

EVOLUTION OF ANTIFUNGAL-DRUG RESISTANCE: MECHANISMS AND PATHOGEN FITNESS

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Abstract | Like other microorganisms, fungi exist in populations that are adaptable. Under the selection imposed by antifungal drugs, drug-sensitive fungal pathogens frequently evolve resistance. Although the molecular mechanisms of resistance are well-characterized, there are few measurements of the impact of these mechanisms on pathogen fitness in different environments. To predict resistance before a new drug is prescribed in the clinic, the full spectrum of potential resistance mutations and the interactions among combinations of divergent mechanisms can be determined in evolution experiments. In the search for new strategies to manage drug resistance, measuring the limits of adaptation might reveal methods for trapping fungal pathogens in evolutionary dead ends.

RESISTANCE

The ability of a pathogen to continue to reproduce in the presence of an antifungal agent, here measured either as minimum inhibitory concentration or as fitness.

The evolution of **RESISTANCE** to antimicrobial agents that are used to control pathogens in medicine and agriculture is a well-documented problem. Antifungal drug therapy is no exception — resistance to many of the antifungal agents now in use has emerged. Although antifungal-drug resistance does not seem to be as much of a problem as resistance to antibacterial agents in bacteria¹, one long-term concern is that the number of fundamentally different types of antifungal agents that are available for treatment remains extremely limited (TABLE 1; FIG. 1). This is because humans and their fungal pathogens belong to sister clades in the tree of life² and potential drug targets that are unique and important to the fungus, but not to the host, are few.

Although there is a wealth of information on the molecular mechanisms of resistance to antifungals^{1,3,4} (BOX 1), this is only half of the picture that is needed for optimal long-term management of antifungal resistance. It is the evolutionary processes, that is, how divergent mechanisms arise through mutation, how these mechanisms impact on pathogen **FITNESS** in various environments and how the resistance mechanisms interact with each another in combination (and respond to natural selection), that collectively determines

the fate of resistance in fungal pathogen populations. The fitness impact of resistance is undoubtedly affected by the complex changes in gene expression that accompany simple mutations that encode resistance^{5–8}. In managing antifungal resistance, these changes in the ‘hard wiring’ of the fungal cell might include hidden targets that, when inhibited or deleted, might reduce the ability of the pathogen to evolve increased resistance.

This review focuses on the yeast model system *Saccharomyces cerevisiae* and the important human pathogenic yeast *Candida albicans* as a paradigm for the emergence of antifungal-drug resistance through mutation, with subsequent transmission of the resistance phenotype to descendants.

Measuring antifungal-drug resistance

Antifungal-drug resistance is usually quantified using the minimum inhibitory concentration (MIC), in which growth in the presence of a range of drug concentrations is measured over a defined time period according to a standard protocol⁹. The lowest drug concentration that results in a significant reduction of growth (usually either 50% or 90% reduction of growth compared with growth in the absence of the drug) is the MIC.

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Table 1 | Antifungal drugs and their targets

Compound class	Example drug	Target
Allylamines	Terbinafine	Ergosterol synthesis, squalene epoxidase
Antimetabolites	Flucytosine	Fungal nucleic acid (RNA and DNA)
Azoles	Fluconazole	Ergosterol synthesis, lanosterol demethylase
Echinocandins	Caspofungin	Cell wall, β -1-3-glucan synthesis
Polyenes	Amphotericin B	Ergosterol
Other	Griseofulvin	Fungal mitotic apparatus

Source: <http://www.doctorfungus.org>

When the transition from growth to no growth that is observed with increasing drug concentration is clear, scoring the MIC is straightforward. When this transition is not clear, however, as is the case for many strains that show strong TRAILING with fluconazole, the MIC can be difficult to assess. In this case, fitness measurements at specified drug concentrations might provide a better measure of resistance.

One well-known limitation of the MIC as a measure of resistance is that it does not always predict the clinical outcome of antifungal therapy¹. Because disease is the result of the complex interactions between the pathogen and host¹⁰, failure to control fungal pathogens that are classified as drug sensitive by MIC and successes in controlling fungal pathogens that are classified as drug resistant by MIC should not be surprising.

An even more fundamental limitation of the MIC as a predictor of the progression of disease caused by a fungal pathogen is that it is sometimes wildly inaccurate in predicting the fitness of the infecting microorganism, owing to strong environment/genotype interactions^{11,12}. Whether fungal fitness measures,

such as population doubling time during the exponential growth phase or assessing the number of doublings required to attain maximum cell density over a range of drug concentrations, might be better predictors of therapeutic outcome has not been evaluated. Also, in some cases, TOLERANCE might be important to the outcome of treatment. Tolerance is measured in time-kill assays and tests of minimum lethal concentration of drugs¹³. Drug tolerance and drug resistance are not always positively correlated.

Antifungal drugs and their targets

Many antifungal drugs target ergosterol or the steps in its synthesis (TABLE 1). Ergosterol, the functional fungal analogue of cholesterol in animal cells, is found in the fungal cell membrane, where it has essential roles in the modulation of membrane fluidity and as a signal for cell division. Because ergosterol has different chemical properties from cholesterol, it has been exploited as a target for polyene-compound drugs, such as amphotericin B, which are incorporated into fungal cell membranes containing ergosterol, but are less readily incorporated into host cell membranes, which contain cholesterol. Amphotericin B, in association with ergosterol, is thought to form membrane-spanning channels with hydrophilic interiors¹⁴ that allow the leakage of essential components, which ultimately results in fungal cell death. In addition to ergosterol itself, there are several enzymatic steps that are unique to ergosterol synthesis and are targets for several other antifungal drugs, including terbinafine and the azoles¹⁵. However, apart from ergosterol and its biosynthesis, there are few targets that can be exploited in antifungal therapy.

