius (RAD) of the zone of inhibition, a parameter that relates to the MIC, and the fraction of growth (FoG) within the zone of inhibition, a parameter that measures tolerance. We found that a range of adjuvant drugs, used in combination with FLC, increased drug cidality by reducing FoG and not MIC and are effective both in vitro and in vivo at inhibiting strains with high (and not low) FoG levels. Finally, highly tolerant isolates are associated with persistent candidemia, suggesting that knowing the tolerance level of an infecting isolate may have important clinical implications and may inform treatment options.

Results

Tolerance measured as FoG is distinct from drug resistance. We quantified drug responses in C. albicans isolates from different genetic backgrounds and types of infections using disk-ImageR, a quantitative analysis tool that measures RAD of the zone of inhibition, an indicator of susceptibility that relates to MIC, and FoG within the zone of inhibition, relative to the maximum possible growth. A screen of 219 clinical isolates revealed that FoG levels ranged widely from 0.10 to 0.85 and did not correlate with RAD levels, indicating that FoG and RAD measure independent drug responses. FoG was detected in response to other drugs including fungistatic antifungals (azoles, 5-fluorocytosine) and, to a lesser degree, to fungicidal agents (echinocandins, polyenes). Antifungal responses varied widely in different Candida species as well as in Saccharomyces cerevisiae. For example, Candida glabrata exhibited the highest tolerance to azoles, followed by Candida tropicalis and Candida krusei, while S. cerevisiae had low tolerance to azoles and intermediate tolerance to echinocandins and polyenes.

Broth microdilution assays define the MIC50—the lowest drug concentration that inhibits 50% of growth at 24 h (termed MIC throughout the manuscript). Most reports of "trailing growth" or "tolerance" monitor growth at 48 h. We quantified super-MIC growth (SMG) at 48 h (average growth per well above the MIC50 normalized to growth without FLC; Fig. 1d). Most isolates exhibited some level of SMG at 48 h, while MIC levels remained unchanged between 24 and 48 h. Thus, SMG provides a parameter that, like FoG, is distinct from MIC.

Importantly, cells exhibiting FoG in disk assays or SMG in broth assays are not due to the emergence of drug resistance. Thus, for a given isolate, FoG and SMG levels were reproducible for cells taken from inside or outside the zone of inhibition, or for cells taken from wells above or below the MIC (Supplementary Fig. 2a, b). These cells yield progeny indistinguishable from other cells in the population, and thus are the result of phenotypic heterogeneity rather than genetic alteration. Consequently, tolerance is due to growth heterogeneity in the population and is stable for a given isolate.

While SMG and FoG correlated well with each other ($R^2 = 0.82$, $P < 0.01$), there was no relationship between FoG and RAD ($R^2 < 0.01$), or SMG and MIC ($R^2 = 0.25$, $P < 0.01$, Supplementary Fig. 2c). FoG and SMG therefore reflect similar features of growth at supra-MIC concentrations that are distinct from drug
**Fig. 1** Measuring drug responses in disk diffusion assays (DDAs) and in liquid broth microdilution assays (BMDAs). **a** diskImageR analysis measures pixel intensity corresponding to cell density, along 72 radii every 5°. The average radius (RAD) represents the distance in mm corresponding to the point where 20%, 50%, or 80% growth reduction occurs (light, medium, dark blue dots). The fraction of growth inside the zone of inhibition (FoG) is the area under the curve (red) at the RAD threshold, divided by the maximum area. **b** Range of FoG levels in 219 *C. albicans* clinical isolates. Red, blue, and green lines estimate high, medium, and low FoG levels, respectively. Unless otherwise specified, DDAs were performed using a single 25 µg FLC disk and analyzed after 48 h at 30 °C. **c** Comparison of FoG20 and RAD20 for the 219 isolates. **d** Illustration of MIC and supra-MIC growth (SMG) calculations. MIC50 was calculated at 24 h as the FLC concentration at which 50% of the growth was inhibited, relative to growth in the absence of drug. SMG was calculated as the average growth per well above the MIC divided by the level of growth without drug. FLC was used in two-fold dilutions (0, 0.125 to 128 µg/ml). **e** Heatmaps illustrating OD600 levels for concentrations below the MIC (yellow bar) in cyan and above the MIC in yellow for the isolates from Fig. 1c. Maps show OD600 values at 24 and 48 h. **f** Effect of incubation time on RAD/MIC and FoG/SMG values. diskImageR analysis (f) and corresponding MIC and SMG levels (g) measured at 24 and 48 h for strains as in d. Of note, for truly drug-resistant strains such as T101 (MIC = 64, g), the small zone of inhibition makes FoG measurements less accurate than SMG levels. **h** RAD is concentration-dependent and FoG is concentration-independent as illustrated for strain SC5314 exposed to disks containing increasing concentrations of FLC (25, 50 and 300 µg) (left); RAD (middle) and FoG (right) levels for strains as listed. For all panels, n ≥ 2; **P < 0.01; *P < 0.05**
susceptibility/resistance, as measured by RAD or MIC, which are concentration-dependent parameters. Accordingly, whereas RAD increased with increasing drug concentrations in the disk (Fig. 1h), FoG remained similar irrespective of drug concentration. Similarly, SMG levels remained constant across a broad range of supra-MIC concentrations (Fig. 1e). Thus, tolerance measured as FoG or SMG is not dependent upon drug concentration, highlighting the distinctive nature of tolerance relative to susceptibility/resistance.

