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EVOLUTION OF DRUG RESISTANCE IN *CANDIDA ALBICANS*

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■ **Abstract** The widespread deployment of antimicrobial agents in medicine and agriculture is nearly always followed by the evolution of resistance to these agents in the pathogen. With the limited availability of antifungal drugs and the increasing incidence of opportunistic fungal infections, the emergence of drug resistance in fungal pathogens poses a serious public health concern. Antifungal drug resistance has been studied most extensively with the yeast *Candida albicans* owing to its importance as an opportunistic pathogen and its experimental tractability relative to other medically important fungal pathogens. The emergence of antifungal drug resistance is an evolutionary process that proceeds on temporal, spatial, and genomic scales. This process can be observed through epidemiological studies of patients and through population-genetic studies of pathogen populations. Population-genetic studies rely on sampling of the pathogen in patient populations, serial isolations of the pathogen from individual patients, or experimental evolution of the pathogen in nutrient media or in animal models. Predicting the evolution of drug resistance is fundamental to prolonging the efficacy of existing drugs and to strategically developing and deploying novel drugs.

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INTRODUCTION

The emergence of drug resistance in all pathogenic microorganisms, including fungi, is an evolutionary process initiated by exposure to antimicrobial agents (65, 118). Resistance evolves because antimicrobial agents are rarely deployed in a way that completely eradicates the pathogen population, with survivors subject to natural selection. Whenever the pathogen population remains large enough over the course of drug treatment, the evolution of resistance is all but inevitable.

The evolution of drug resistance depends on genetic variability, the ultimate source of which is mutation. Once a mutation conferring resistance arises in the pathogen, its fate is determined by key population processes, such as selection, genetic drift, recombination, and migration (including transmission between hosts). The emergence and spread of drug resistance depends on more than the variety of different possible mutations that enable the pathogen to avoid, remove, or inactivate a drug. These resistance mutations interact with the rest of the genome to determine the composite phenotype (48, 131). In the evolutionary process the most important component of a resistance phenotype is reproductive output, or fitness. The relative fitness of sensitive and resistant genotypes determines how quickly resistance will spread in a pathogen population exposed to a drug and whether resistance will persist in the absence of the drug. Although biochemical and genetic mechanisms of antifungal drug resistance are well documented, the evolutionary processes by which these mechanisms spread and persist in pathogen populations await investigation.

Fungi are major pathogens of agricultural plants (86) and important opportunistic pathogens of humans (37, 81, 89), recently ranking as the seventh most common cause of infectious disease–related deaths in the United States (76, 94). Opportunistic fungal pathogens may cause superficial or invasive infections. The majority of invasive mycoses are caused by *Cryptococcus neoformans* and species of *Candida* and *Aspergillus* (94). Despite the increasing importance of opportunistic fungal pathogens, the number of effective antifungal drugs remains limited, with resistance compromising the effectiveness of all but the newest (37, 132).

Antifungal drug resistance has been studied most extensively with the diploid pathogenic yeast *Candida albicans*. *C. albicans* is a ubiquitous commensal, residing on the mucosal surfaces of the mouth, digestive tract, or genitourinary system of 15%–60% of healthy humans, depending on the sample group (82). *C. albicans* is also a good example of an opportunistic pathogen, causing both superficial infections and invasive fungal disease in immunocompromised individuals (32, 51, 103). Species of *Candida* are the fourth most common cause of nosocomial bloodstream infections in the United States, with *C. albicans* the most commonly encountered (91, 98).

C. albicans has been the fungal pathogen of choice for studying drug resistance because it is more easily manipulated and contained than other medically important fungal pathogens, such as *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Histoplasma capsulatum* (27). *C. albicans* is a wet yeast, classified as

a relatively low risk to research personnel. In common with other countries in the American Biological Safety Association (http://www.absa.org/riskgroups/fungi.htm), Health Canada considers *C. albicans* to be a Risk Group 2 agent, unlikely to be a serious hazard to healthy laboratory workers, the community, livestock, or the environment (http://www.hc-sc.gc.ca/hpb/lcdc/biosafty/docs/lbg4_e.html#4.6.2). As a research system, *C. albicans* also offers a range of molecular-genetic tools (27), a complete genome sequence (123), and a sufficiently close phylogenetic relationship to the model yeast system, *Saccharomyces cerevisiae*, that many genes and pathways have highly similar counterparts in both yeasts. Unfortunately, *C. albicans* is, as yet, not amenable to conventional genetic analysis. Although *C. albicans* cells rendered homo- or hemizygous for mating-type-like genes are able to mate, meiosis has not been observed (50, 74). This disadvantage can be overcome with complementary studies using *S. cerevisiae* as a genetic stand-in. It is notable that some of the key studies of drug resistance (18, 52, 56, 57, 99, 109, 115), dimorphism, and virulence (58, 68, 78, 133) have exploited this synergy. *S. cerevisiae* stands on its own as a pathosystem based on recent reports of its clinical isolation from a variety of body sites and patient groups, with subsequent successful infection using these isolates in animal models (44).