FITNESS

The extent to which an individual contributes genes to future generations, here measured as the number of generational doublings by a given fungal strain in a given environment in a set period of time or as an instantaneous rate of reproduction. In nature, ability to tolerate adverse environments, efficiency of sporulation and viability of offspring might also be important components of fitness.

TRAILING

The ability of a fungal strain to grow at drug concentrations above the minimum inhibitory concentration (MIC) in MIC tests.

TOLERANCE

The ability of the pathogen to survive while under inhibition by an agent.

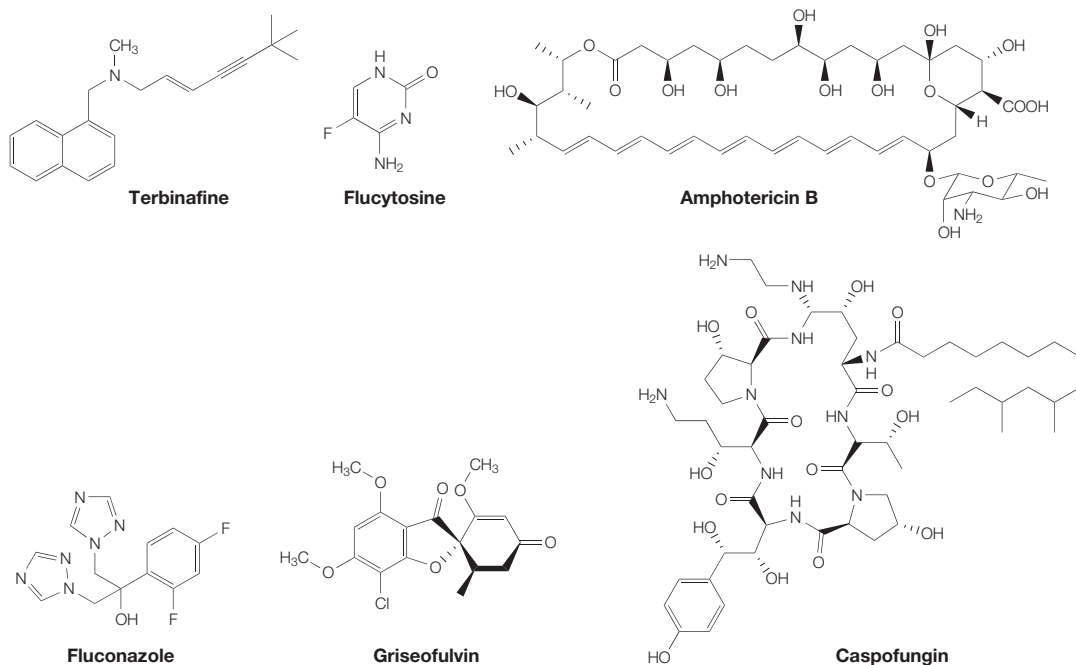


Figure 1 | Chemical structures of some antifungal drugs and their targets. Selected examples of antifungal drugs (see TABLE 1 for targets).

Box 1 | **Antifungal-drug-resistance mechanisms****Increased efflux**

Overexpression of ABC (ATP-binding cassette) transporters or major facilitator proteins in the cell membrane. Often caused by mutations in transcriptional regulators. Represents a broad mutational target, as many non-synonymous changes in the regulators confer a resistant phenotype. See the second case study in BOX 3 for the example of the transcriptional regulator *PDR1* of *Saccharomyces cerevisiae*.

Alteration of target enzyme

Changes in target protein either prevent binding of the antifungal drug or prevent the allosteric inactivation of the target after the inhibitor binds. This is a relatively small mutational target, as only a few specific amino-acid changes confer resistance. Alternatively, the target protein might be overexpressed, resulting in sufficient activity in the presence of the drug.

Alteration of metabolism

Loss of enzyme activity prevents the accumulation of a toxic product in the presence of the drug. This is a relatively broad mutational target, as myriad amino-acid changes result in loss of function and a resistant phenotype. See experiment 2 (BOX 4) for the example of the loss of function in the sterol-biosynthesis gene *ERG3* of *S. cerevisiae*.

Several excellent reviews^{1,3,4} have documented these mechanisms in detail. Known mechanisms of resistance do not account for all observed resistance. Additional mechanisms undoubtedly await discovery.

Evolution of antifungal-drug resistance

If the use of an antimicrobial drug always carries the risk that resistance will evolve in the pathogen, what are the main factors that affect the likelihood of resistance in microbial populations? First, as in most evolutionary processes, population size is of paramount importance. Each surviving fungal PROPAGULE represents an independent ticket in the mutational lottery for resistance. The number of fungal survivors depends, to some extent, on how the antifungal therapy is applied, so the best and most obvious strategy is to reduce the pathogen population size to the maximum extent possible and to prevent subsequent increases. Along with population size, the overall rate of mutation to yield resistance is the other main determining factor. The mutation rate specifies the odds associated with each lottery ticket. In contrast to population size, the rate of mutation to yield resistance is specific to the drug, the drug target and the fungal pathogen. Consequently, this factor would be more difficult to manipulate than population size.