Environmental modulation of drug responses. FoG and SMG levels were reduced at pH 4.5 relative to pH 7, while RAD/MIC levels remained relatively stable (Supplementary Fig. 3a, b). FoG/SMG values also decreased with higher temperatures (37–41 °C), while RAD/MIC levels showed less variation with increasing temperature (Supplementary Fig. 3c, d). For some strains, including SC5314, RAD and/or FoG levels varied considerably depending on the growth media (Supplementary Fig. 3e). A series of strains derived from SC5314 by passaging were particularly sensitive to media differences, with lower RAD and higher FoG levels on rich medium (YPD) than on casitone medium (Supplementary Fig. 3f), emphasizing that assays must be performed under consistent conditions to ensure reproducible results. Importantly, differences in genetic background can have a major effect on how C. albicans strains respond to environmental conditions.

Subpopulation growth dynamics. To ask if tolerance reflects the size of a dividing subpopulation at supra-MIC concentrations, we compared the number of cells that form colonies (colony-forming units (CFUs)) in the presence of supra-MIC FLC concentrations to total CFUs without drug. CFUs on drug ranged in size of a dividing subpopulation at supra-MIC concentrations, we estimated cell viability in the presence of supra-MIC FLC in liquid cultures did not change with inoculum size (Supplementary Fig. 3g), indicating that FoG/SMG is independent of cell density. Importantly, these results establish that FoG levels represent the proportion of the population that can form colonies or grow above the MIC.

Growth dynamics were also monitored using microcolony assays, in which time-lapse microscopy follows colony area/size (Fig. 2b)34. As expected, exponentially growing cells plated on 10 µg/ml FLC did not stop growing immediately. Rather, most cells continued dividing for ~5 h, and then slowed or stopped dividing to different degrees (Fig. 2c, d and Supplementary Movies 1–4). In high FoG isolates (e.g., SC5314), a large proportion of the cells yielded microcolonies, while in low FoG isolates (e.g., P87), more cells stopped dividing (black dots in Fig. 2b). Differences in growth rates became apparent after 10–15 h in FLC (Fig. 2d), supporting the idea that different subpopulations have different growth dynamics, and that high FoG strains produce a larger subpopulation of growing colonies than low FoG isolates.

The dynamics of colony growth on agar plates was also examined using ScanLag, a flattened scanner/image analysis pipeline35 that we adapted for use with C. albicans. Here, the area occupied by light pixels and the change in this area over time are proxies for colony size and growth rate, respectively35. Different C. albicans isolates exhibited different initial time of colony appearance (ToA) on medium without drug (Supplementary Fig. 4). At 10 µg/ml FLC, the ToA was delayed relative to the drug-free condition (except for resistant strains such as T101, Fig. 2e, f). Notably, the ΔToA (ToA on FLC – ToA without FLC) of high FoG isolates was shorter than that of low FoG isolates (Fig. 2f), and correlated with overall FoG levels (Fig. 2g). This suggests that high FoG strains overcome the inhibitory pressures of the antifungal more efficiently than low FoG strains. Consistent with this, in liquid assays with drug, highly tolerant isolates also displayed shorter lag times than related isolates with low tolerance levels (Supplementary Fig. 5a).

A number of C. albicans growth parameters did not correlate with FoG levels. These include cell viability in the presence of drug (Fig. 2h, Supplementary Note 1 and Supplementary Table 1), consistent with FLC being fungistatic rather than fungicidal. Unlike tolerant bacteria, which display reduced growth rates and longer colony appearance times1,36, antifungal tolerance did not correlate with slower growth in three sets of related C. albicans strains grown in liquid without drug, including: (1) passaged derivatives of SC5314 with altered FoG levels, (2) a slow-growing clinical isolate (P37005) and faster growing derivatives37 and (3) a cib4 mutant38 with delayed cell cycle progression relative to control strains (Supplementary Fig. 5b). Furthermore, exponential and stationary phases yielded similar FoG levels (Supplementary Fig. 5c), unlike phenotypic drug resistance in bacteria39. Growth parameters (colony size and growth rate, liquid growth rate, and lag phase duration) also failed to show any correlation with FoG levels (Supplementary Fig. 5d, e). This implies that tolerance is not simply a reflection of faster growth in the presence or absence of drug. Rather, it reflects two aspects of C. albicans growth dynamics: a larger subpopulation able to form colonies at supra-MIC drug concentrations (Fig. 2a), and a faster relative recovery in response to fungistatic pressure.