This review focuses on the evolution of antifungal drug resistance [for reviews of clinical, molecular, and biochemical aspects see (1, 28, 37, 56, 88, 109, 132)]. The evolution of antifungal drug resistance in the pathogen proceeds on temporal, spatial, and genomic scales. Temporal scales of evolution range from ancient events associated with speciation to contemporary changes occurring within one patient under treatment. Spatial scales range from large-scale global or regional populations of patients to fine-scale niches within the body of one patient. Genomic scales range from the small-scale interactions of primary resistance mutations with one or a few genes to large-scale interactions with many genes. Large-scale interactions result in substantial changes in genome-wide patterns of gene expression in the pathogen. The effects of small- and large-scale interactions are detected as changes in fitness. The introduction and spread in pathogen populations (recruitment) of genetic mechanisms conferring antifungal drug resistance on different temporal, spatial, and genomic scales can be observed through both epidemiological and population-genetic studies. Population-genetic studies are implemented with samples of the pathogen from patient populations, with serial isolations of the pathogen from single patients or, alternatively, through experimental evolution of the pathogen in batch cultures, chemostats, or animal models. In this review we consider the sources and recruitment of antifungal drug resistance in pathogen populations on these temporal, spatial, and genomic scales.

MECHANISMS OF ACTION OF ANTIFUNGAL DRUGS

There are a limited number of antifungal drugs, especially compared with the number of antibacterial drugs (29, 39, 110). Drug targets that distinguish pathogen from host are more difficult to identify in fungi than in bacteria, at least in part because

fungi and animals are relatively closely related as crown eukaryotes, whereas bacteria are much more distantly related to their human hosts (8). Because many potential antifungal drug targets have homologs of similar function and susceptibility to inhibition in humans, toxic side effects dramatically reduce the number of antifungal agents that can be used therapeutically. Currently available antifungals have only a small number of targets, including ergosterol and its biosynthesis, nucleic acid synthesis, and cell wall synthesis [for reviews of mechanisms of action of antifungal drugs see (28, 37, 56, 120, 125, 130)]. We summarize the salient features of the main classes of drugs.

Of the five classes of systemic antifungal compounds currently in clinical use, the polyenes, the azoles, and the allylamines all target ergosterol, the major sterol in fungal cell membranes that is not present in animals, whereas the fluoropyrimidines and the echinocandins have other targets (37). The polyenes complex with ergosterol in the fungal cell membrane and compromise the integrity of the cell membrane. Drugs in this class are fungicidal and have the broadest spectrum of activity of any clinically useful antifungal (37). The only systemic polyene in clinical use is amphotericin B, which has both acute and chronic side effects (28, 132). The azoles inhibit the cytochrome P450 enzyme, lanosterol demethylase, in the ergosterol biosynthesis pathway (56, 109). Ergosterol depletion and the accumulation of intermediates in the pathway disrupt the structure of the cell membrane, alter the activity of several membrane-bound enzymes, and may interfere with the hormone-like sparking function in the cell cycle (132). The azoles are fungistatic and have a broad spectrum of activity. Azoles include both the imidazoles (e.g., ketoconazole, as well as miconazole, which is now approved only for topical use) and the triazoles (e.g., fluconazole, itraconazole, and voriconazole), the group of antifungals most intensively under development (37, 110, 120). Systemic azoles are well tolerated and are generally free of serious host toxicities (28). The allylamines inhibit the enzyme squalene epoxidase in the ergosterol biosynthesis pathway. This class of drugs is fungicidal and has a broad spectrum of in vitro activity but has limited clinical efficacy owing to poor pharmacokinetics. Terbinafine is the only systemic antifungal in this class in clinical use (37). The fluoropyrimidines inhibit nucleic acid synthesis. Five-fluorocytosine (5-FC) is the only systemic antifungal drug in this class in clinical use and is fungicidal, with a limited activity spectrum (29). The echinocandins, the newest class, inhibit β-(1,3)-glucan synthesis, resulting in disruption of the fungal cell wall. Caspofungin is the only compound in this class approved for clinical use and is fungicidal with minimal host toxicities (38, 120).

Conventional drug screening procedures have resulted in the discovery and development of few compounds with unique modes of action, with caspofungin being the only one that has been commercially developed (38, 108, 120). Although the identification of novel drug targets has been facilitated by genomics technology (43, 75), resistance to all of the antifungal drugs that have been widely deployed is well documented.

A major public health concern is that the availability of antifungal drugs may not keep pace with the growing need. With the trend of increasing numbers of

immunocompromised individuals, especially as a result of HIV infection (79), the epidemiology of opportunistic fungal infections is volatile. While the introduction of treatment therapies for HIV, including protease inhibitors, has reduced the incidence of mucosal candidal infections among treated patients (30), these HIV therapies are associated with high toxicity (31, 49), may have negative interactions with antifungal therapies (29), and are vulnerable to resistance (36). There are two facets to the impact of this resistance on the incidence of fungal infections. Resistance in the virus, with resulting decline in the immune status of the host and associated increase in susceptibility to opportunistic infection is well documented (36). Surprisingly, resistance in the fungus may also be a factor. These same protease inhibitors also reduce the activity and production of *C. albicans*–secreted aspartyl proteases (19, 55), reduce adherence and invasion of *C. albicans* to epithelial cells (10, 55), and reduce growth of *C. albicans* both in vitro and in vivo (19). Still unknown is the evolutionary potential of the fungus to overcome the inhibitory effects of these antiviral drugs. The severity of the AIDS epidemic is compounded by political and economic circumstances restricting access to effective drugs (12). With limited access to both drugs and medical supervision, even those patients receiving treatment may not undergo a complete course of therapy, creating conditions that are favorable for the evolution of resistance. Management of opportunistic fungal infections is a challenging problem, the solution of which will depend on the discovery of new and effective drugs.