An antifungal drug prone to resistance. Of the agents that target ergosterol synthesis, the azole drug fluconazole is highly effective, has few side effects and, along with amphotericin B, has been a mainstay of antifungal therapy. Fluconazole is vulnerable to the development of resistance for two reasons. First, fluconazole is fungistatic, not fungicidal, and can leave large populations of survivor cells that are exposed to strong DIRECTIONAL SELECTION for resistance. Second, because of the many different mechanisms of resistance (BOX 1), the overall rate of mutation to yield a resistant phenotype is probably high. Precise estimates for overall, per-generation rates of mutation that result in fluconazole resistance, however, are difficult to obtain. This is because even the sensitive fungal

types can reproduce to some extent in the presence of high concentrations of fluconazole. The resistance phenotype, therefore, is not always sharply defined and is not easy to quantify in mixtures of resistant and sensitive types. Nonetheless, the net effect of pathogen population size and mutation rate together is that fluconazole resistance can emerge rapidly and repeatedly within the host environment. The problem of resistance is compounded by long-term treatment regimens typically lasting weeks or months.

In contrast to fluconazole, resistance to the newest antifungal drug, caspofungin, is, at present, not commonly observed in patients, probably because caspofungin is fungicidal and because the rate of mutation to yield resistance to caspofungin is lower than fluconazole. The gene encoding the target of caspofungin might contain fewer possible sites for mutation to resistance and there might be fewer mechanisms of resistance to caspofungin than fluconazole, although this remains to be determined. The relative rarity of resistance to caspofungin to date in patients, however, should not be taken as a guarantee that resistance will never become common. Examples of resistance to caspofungin have been observed both *in vitro*¹⁶ and *in vivo*¹⁷.

Fate of resistance determinants

Once resistance arises, the transmission of resistance determinants in fungi differs in at least one main way from the transmission of drug resistance in bacteria. In bacteria, horizontal transfer of mobile elements that include plasmids, prophages, integrons and gene islands containing antibiotic resistance occurs extensively across widely divergent taxa¹⁸. These resistance determinants, such as those that degrade or modify the antibiotic, often have no previous counterpart in the genome of the new bacterial host and are newly acquired genetic material. This is not the case for fungal resistance.

Horizontal transmission versus local evolution. In fungi, drug-resistance and other genes are generally not spread horizontally among widely divergent taxa. Unlike bacterial cells, intact fungal cells do not readily take up exogenous DNA. Even infectious extrachromosomal elements in fungi, such as plasmids, mitochondrial DNA elements and mycoviruses, require cell fusion and cytoplasmic contact before they can spread¹⁹.

If ongoing, horizontal transmission is not prevalent in fungi, how does antifungal-drug resistance arise within populations? In fungi, as in bacteria, some pathogenic species are inherently, uniformly resistant to selected antifungal drugs. For example, the proportion of isolates of *Candida krusei* and *Candida glabrata* that are resistant to several antifungal agents tends to be higher compared to other *Candida* species. In drug-sensitive fungal species, resistance phenotypes can be attributed to local mutation(s). The prevailing pattern suggests that antifungal-drug resistance repeatedly evolves in isolated populations. Furthermore,

PROPAGULE

Any fungal structure capable of dissemination and reproduction, including hyphal fragments, yeast cells or spores.

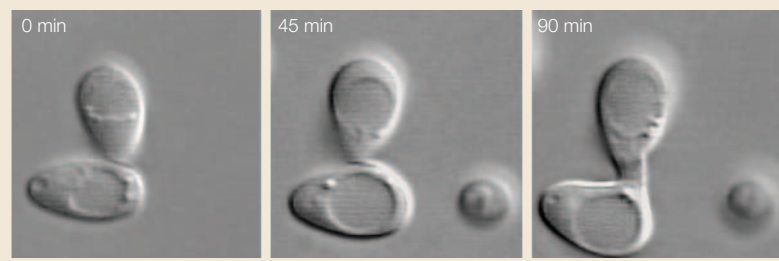
DIRECTIONAL SELECTION

Occurs when fitness increases with the phenotypic value of a trait, for example, the higher the resistance, the higher the rate of reproduction.

Box 2 | Recognition and genetic exchange among fungal species

In filamentous fungi, programmed cell death, which is associated with heterokaryon incompatibility, prevents general genetic exchange and/or reduces the transmission of infectious cytoplasmic genetic elements^{54,55} through HYPHAL ANASTOMOSIS. These barriers to the survival of non-self fusion cells are prevalent in higher fungal taxa, including filamentous ascomycetes such as *Aspergillus fumigatus*. These barriers, however, are not always absolute and 'leakage' of cytoplasmic determinants can occur with some genotype combinations⁵⁶. Unlike the hyphae of higher fungi like *Aspergillus* or *Neurospora* species, the yeasts, such as *Candida* species, do not generally undergo vegetative cell fusion and, consequently, heterokaryon incompatibility systems have not been described for yeasts.

Unlike heterokaryon compatibility, sexual compatibility occurs when strains carry different mating type determinants^{57–60}. Although many yeast-like fungi seem to lack a sexual life cycle, recent research shows that this cannot be taken as proof of its absence. In *Candida albicans*, an intact, functional mating system (see figure) was recently discovered^{62–65}; meiosis has not been observed. Although the signature of past recombination is clear in *C. albicans*, the evidence might indicate that exchange is infrequent^{24,26}. Moreover, the cellular signs of mating and meiosis are generally not observed in fungal pathogens *in vivo*. Also, where antifungal-drug-resistance mechanisms are strongly antagonistic, genetic exchange of the resistance determinants, if it occurs at all, would only produce genotypes that are at a fitness disadvantage in the presence of the drug (see the second case study, BOX 3)¹⁴. Image reproduced with permission from REF. 61 © (2003) American Society for Microbiology.



evidence for dissemination of resistance back into the environment of the potential patient population is scant. Nosocomial infections do occur, but are mainly associated with intensive-care units within hospitals and with the hands of health-care workers²⁰. In this scenario, antifungal-drug resistance is most often a local adaptation that confers no general advantage to the larger pathogen pool. Also in this scenario, resistance to antifungals involves modification of existing genes instead of introduction of new genes from distantly related taxa.