Decreased intracellular drug levels underlie increased tolerance. A recently developed fluorescent azole probe, FLC-Cy540, provides a powerful tool to monitor intracellular drug levels and uptake rates using flow cytometry (Fig. 3a). The initial rate of FLC-Cy5 uptake per cell varied between strains and showed a weak inverse correlation with FoG levels ($R^2 = 0.64$, $P = 0.12$, Fig. 3b). By contrast, steady-state FLC-Cy5 levels at 24 h correlated inversely with FoG levels ($R^2 = 0.8$, $P < 0.01$, Fig. 3c). Cells were categorized as having low, mid, and high levels of FLC-Cy5 (at 24 h). FoG levels inversely correlated with the proportion of cells that contained high FLC-Cy5 levels ($R^2 = 0.82$, $P = 0.04$) and positively correlated with the proportion of cells containing mid FLC-Cy5 levels ($R^2 = 0.81$, $P = 0.05$, Fig. 3d, e).

Based on the assumption that the steady-state drug levels are influenced by both drug efflux and uptake, we measured efflux of Rhodamine 6G (R6G), a dye removed primarily via the ABC class of transporters41. In general, the rate of R6G efflux was higher in the presence, than in the absence, of FLC, with SC5314 as the notable exception (Fig. 3f and Supplementary Fig. 6a). Yet, efflux rates did not correlate with FoG under any of the conditions tested (Supplementary Fig. 6b, $R^2 < 0.2$). Thus, the determinants of steady-state intracellular FLC levels are complex, and include uptake and efflux rates, as well as other factors (Fig. 3g).

Adjuvant FLC combinations clear tolerance and do not alter resistance. Combination therapies have the potential to extend the lifespan of the few available antifungal drug classes12. We tested a series of adjuvant drugs to determine their quantitative effect on fungal responses when used together with FLC. Most adjuvants significantly reduced FoG. This includes geldanamycin and radicicol, which inhibit heat shock protein 90 (Hsp90) activity13,42, cycloporsone A and FK506, which inhibit calcineurin42,43; and staurosporine, which inhibits protein kinase C1 (PKc1) activity44. In addition, other adjuvants not directly connected to Hsp90 function also reduced FoG, including aur-ebosidin A, which inhibits sphingolipid biosynthesis45 and rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR) signaling pathway46; tunicamycin, an inducer of the
**Fig. 2** Analysis of cells growing at supra-MIC concentrations. All tested strains, except for T101 (MIC = 64 µg/ml), had FLC MIC values below 10 µg/ml. 

a. FoG correlates with the proportion of colonies that grow on 10 µg/ml FLC relative to growth on plates without drug. Black dots are additional strains from Fig. 1c.

b. Microcolony analysis at supra-MIC concentrations of FLC (10 µg/ml). Symbols indicate cells that have stopped dividing in the presence of drug over 24 h, scale bars = 100 µm. On average, strain AM2 formed fewer microcolonies than strain SC5314, but these were larger than those formed by SC5314. Time-lapse videos are available as Supplementary Movies 1–4.

c. Growth analysis of cells growing at supra-MIC FLC (10 µg/ml) during 0–5 h (c) and 10–15 h (d).

d. schematic of ScanLag analysis that measures time of colony appearance (ToA), colony growth rate, and colony size using desktop scanners.

**e.** ToA on medium without drug (blue) or with 10 µg/ml FLC (red) for resistant isolate T101, and isolates with different FoG levels. Graphs show the number of colonies (y-axis) at each time point (x-axis). Additional isolates are included in Supplementary Fig. 4.

f. Correlation between FoG and the difference (Δ) in the ToA of colonies in the presence vs. absence of FLC (ΔToA = ToA with FLC - ToA without FLC). Black dots are additional strains from Fig. 1c.

h. Cells that grow within the zone of inhibition are viable, as seen by replica plating of disk diffusion assays grown on casitone without FLC and incubated at 30 °C for 48 h. For all panels, n ≥ 2; ***P < 0.001; error bars denote standard deviations.
unfolded protein response pathway,47,48 fluoxetine, a serotonin inhibitor,6 and FNZ, an antipsychotic drug that stimulates ABC transporter expression and indirectly inhibits calcineurin via calmodulin14,50. FLC + adjuvant cleared tolerance for all seven clinical isolates tested (Fig. 4a, b), as well as for a longitudinal series from a single HIV patient51 that had acquired resistance over time, and for a set of persistent and NP clinical isolates (Supplementary Fig. 7a–d). Importantly, most adjuvants had little or no effect on RAD/MIC levels (Fig. 4a, b and Supplementary Fig. 7a–d), and thus are not expected to affect resistance in standard susceptibility assays52.