Genetic exchange and recombination in fungi and resistance. Although horizontal gene transfer between distantly related species is not observed in fungi, local recombination within species or between closely related species might occur through a mating process (BOX 2) or, in the case of filamentous fungi (but not generally in yeasts), through vegetative cell fusion and subsequent parasexual shuffling of genes²¹. Although fusion of vegetative cells is a common and constitutive process in most higher fungi, genetic exchange is curtailed to some extent by the widespread occurrence of heterokaryon incompatibility barriers (BOX 2) that function in self/non-self recognition in a manner analogous to that of tissue rejection systems in animals.

HYPHAL ANASTOMOSIS

A constitutive process in which the vegetative cells of filamentous fungi of the same, or closely related, species grow together and fuse with one another.

Genetic exchange and recombination might give rise to recombinant genotypes that contain multiple mechanisms of resistance of independent origin. One important question is the extent of the total pool of fungal individuals that might potentially share resistance mechanisms. The answer to this question can be found in basic investigations of the population genetic structure of a wide variety of fungi, including pathogens and non-pathogens^{22,23}. Surprisingly, the signature of past genetic exchange and recombination within fungal species is strong even within species that no longer seem to have a complete sexual cycle^{24–27}. This signature of recombination has been proposed as a way of recognizing biological units as species²⁸. Proceeding from the roots to the tips of multiple gene genealogies, the transition from concordance to conflict establishes the groups within which genetic exchange and recombination either occur presently or have occurred in the past. These groups are considered to be fungal species. This operational species concept works because genes are generally not exchanged horizontally among different fungal species and, relative to past speciation events, different genes all have the same history of descent. Within species, however, recombination shuffles genes; different genes therefore have conflicting histories of descent in the recent past. Fungal species defined in this way are the biological units within which sharing of independently derived resistance mechanisms might occur, but only if the capacity for genetic exchange and recombination is ongoing. This species concept is especially useful where morphological characteristics are not diagnostic and mating tests are not possible, and it might be effective in identifying emerging opportunistic fungal pathogens.

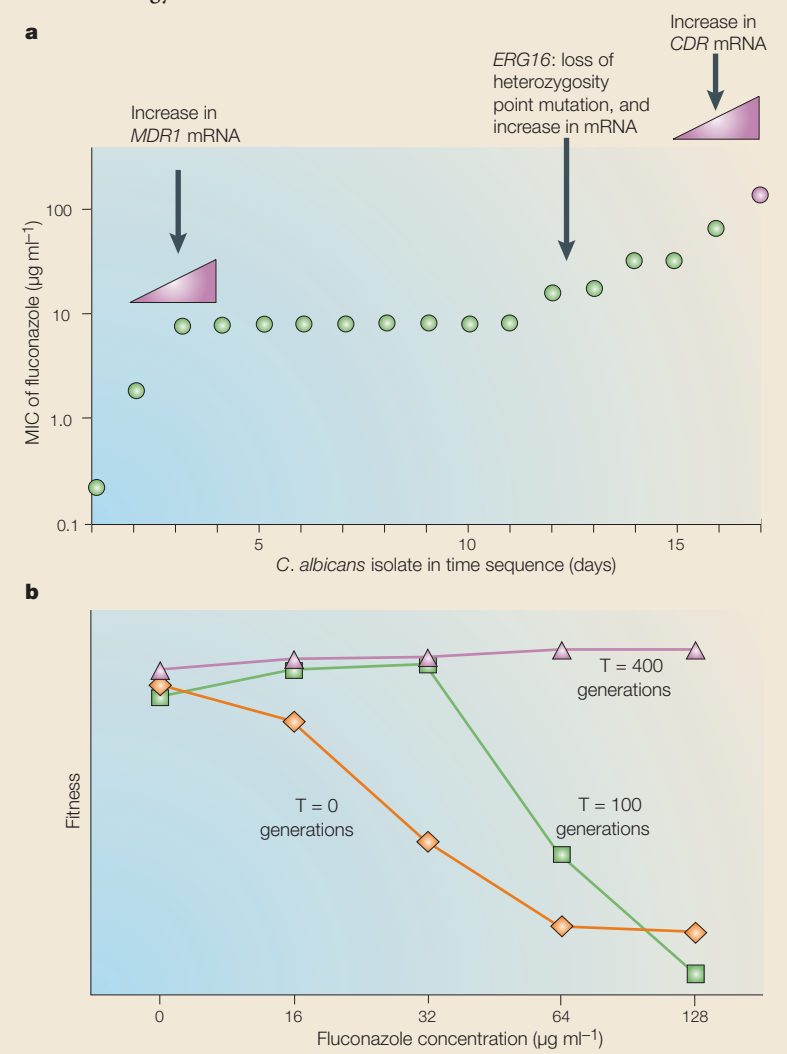
Experimental evolution of resistance

The evidence for the evolution of resistance in local populations in real time comes from two different types of study. First, several studies have monitored fungal pathogen populations over time in patients undergoing antifungal drug therapy^{15,29} (BOX 3, first case study). These studies show that resistance phenotypes accumulate over time, starting from a single genotype. Although these *in vivo* studies accurately document the emergence of fungal resistance in human patients, they are difficult to replicate and cannot control for important factors such as population size, temporal sequence of mutations and undetected reservoirs of pathogen genotypes that might contribute to the evolutionary outcome. Nonetheless, studies of pathogen populations in the patient have indicated that there is a microevolutionary progression towards resistance, that the same mechanisms of resistance found in the clinic are recruited locally, and that certain mechanisms of resistance can potentially combine to yield higher levels of resistance.

The second type of study monitors the evolution of resistance in artificial populations in real time (BOX 3, second case study). Experimental evolution has

Box 3 | Case studies of experimental evolution of drug resistance

In one case study (figure part a), *Candida albicans* isolates were obtained from a patient undergoing azole treatment over time^{15,29}. The minimum inhibitory concentration (MIC) of fluconazole increased over time, concomitant with the accumulation of several different resistance mechanisms that together result in greater resistance. In a second case study (figure part b), the experimental evolution of fluconazole in *Saccharomyces cerevisiae* was assessed¹². The mean fitness for three different populations of *S. cerevisiae* that were propagated through serial transfer between batch cultures for 400 generations was assessed in the presence of increasing concentrations of fluconazole (16, 32, 64 and 128 $\mu\text{g ml}^{-1}$ for each 100 generations). It is important to note that an MIC of 16 $\mu\text{g ml}^{-1}$ might be considered resistant in some pathogens; in this example with *S. cerevisiae*, resistance in an evolved strain is relative to that of the ancestor. All three populations showed similar evolutionary trajectories; the standard error for each mean is smaller than the display symbol. At 100 generations, each population had fixed a mutation for resistance in *PDR1*, resulting in enhanced fitness at 32 and 64 $\mu\text{g ml}^{-1}$, but, unexpectedly, reduced fitness at 128 mg ml^{-1} , with respect to the progenitor. This fitness deficit in the highest drug concentration was a true antagonistic pleiotropy associated with the *PDR1* mutations. At 400 generations, each population had accumulated an additional resistance determinant in an unidentified gene (*UNK1*) and each showed enhanced fitness at all drug concentrations. *MDR1*, multidrug efflux pump of the major facilitator superfamily; *CDR*, multidrug transporter of the ATP-binding cassette superfamily. Figure part a reproduced with permission from REF. 29 © (1997) American Society for Microbiology.



emerged as a new method for studying adaptation, either to general environments or specific inhibitors³⁰. The advantage of experimental evolution is that experiments are easy to replicate and strict control of conditions such as the initial genotype(s), population size, strength of selection, degree of physical structure in the environment (such as planktonic or biofilm growth) and the presence or absence of sex can be maintained. In most cases, the genetic variation that is favoured by selection arises only from mutation(s) that occurs during the course of the experiment. Yeasts are particularly amenable to experimental evolution³¹. The main disadvantage of evolution experiments is that the relevance of these *in vitro* experiments to 'real' situations is always open to question. So far, however, the mechanisms of resistance arising in experimental populations of *C. albicans* and the non-pathogenic *S. cerevisiae* are fully consistent with the types of resistance that have been found in 'natural' populations^{5,12}. So, experimental evolution of antifungal-drug resistance might be predictive of the evolution of fungal pathogens in their hosts—it is the resistance determinants themselves and not the many differences between the artificial media and the host environment that specify the evolutionary outcome with respect to antifungal drugs.

Resistance mechanisms and fitness

After resistance arises, the fitness effects of the different resistance mechanisms in various environments indicates whether resistance is maintained in the local population or not³². In most bacteria, resistance comes with a fitness cost in the absence of the drug. Although this fitness cost might be expected to result in the decrease, or loss, of resistant types in the absence of a drug, this is not usually the case. After resistance arises, further evolution involves the appearance of subsequent compensatory mutations that alleviate the fitness cost of resistance^{33,34}.

Resistance and fitness costs. The general ability of fungi to accommodate resistance mutations without a devastating loss of fitness is largely unknown, but in early studies the appearance of antifungal-drug resistance was not generally accompanied by an immediate fitness cost in the absence of the drug^{11,12}. In all cases, antifungal-drug resistance, even where resistance involves single mutations, involves complex, genome-wide changes in gene expression^{5–8} that become entrenched even in the absence of the drug. Many of the mutations that result in drug resistance are consistent with a drug-resistant phenotype, for example, overexpression of genes involved in drug efflux, lipid metabolism or oxidative stress can mediate resistance, but changes in the expression of other genes seem to have little relevance to drug resistance. However, which of the changes in gene expression might impose a cost on fitness under different conditions is not yet known. One clue comes from one of six experimental populations of *C. albicans*, in which there was an initial fitness cost that was reduced over time.

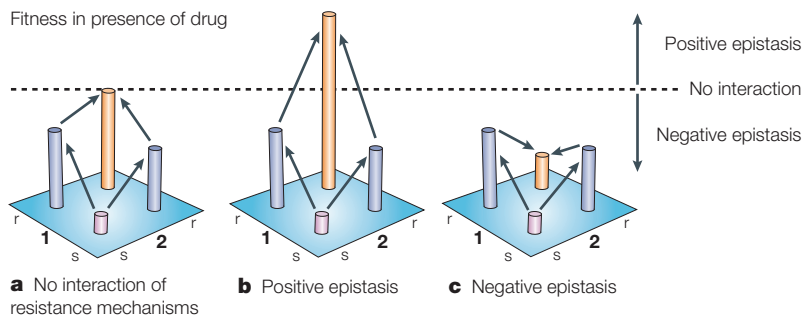


Figure 2 | Fitness landscapes for antifungal-drug-resistance mechanisms. Each vertical bar represents the combination of resistance (*r*) versus sensitivity (*s*), which are plotted on the two horizontal axes. The horizontal axes represent different resistance mechanisms (resistance mechanism 1 and resistance mechanism 2). **a** | The resistance mechanisms do not interact; the fitness associated with the combined mechanisms (orange) is the product of the fitness gains in the presence of the drug for each mechanism separately (no epistatic interaction between mechanisms). **b** | The two mechanisms interact synergistically (positive epistasis, shown in the orange bar). **c** | The two resistance mechanisms are antagonistic (negative epistasis, shown in the orange bar). Arrows indicate the alternate pathways by which a drug-sensitive ancestor (pink) might accumulate both resistance mechanisms.