Hsp90-dependent responses affected tolerance and not susceptibility/resistance measured as MIC (at 24 h). Consistent with this, the effects of temperature and Hsp90 inhibitors correlated well with FoG (R² = 0.93, Supplementary Fig. 7e) and only partially with RAD (R² = −0.61, Supplementary Fig. 7f). Thus, temperature inhibition of Hsp90 affects tolerance and not resistance per se. Furthermore, FoG levels were not affected by Hsp90 steady-state protein levels, as the amount of Hsp90 did not correlate with FoG (R² = 0.07, Supplementary Fig. 7g). Because inhibitors of calcineurin and Hsp90 are known to affect FLC cidality10,12,13,28,32, we asked whether other adjuvant drugs have a similar effect. All tested combinations were cidal (Fig. 4c and Supplementary Fig. 7f), indicating they have a profound effect on cell viability at supra-MIC FLC concentrations and implying that stress pathways make essential contributions to tolerance.

Consistent with the adjuvant drug responses, genes involved in several pathways, including calcineurin53 and PKC signaling44,
primarily affected FoG and not RAD (Fig. 4d, Supplementary Fig. 8, detailed information on genes tested in Supplementary Notes 2–4 and Supplementary Tables 2 and 3). UPC2, which encodes a major transcriptional regulator of ergosterol biosynthesis, affected both RAD and FoG levels significantly, while two regulators of efflux pump transcription had no significant effect, consistent with the redundancy of efflux mechanisms (Fig. 4e). In addition, IRO1 (Supplementary Note 2), which is involved in iron homeostasis, and VPS21, which is involved in vacuolar trafficking, affected FoG and not RAD. Thus, many genetic pathways affect the ability of subpopulations of cells to grow in the presence of supra-MIC...
FLC concentrations, including Hsp90-dependent and Hsp90-independent responses that are due to tolerance rather than resistance.

**Adjuvant drugs improve FLC efficacy against high FoG strains in vivo.** Given that the beneficial effects of adjuvant drugs are more evident on high FoG strains, we asked if adjuvant therapy would distinguish between levels of tolerance in an infection model. *Galleria mellonella* larvae were used to test potential in vivo differences between isolates with different tolerance levels, both with respect to their ability to cause disease and with their ability to respond to therapy with FLC alone or FLC plus the adjuvant FNZ (Fig. 4a). A series of isolates was derived by passing the SC5314 strain in the presence or absence of FLC. The resulting isolates had indistinguishable RAD but distinct FoG levels relative to the parental strain (Supplementary Fig. 9a and Supplementary Data 1).

*G. mellonella* larvae infected with fungal cells were treated with either phosphate-buffered saline (PBS) (control), FLC, or combination therapy (FLC and FNZ) (Fig. 5a). SC5314 and derived strains with similar FoG levels killed all larvae within 9–14 days. Importantly, these lower FoG isolates had similar responses to FLC and to the FLC + FNZ combination: they rescued up to 17% of the infected larvae (Fig. 5b, c and Supplementary Fig. 9b). By contrast, high FoG strains displayed delayed killing and decreased virulence in the larvae, and did not respond to FLC alone (Fig. 5d and Supplementary Fig. 9b). Notably, the FLC + FNZ combination significantly improved the outcomes for high FoG strains relative to FLC alone and PBS-control groups: larval death was delayed and up to 50% of larvae infected with high FoG strains survived the experiment (Fig. 5d and Supplementary Fig. 9b). Similar results were obtained with a low FoG clinical isolate (readily cleared by FLC therapy) when compared with a high FoG isolate (that persisted in the bloodstream during multiple rounds of FLC therapy) (Supplementary Fig. 9c). Presumably, FLC + FNZ significantly decreased larval killing by rendering FLC cidal for those cells growing at supra-MIC concentrations. Thus, in vivo responses to azoles and to combination therapies differed for different isolates according to their tolerance levels.

**Persistent candidemia is associated with highly tolerant isolates.** Echinocandins are considered the optimal line of therapy for patients with candidemia, yet FLC remains a frontline drug for systemic *C. albicans* infections, especially in low income countries. Treatment with FLC often fails despite isolates being drug susceptible when tested in vitro, and rates of persistent candidemia are often higher in patients treated with FLC rather than with echinocandins. To examine possible connections between susceptibility and tolerance levels, we collected sets of clinical isolates from patients that were either efficiently cleared by a single course of FLC treatment (NP) or that persisted in the host despite extended FLC therapy (Fig. 6a, Supplementary Fig. 10a, see detailed isolate description in Methods and Supplementary Data 2).

The drug responses of persistent and NP isolates and several control strains displayed similar RAD and MIC levels (0.25–1 µg/ml), well below those of resistant strains (clinical MIC breakpoint = 4 µg/ml), when assayed at both 24 and 48 h (Fig. 6b and Supplementary Fig. 10b, c). Strikingly, FoG/SMG levels differed significantly between the two groups of clinical isolates, especially when compared at 48 h (Fig. 6b, c) and, as expected, correlated well with one another (Supplementary Fig. 10d, R² = 0.62). Importantly, persistent candidemia isolates displayed higher FoG/SMG levels than those readily cleared upon FLC treatment. In fact, many persistent isolates had FoG levels higher than 0.5, indicating that over half of the cells in the population could grow, albeit slowly, in the presence of FLC (Fig. 6b). Taken together, these experiments demonstrate that tolerance is an intrinsic property distinct from drug susceptibility, and that tolerance levels correlate with infections that resist azole treatment in the clinic.

**Discussion**

Currently, decisions about therapeutic strategies to treat *Candida* infections are based upon patient status, infecting species, and antifungal susceptibility, with clinical MIC assays designed to avoid the detection of tolerance. Here, quantification and characterization of tolerance across *C. albicans* isolates found that many of them exhibit tolerance, defined as the ability of a subpopulation of cells to grow slowly at supra-MIC concentrations. Tolerance is related to phenomena previously described as “trailing growth” or “slow killing,” and correlates with intracellular drug levels; (4) dependent upon stress response pathways; and (5) mechanistically distinct from resistance. Several adjuvants completely cleared FoG without altering susceptibility (MIC) and, when combined with the fungistatic drug FLC, yielded a fungicidal cocktail. Consistent with this, combination therapy was most effective in vivo at treating infections by high tolerance strains, whereas the adjuvant did not improve FLC efficacy against low FoG isolates. Furthermore, *C. albicans* isolates that cause persistent candidemia exhibited significantly higher tolerance than isolates readily cleared by FLC. Thus, quantitatively measuring tolerance levels of infecting isolates may provide important prognostic insights concerning both the success of FLC therapy and the potential efficacy of combination therapies.

The subpopulation effect of tolerance is readily detected on agar plate assays, where individual cells and their clonal progeny are evident; in liquid assays, cells are mixed and subpopulation differences relative to corresponding parental strains. For all panels, n ≥ 2; ***P < 0.001; **P < 0.01; *P < 0.05, error bars denote standard deviations.
effects are more difficult to distinguish. Growth dynamics in colonies, microcolony formation, and liquid growth assays all suggest tolerance correlates with the degree of growth after drug exposure. This implies that a subpopulation of cells is inherently more able to adapt to the drug than the rest of the population. We do not rule out the possibility that aneuploidy could contribute to tolerance in some strains; however, isolates analyzed here include those with euploid genomes yet a wide range of tolerance levels. It is therefore unlikely that aneuploidy represents the main mechanism underlying tolerance. Moreover, the fact that tolerance occurs in a subpopulation of cells and that the size of this subpopulation is retained when cells are retested also argues against aneuploidy being a major driver of tolerance. Since the size of such subpopulations is stable (FoG levels do not change with extended passaging in the absence of FLC) tolerance is predicted to be a function of cell physiology and environmental responsiveness that differs between genetic backgrounds and growth conditions.

We show that isolates with higher tolerance levels have more cells with lower intracellular drug levels, but that tolerance is not a function of either uptake or efflux rates, despite the well-known role of efflux in drug resistance. SC5314-derived high FoG isolates also appeared to be less virulent than isogenic low FoG strains when no drugs were administered (Fig. 5c, d and Supplementary Fig. 9b), although this correlation was not evident for the non-isogenic isolates and was not determined. SC5314-derived high FoG isolates also appeared to be less virulent than isogenic low FoG strains when no drugs were administered (Fig. 5c, d and Supplementary Fig. 9b), although this correlation was not evident for the non-isogenic isolates and was not determined. SC5314-derived high FoG isolates also appeared to be less virulent than isogenic low FoG strains when no drugs were administered (Fig. 5c, d and Supplementary Fig. 9b), although this correlation was not evident for the non-isogenic isolates and was not determined.