This reduction was accompanied by modification of the expression of many genes from altered to normal levels⁵. At the other extreme, at least one form of resistance in *C. albicans* that involves constitutive overexpression of ABC (ATP-binding cassette) transporters and several other genes is accompanied by a gain in fitness both in the presence and in the absence of the drug¹¹. Although the effects of this kind of resistance on the ability of the fungus to colonize the body is not known, it raises the possibility that some resistant genotypes might actually increase in frequency even when the drug is not present in the environment. A problem, however, is that this increase would have to be accompanied by the ability of the resistant fungus to disseminate from the patient in which it initially arose. This phenomenon has not been observed with any pathogenic fungus, but the *in vitro* evolution experiments show that it is possible.

Combinability of resistance mechanisms. Whether resistance evolves through genetic exchange and recombination or by the stepwise accumulation of resistance determinants in fungal populations that form clonal lineages, the combinability of resistance mechanisms is the main factor determining the maximum level of resistance possible. The possibilities can be visualized on an adaptive landscape in which fitness in the presence of the drug is a function of genotype (FIG. 2). In purely evolutionary terms, there are three possibilities. First, the combined resistances might behave independently of one another, resulting in a fitness increase equal to the product of the proportional increases due to each mechanism separately. The other two possibilities are that the combined resistances show a synergistic interaction (positive epistasis), in which the fitness is higher than the expectation under independence, or show an antagonistic interaction (negative epistasis), in which the fitness is lower than the expectation under independence. This framework for interactions

might not work well in practice for antifungal-drug resistance, because the fitness increments relative to those found for general adaptation (for example, to a particular growth medium³⁵) are so large that the expectation for the combined resistances might be outside of the possible fitness range for the fungus under any condition. A more informative framework might be one in which the combined resistances result in fitness either greater or lesser than the fitness for each mechanism separately. In the former case, which might include independence or various degrees of synergism or antagonism, genetic exchange might lead to recombinant genotypes of higher fitness than were present before. In the latter case, which includes only strong antagonism, sex would represent a dead end with respect to drug resistance, as no further fitness gains in the presence of the drug would be realized with recombination.

To date, the evidence for the combinability of resistance mechanisms is mixed. The accumulation of multiple kinds of resistance of *C. albicans* in patients and *in vitro*, and of *S. cerevisiae in vitro*, supports a fitness gain when divergent mechanisms accumulate in the same lineage^{12,36} (BOX 3, first case study). This might have been expected, as only mechanisms that increase fitness over those already present would be favoured by natural selection. The other possible interaction, lower fitness than that associated with either mechanism separately, or strong antagonism, has emerged when resistances first evolve in separate lineages and then are combined through sexual crosses into the same background (BOX 4, second case study). Here, strong antagonism is observed for dominant regulatory mutations for overexpression of the ABC transporters and the alteration in sterol synthesis caused by loss of function at *ERG3* (REF. 12). The probable mechanism of antagonism is alteration of the sterol content of the cell membrane, where the efflux pumps reside. Many more combinations of drug resistance mechanisms need to be examined before the general patterns of interaction (or lack thereof) can be established. Surprisingly, the fitness effects of combining resistance mechanisms that arose independently in pathogens that infect patients have not yet been examined.

Predicting antifungal-drug resistance

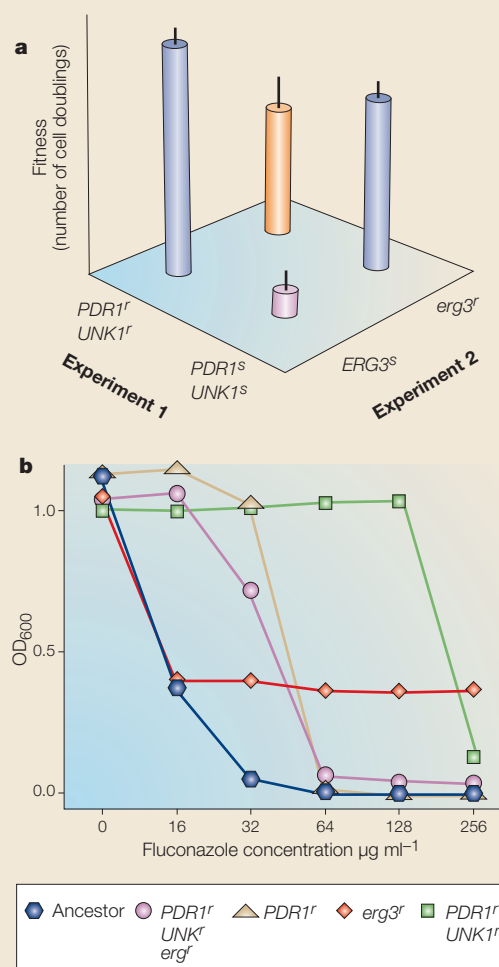
In the development of new antifungal drugs, how can the potential for resistance be evaluated?