A novel insight from this work is that inhibitors of cellular stress pathways improve FLC efficacy primarily via their effect on tolerance and not resistance. This has two important implications. First, the subpopulation nature of tolerance suggests that these stress pathways must exhibit cell-to-cell variation. Second, the contributions of stress response pathways differ considerably between isolates, albeit in a manner that maintains population heterogeneity (and the size of the tolerant subpopulation) as a heritable feature of the given strain. Tolerance is clearly related to the Hsp90-dependent response and distinct from bona fide resistance. Hsp90 and calcineurin are well known to affect fungal drug responses, including the cidality of azoles, drug resistance, and calcineurin are well known to affect fungal drug responses, including the cidality of azoles, drug resistance, and calcineurin are well known to affect fungal drug responses, including the cidality of azoles, drug resistance, and calcineurin are well known to affect fungal drug responses, including the cidality of azoles.

How the TOR pathway, the unfolded protein response, PKC signaling, sphingolipid synthesis, and iron homeostasis, as well as fluoxetine and FNZ affect drug responses and increase FLC cidality remains to be determined.

The distinction between resistance and tolerance is consistent with the mechanisms that impact them: resistance mechanisms directly affect the drug target or its concentration in the cell, thereby enabling efficient growth in the presence of the drug. By contrast, tolerance reflects stress response strategies that are indirect and may enable survival despite the continued ability of the drug to interact with its target, to remain in the cell (albeit at lower average concentrations) and to affect cell growth. Stress responses can modulate physiology, for example, membrane integrity and the aggregation of proteins into stress granules or other physiological switches, thereby minimizing deleterious cellular responses to the drug.

The growth properties of cells at supra-MIC concentrations measured here with FLC, a fungistatic drug, contrast with those measured for antibacterial tolerance in bactericidal drugs. This likely reflects distinct modes of action between static and cidal drugs, as well as different molecular mechanisms of stress responses in eukaryotes and bacteria. Antibacterial tolerance generally involves a longer lag phase for the majority of the
population. By contrast, antifungal tolerance involves the earlier appearance of a subpopulation of cells that continue to grow in the presence of drug and, unlike "phenotypic resistance" described for bacteria, is not dependent upon growth phase or cell density.

This study revisited the question of whether supra-MIC growth is clinically relevant and, by inference, whether the stress pathways that mediate it represent important drug targets. Previous work suggested that trailing growth was not important for virulence in either mouse models or infection outcomes in the human host. However, these studies analyzed short-term responses, rather than persistence or recurrence of Candida infection over longer time frames. Here, both in vivo and clinical studies provide a proof of principle suggesting that retrospective and prospective clinical studies that measure tolerance are warranted. We posit that monitoring FoG/SMG, in addition to RAD/MIC, in standard clinical assays may have prognostic value for the likelihood of a persistent infection, for the responsiveness of a given isolate to FLC, as well as for the synergism of an adjuvant drug with FLC. Furthermore, in developing countries where azoles remain the major antifungals in use, measuring tolerance, in addition to susceptibility, may be especially relevant for predicting clinical outcomes and the addition of low cost adjuvant drugs could significantly impact treatment efficacy.
Understanding the contribution of tolerance to the progression of fungal infections not only can provide fundamental insights into the biology of fungal subpopulation behaviors but also has the potential to inform clinical practices.

**Methods**

*Candida albicans* isolates. All strains (Supplementary Data 1) were streaked onto rich media (YPD) plates and grown for 24 h at 30 °C. A single colony from each strain was grown on YPD and frozen in 50% glycerol and stored at −80 °C for all assays. For each mutant tested, we used genetically matched parental control strains and, where appropriate, nutrient supplements were used to compensate for auxotrophies.

Isogenic SC5314 low and high FoG isolates were obtained by sequentially passaging SC5314 every 24 h in YPD (1/100 dilutions, ~84 generations) either with or without 1 µg/ml FLC. Persistent and NP clinical isolates were obtained from patients with candidemia that were investigated by one of us (A.L.C.) during several candidemia surveys. Fungal isolates were obtained from two groups of patients: patients with a single episode of candidemia, for which infection was resolved on first-line treatments; and patients with persistent or recurrent candidemia. Persistent candidemia was defined here as two or more blood cultures positive for *C. albicans*, on one or more days apart, despite at least 3 days of antifungal therapy with FLC. NP isolates (NPI–7) were cleared from the bloodstream soon after FLC treatment was initiated and treatment was continued for an additional 16 days. In contrast, persistent candidemia in 12 patients was not resolved on first-line treatments, in which case either caspofungin or amphotericin B was added to intravenous FLC as second-line antifungal therapy was continued with either caspofungin or amphotericin B. While each patient had distinct clinical backgrounds and trajectories, the two groups were matched in terms of age, underlying conditions, time of central venous catheter removal, and first line of antifungal therapy. Patients received intravenous FLC as first line of therapy and the initial time of therapy did not differ between persistent and NP infections. In some cases of persistent candidemia, antifungal therapy was continued with either caspofungin or amphotericin B (Supplementary Fig. 10a). Persistent candidemia in these patients occurred despite clinical MIC susceptibility testing were followed with slight modifications. In brief, strains were streaked from glycerol stocks onto YPD agar and incubated for 24 h at 30 °C. Colonies were suspended in 1 ml PBS and diluted to 10^7 cells/ml in a 96-well plate with casitone containing a gradient of two-fold dilutions per step of FLC, with the first well containing no drug. For persistent and NP clinical isolates, cells were grown overnight in YPD at 30 °C and diluted to 10^5 cells/ml in YPD containing a gradient of two-fold dilutions per step of FLC. MIC<sub>50</sub> levels were determined after 24 or 48 h by taking an optical density reading (OD<sub>600</sub>) by a Tecan plate reader (Infinite F200 PRO, Tecan, Switzerland). MIC<sub>50</sub> levels (shown as yellow lines on broth micro-dilution assays heatmaps) were determined at the point at which the OD<sub>600</sub> had been reduced by ≤50% compared to the no-drug wells.