Evolution of target genes. First, if the target genes are known, they could be subjected to the Barlow–Hall³⁷ procedure for predicting the evolutionary potential of a gene to develop resistance. This procedure uses mutagenic PCR and artificial recombination, so that many more sequence variants (differing from one another by one to several mutational steps) than could possibly occur in nature are evaluated. The resulting libraries of variant genes could be tested for resistance in a suitable fungal background, such as *S. cerevisiae*, in which the available molecular genetic background and genomics resources provide the means for

Box 4 | **A case study of combining drug resistance mechanisms**

In a companion experiment to the second case study described in BOX 3, in which populations were grown in the presence of increasing concentrations of fluconazole (see figure part a, in which the case study outlined in BOX 3 figure part b is denoted experiment 1), populations were grown in the presence of a single (high) concentration ($128 \mu\text{g ml}^{-1}$) of fluconazole and asked to adapt in one step (see figure part a, denoted experiment 2). In this environment, adaptation consistently occurred through loss-of-function mutations (*erg3^r*). Each vertical bar represents the combination of alleles on the two horizontal axes in a heterozygous, diploid genotype. Collectively, these two experiments revealed that the different regimens of fluconazole selection favoured completely different types of resistance¹². The divergent types of resistance from different regimens of selection were strongly antagonistic to one another. Diploid genotypes that are homozygous for the resistance determinants *PDR1^r* and *UNK1^r* and those that are homozygous for the resistance determinant *erg3^r* are fitter than hybrid diploid genotypes heterozygous for all three gene loci. The two axes in the figure (part a) represent the resistance determinants that accumulated in experiments 1 and 2. The ancestor is homozygous for the sensitive alleles for all three genes (*PDR1^s*; *UNK1^s* and *ERG3^s*). The combinability of the resistance mechanisms was also assessed (see figure part b).

The meiotic offspring of the diploids are heterozygous for resistance determinants at all three gene loci (*PDR1^s/PDR1^r*, *UNK1^s/UNK1^r*, *erg3^s/ERG3^r*). In this case, mating between separately evolved resistance genotypes probably represents an evolutionary dead end, because the hybrids and recombinant offspring genotypes suffer a reduction in fitness relative to the parents in the presence of the drug.



high-throughput tests. Candidate resistance alleles could then be evaluated in the background of the original pathogen. In this way, most or all of the specific mutations and combinations of mutations that lead to antifungal-drug resistance could (in theory) be characterized before they actually arise in patients. Prime candidate genes for this kind of systematic procedure in *S. cerevisiae* are *ERG11*, which encodes the target of the azoles and in which many mutations have been described, and in some cases evaluated for their contribution to resistance^{1,4,15,38,39}, and *FKS1*, which encodes the target of caspofungin⁴⁰. The expectation is that the Barlow–Hall procedure might recover all previously described mutations in these genes that confer resistance, plus others that might yet be found in fungal pathogen populations exposed to these drugs. This approach has not yet been applied to the target genes of antifungal drugs.

Evolution of the pathogen. The fungal pathogen could be tested for the evolution of resistance *in vitro*, using the framework of experimental evolution³⁰. In the search for potential resistance mechanisms, the goal is exactly the opposite of antifungal chemotherapy.

In these experiments, population size, mutation rate and the strength of selection through the dose of the drug can all be varied to maximize the chances of recruiting as many diverse resistance mechanisms as possible in fungal populations. Depending on the fungus, these experiments can be done with or without genetic exchange and recombination through a sexual cycle. With sexual reproduction, the speed of adaptation to the drug might be increased relative to exclusively asexual reproduction, whenever the individual mutations for resistance are rare and likely to occur in separate lineages within the population. This conforms to one of the hypothesized general benefits of sex in evolution⁴¹. As in the Barlow–Hall procedure, patterns of resistance with experimental populations could be found with minimal expense and high efficiency before they ever become apparent in patients.

Managing antifungal-drug resistance

Given what is already known about the fitness landscape for antifungal-drug resistance, how might the evolution of resistance be channelled in a less damaging direction? There are three main possibilities, including combination therapy, which is already in use.

Combination therapy. Combination therapy uses two different antifungal drugs, simultaneously or in succession. The degree of interaction between the drugs that affect the fungus is commonly measured by the fractional inhibitory concentration index, which is determined by deploying both drugs in a checkerboard pattern in 96-well plates. Here, the effects might be categorized as synergism, indifference (that is, independence or absence of interaction) or antagonism⁴². The interactive effect of the drugs on the fungus can also be evaluated in time-kill studies.

Beyond the immediate effects on the growth of the fungal pathogen, combination therapy might impede the evolution of resistance in two fundamental ways. First, where the combination of drugs has a greater inhibitory or killing effect on the fungus than either agent separately, the pathogen population size will be further reduced. Second, the overall rate of resistance to both agents will ideally approach zero, as the probability of simultaneously overcoming two different types of inhibition is the product of the probabilities of developing resistance to either agent when used on its own. An extreme example of using combination therapy to prevent resistance is flucytosine, which is only used in combination with other drugs because a high rate of resistance results when this drug is used alone.