**Disco diffusion assays**. The CLSI M44-A2 guidelines with minor modifications as follows. Strains were streaked from glycerol stock onto YPD agar and grown for 24 h at 30 °C. Colonies were suspended in 1 ml PBS and diluted to 10^7 cells/ml in a 96-well plate with casitone containing a gradient of two-fold dilutions per step of FLC, with the first well contain no drug. For persistent and NP clinical isolates, cells were grown overnight in YPD at 30 °C and diluted to 10^5 cells/ml in YPD containing a gradient of two-fold dilutions per step of FLC. MIC<sub>50</sub> levels were determined after 24 or 48 h by taking an optical density reading (OD<sub>600</sub>) by a Tecan plate reader (Infinite F200 PRO, Tecan, Switzerland). MIC<sub>50</sub> levels (shown as yellow lines on broth micro-dilution assays heatmaps) were determined at the point at which the OD<sub>600</sub> had been reduced by ≤50% compared to the no-drug wells.

**Enzyme-linked immunosorbent assay (ELISA)** protocol was modified from ref.<sup>75</sup>. Brieﬂy, *C. albicans* cells were grown overnight in 1 ml YPD (250 rpm, in the absence of 10 µg/ml FLC (Sigma–Aldrich, St. Louis, MO, USA) and harvested at exponential phase (OD<sub>600</sub> 0.6–0.8). Cells were centrifuged for 10 min (3000 rpm, 4 °C), and washed once with ice-cold PBS. Pellets were resuspended in 200 µl lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, protease inhibitor cocktail (Roche Diagnostics)) together with acid-washed glass beads. Cells then were mechanically disrupted by vortexing for 30 min at 4 °C. Cell lysates were diluted 1:10 in PBS and subjected to BCA analysis (Pierce Biotechnology, Rockford, IL, USA) to determine protein concentrations. Cell lysates at 10 µg/ml concentration were incubated in wells of a 96-well ELISA plate for 18 h at 4 °C. Wells were washed and coated with PBS (PBS – 0.02% Tween-20), block with 1% skim milk in PBS for 2 h at 37 °C, and washed. Rabbit polyclonal anti-Hsp90 antibody (Dundee Cell Products, Scotland) was diluted 1:1000 in blocking buffer applied overnight at 4 °C. Horseradish peroxidase–coupled donkey anti-rabbit IgG (1:1000 dilution, Jackson Immunoresearch Laboratories, West Grove, PA, USA) was incubated for 1 h at 37 °C followed by washing. Detection was done with TMB-tetramethylbenzidine). The reaction was terminated with 0.16 M sulfuric acid and absorbance was measured at 450 nm in an ELISA plate reader.

**FSC-Flow** uptake measured by flow cytometry. *Candida albicans* cells were grown overnight in YPD media at 30 °C. Cultures where diluted 1:100 in 3 ml casitone medium and incubated for 3 h at 30 °C. Drugs were added to a final concentration of 10 µg/ml for FLG and 1 µg/ml for FLC-Cy5<sup>40</sup>. Cells were harvested every 30 min and analyzed in 50% SSC (50 mM Tris, pH 8, 5 mM EDTA). Data were collected from 25,000 to 35,000 cells per time point using 561 mEx and 661/20 mEm in a MACSQuant flow cytometer and gated to by SSC<sub>c</sub>: 10<sup>4</sup> A.U. and 20<sup>5</sup><sup>4</sup> A.U. (in SSC vs. FSC) to eliminate small debris particles. Analysis was done using the FlowJo 8.7 software.

**Efflux of R6G as measurement of drug efflux capacity**. The assay was adapted from ref. 79 with minor modifications. In brief, strains were streaked from glycerol stock onto YPD plates and incubated for 24 h at 30 °C. Colonies were resuspended in 3 ml casitone medium and grown overnight in 30 °C. Cultures were diluted 1:100 in 5 ml casitone medium and incubated for 3 h at 30 °C. Drugs were added to a final concentration of 10 µg/ml for FLG and 1 µg/ml for FLC-Cy5<sup>40</sup>. Cells were harvested every 30 min and analyzed in 50% SSC (50 mM Tris, pH 8, 5 mM EDTA). Data were collected from 25,000 to 35,000 cells per time point using 561 mEx and 661/20 mEm in a MACSQuant flow cytometer and gated to by SSC<sub>c</sub>: 10<sup>4</sup> A.U. and 20<sup>5</sup><sup>4</sup> A.U. (in SSC vs. FSC) to eliminate small debris particles. Analysis was done using the FlowJo 8.7 software.
*Galleria mellonella* virulence assays. G. larvae were obtained from Vanderhorst Wholesale (Saint Marys, OH, USA) and the infection protocol was adapted from Li et al.\(^4\). Larvae were kept at 15 °C and used within 1 week of delivery. Larvae were randomly selected for each experiment in groups of 12 and those showing signs of melanization were excluded. *C. albicans* inoculums were prepared from cultures grown overnight at 30 °C in YPD and washed twice with sterile PBS. Cells were counted using a hemocytometer and adjusted to $3 \times 10^5$ cells/ml in sterile PBS. Each larva was initially injected with $3 \times 10^5$ cells via the left last pro-leg using a 10 µl glass syringe and a 265 g needle (Hamilton, 80300). A second injection with either FLC (1 mg/ml), FLC + FNZ (FNZ, 10 mg/ml), or sterile PBS was done via the last right pro-leg 1.5 h post infection. The inoculum size was confirmed by plating in duplicate on YPD. Infected larvae and PBS-injected controls were maintained at 37 °C for 14 days and checked daily. Larvae were recorded as dead if no movement was observed upon contact. Virulence assays were performed with strain SC5314, with SC5314-derived isolates passed for 12 days in either YPD alone (2 “low” FoG isolates) or YPD plus 1 µg/ml FLC (4 “high” FoG isolates), and with isolates NPF3 and S12-01 from a clinical isolate set (see Supplementary Data 1). Experiments were performed in duplicate ($n = 24$ larvae) and statistical differences between larval groups were tested using the Mantel–Cox (log–rank) test. Negative controls included PBS, FLC alone or FLC + FNZ for groups of 24 uninfected larvae. No significant killing of larvae was observed in either of these conditions.

**Growth assays.** To determine growth parameters (doubling time and lag phase duration), *C. albicans* cells were seeded at a concentration of $2 \times 10^5$ cells/ml into 96-well plates containing YPD media. Plates were incubated for 48 h at 30 °C with continuous shaking in a Tecan plate reader (BioTek) and the optical density reading (OD\(_{600}\)) was recorded every 15 min. Measurements of doubling time and lag phase duration were determined using the BioTek Gen5 software and from three biological replicates, each performed in duplicate.

**Microcolony assays.** Microcolony assays were adapted from ref.\(^34\) with minor modifications. Colonies were resuspended in 3 ml casitone medium and grown overnight in 30 °C. Cultures were diluted 1:100 in casitone medium and incubated at 30 °C until cultures reached logarithmic phase (3–4 h) and diluted to $10^5$ cells/ml in casitone medium. For microscopy, glass-bottomed 24-well plates (De-GROOT, Israel) were coated with 1 ml of a 200 µg/ml concanavalin A solution (Type VI, Sigma-Aldrich, St. Louis, MO, USA) for 3–4 h. Wells were washed twice with 1 ml ddH\(_2\)O and $10^5$ cells/ml in a 2 ml volume were added to wells with or without 10 µg/ml FLC. Plates were sealed with an optically clear film (Axxygen, Coral, Israel) and spun at 1200 rpm for 1 min. Images were captured in 100 fields per well for 48 h in 1 h intervals using a Nikon Ti Eclipse microscope equipped with a full-stage environmental chamber (set to 30 °C) with a Nikon Plan Apo ×10 (0.45 numerical reading) objective using the Nikon Elements AR software. The focusing routine was calculated for 1500–5000 colonies per strain\(^34\) by regressing the natural log of microcolony area over time. Growth rates were calculated separately for two time fields per well for $t < 19$ (2015).

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Author contributions

I.V.E., R.J.B., and J.B. designed the study; A.R., I.V.E., and A.M.D. collected the data; A.R. and I.V.E. analyzed the data; M.B. produced and analyzed the data in Fig. 3a, d; S.Z. performed the experiment in Supplementary Fig. 7g; E.S.S. constructed mutant strains; N.Z. implemented and helped analyze the data in Fig. 2b; A.L.C. provided clinical isolates and clinical input to the paper; and A.R., I.V.E., R.J.B., and J.B. drafted and edited the manuscript.

Additional information

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