An intriguing interaction occurs between certain immunosuppressive agents, such as cyclosporin and FK506, and azole drugs, which have the potential for combination therapy. In *Candida* spp. and *Saccharomyces* spp., the immunosuppressive drugs inhibit the calcineurin pathway, which is an important component of the general response to stress. The calcineurin response is necessary for the fungal pathogen to maintain viability when azole drugs are present in the cell and are bound to their target protein, **Erg11p** (lanosterol 14 α -demethylase). In combination with fluconazole, the combined effect on the pathogen is synergistic and results in loss of viability in the pathogen^{43–45}, essentially because the tolerance response is curtailed. For the interference with calcineurin function to be exploited in combination with other antifungal drugs, however, it will ultimately be necessary to have antifungal molecules that distinguish the fungal calcineurin from the host calcineurin, to avoid modification of the host immune response. Similarly, the molecular chaperone Hsp90 (heat shock protein) is necessary for the general stress response, and a therapeutic antifungal anti-Hsp90 antibody that inhibits this chaperone is currently being tested in clinical trials⁴⁶.

In practice, the usefulness of combination therapy in treating fungal infections is mixed^{42,47–49}. Each potential drug combination requires *in vitro* tests, experiments with animal models, clinical trials and careful consideration of costs and benefits. Along with these direct effects on the pathogen, the patterns of resistance should also be evaluated.

Channelling the evolution of resistance. An entirely different method for managing resistance is at first glance counter-intuitive. **PDR1** (pleiotropic drug resistance) mutations in *S. cerevisiae* have a strong and unexpected antagonistic pleiotropy that is associated with resistance to fluconazole (BOX 3). Although there is no detectable fitness cost when low concentrations of the drug are used, and although resistance is evident at intermediate drug concentrations, an increased sensitivity to fluconazole is found at the highest drug concentrations, possibly owing to a loss of the tolerance that is normally found in drug-sensitive types even at the highest drug concentrations. Here, the drug-sensitive strain actually has improved fitness compared with its drug-resistant derivative at the highest drug concentrations. In the presence of low concentrations of fluconazole, dominant **PDR1** and **PDR3** mutations arise readily through spontaneous mutation^{12,50}, and so the entire population is effectively channelled to a peak of fitness that becomes a dead end if a higher concentration of the drug is subsequently used.

Because of the severe fitness deficit that occurs when the fungi are in the presence of increased drug concentrations, **PDR1** and **PDR3** mutant populations cannot maintain population sizes with the dilution between successive batch cultures, and therefore cannot respond to the natural selection imposed by the drug. For therapy in situations in which at least some resistance is inevitable, it might therefore be advantageous to channel resistance so that the mutant strain that predominates carries a vulnerability that can then be overcome with increased doses of the drug. For this strategy to be contemplated in the treatment of patients afflicted with fungal pathogens, the fitness implications of all the forms of resistance that might arise would need to be known with precision, both *in vitro* and in animal models. There is, however, no impediment to obtaining these kinds of measures with any fungal pathogen in which resistance has been a problem.

Impeding evolvability. An entirely unexplored possibility would be to interfere with the ability of a fungal pathogen to evolve, known as **EVOLVABILITY**⁵¹. Using this approach, companion agents might ultimately be targeted not to impair the viability of the fungal population, but to impair its ability to produce phenotypic variation that might be subject to natural selection in the future. Although lowering the overall mutation rate would reduce the capacity of the pathogen to produce phenotypic variation, it is difficult to imagine how this might be achieved; both the fidelity of DNA replication and the efficiency of repair processes are considerably easier to disrupt or inhibit than to improve. Also, reducing the mutation rate might not have much effect on the emergence of resistance, especially in large populations in which the strong selection imposed by the drug would continue to efficiently amplify the resistance mutations that would still occur, albeit at a lower frequency. There is, however, another level at which the capacity to produce phenotypic

EVOLVABILITY

The capacity of an organism to express variation at the phenotypic level that might then be acted on by natural selection.

variation in the form of resistance might be reduced. The simple, underlying mutations that are responsible for drug resistance are accompanied by complex genome-wide changes in gene expression. Might the expression of certain genes be essential for the ability of the fungus to tolerate these complex downstream changes in gene expression? Candidate genes could be screened for the ability to be essential — not for viability, as an essential gene would be identified in most screens for drug targets — but for the pathogen to evolve resistance, using the same selective conditions in which the wild type always evolves resistance. Short-term assays for those genes that are essential for the evolution of drug resistance already exist⁵⁰. In effect, in backgrounds in which the expression of these genes was curtailed, mutations that conferred resistance would occur at the same frequency, but a resistant phenotype would not result, so there would be no variation on which natural selection could act. Evolution toward resistance in the population would therefore be blocked or limited. Candidate genes that are shown to be essential for the evolution of resistance could then be further tested to determine whether the effect extends to multiple drugs.

Conclusions

The emergence of antifungal-drug resistance is an example of evolution in action. As with other antimicrobial agents for the control of pathogens in medicine and agriculture, the evolution of antifungal-drug resistance in patients is also a direct outcome of Murphy's Law, the principle that anything that can possibly go wrong, will go wrong⁵². Management of antifungal-drug resistance would benefit from a comprehensive picture of the fitness landscape for fungal pathogens and their resistance mechanisms, both when used alone and in combination. As an evolutionary process, drug resistance is amenable to the analysis of the molecular basis for adaptation, because the fitness increases occur in large intervals and the mutations responsible can be easily identified and tested for their fitness effects in various environments, degree of dominance and potential for epistatic interactions. In addition, dominant, gain-of-function mutations, the 'holy grail' of evolutionary studies of adaptation⁵³, are now available. Connecting the evolution of drug resistance with the underlying molecular mechanisms could ultimately reveal how pathogens adapt to new environments and the nature of the constraints on the extent of adaptation.

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The author declares no competing financial interests.

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