WHAT IS ANTIFUNGAL DRUG RESISTANCE?

Drug resistance is a complex manifestation of factors in both host and pathogen. From a clinical perspective, drug resistance may be defined as the persistence or progression of an infection despite appropriate drug therapy. The clinical outcome of treatment depends not only on the susceptibility of the pathogen to a given drug but also on factors including pharmacokinetics, drug interactions, immune status, and patient compliance, as well as several specific conditions such as the occurrence of biofilms on surfaces of catheters and prosthetic valves (132).

Evaluation of how the susceptibility of the pathogen to a drug contributes to clinical outcome of treatment requires that drug susceptibility be measurable and reproducible in vitro. Drug resistance can be measured as the minimum inhibitory concentration (MIC) that curtails the growth of the fungus under standardized in vitro test conditions (104). Before the development of a standardized protocol, MIC determination varied up to 50,000-fold among different laboratories (42). Interlaboratory reproducibility was dramatically improved with the development of the National Committee for Clinical Laboratory Standards (NCCLS) protocol for antifungal susceptibility testing of yeasts (100, 101). Even with a standardized method of susceptibility testing, MICs are only sometimes predictive of clinical outcome. For example, a correlation of in vitro susceptibility to azole drugs with clinical response was observed with mucosal candidal infections in HIV-infected patients (42, 100, 132). In contrast, azole MIC did not correlate with clinical outcome

for patients with candidal infections who were not infected with HIV (41). Despite cases of discordance between MIC and clinical outcome, MICs determined according to the NCCLS protocol have been used to determine interpretive breakpoints for fluconazole and itraconazole susceptibility in a classification system for clinicians (100, 102). Given that host variables, especially the immune status of the patient and the site of fungal infection, are likely to affect the efficacy of drug treatment, it is not surprising that the relationship between MIC and clinical outcome is complex. More surprising is the discordance between MIC and pathogen fitness in the presence of a drug in a simple laboratory environment (discussed in "Experimental Evolution," below).

Differences in antifungal drug susceptibilities among fungal individuals, populations, and species reflect different timescales of evolution (note that in yeast populations the individual is the cell). Antifungal drug resistance has been classified as either primary, when a fungus is resistant to a drug prior to any exposure, or secondary, when an initially sensitive fungus becomes resistant after exposure to the drug (102, 132). The major limitation of this classification is that lack of prior drug exposure can never be determined conclusively. Fungal species display intrinsically different susceptibilities to different drugs, with each species showing a distribution of MICs. For example, *Cryptococcus neoformans* and several species of *Aspergillus* are resistant to 5-FC (132). *C. lusitaniae* and *C. guillermondii*, as well as *Fusarium* and *Trichosporon* species, are more resistant to amphotericin B than is *C. albicans* (132). *C. neoformans* and several *Candida* species, including *C. krusei* and *C. glabrata*, are resistant to many of the azole drugs (37). Not surprisingly, species of *Candida* that are intrinsically more resistant to azoles have emerged as important opportunistic pathogens following the widespread deployment of these drugs (20, 80). Differences in intrinsic antifungal susceptibility between species reflect genetic changes that could have arisen before or after speciation, becoming fixed with continued reproductive isolation (7, 117).

On a more recent evolutionary timescale, many fungal species show a broad range of susceptibilities to a given drug, with genotypes that are sensitive and genotypes that are resistant. Populations within the species may have adapted to the drug in question after exposure to the agent at any point in their history. Depending on the extent of the fitness cost of resistance, the resistance phenotype may persist even in the absence of the drug. Resistant genotypes can be transmitted, as reported for fluconazole-resistant *C. albicans* strains among patients in the same hospital, as well as between sexual partners (132). On the most contemporary evolutionary scale is the emergence of drug resistance in an initially sensitive population of yeast cells that adapts to the drug in a patient over the course of treatment (discussed in "Serial Isolations from Individual Patients," below).

Initially sensitive fungal pathogens have become resistant to the clinically important antifungal drugs by a variety of molecular mechanisms summarized here [for reviews see (37, 56, 88, 109, 124, 132)]. Resistance to amphotericin B has emerged in both *Candida* and *Cryptococcus* species after exposure to the drug, but this is relatively rare (37). Fungal resistance to polyenes in general is associated

with altered membrane lipids, especially the sterols (37). Based on the number of reports, azole resistance is relatively widespread, especially in *C. albicans* and *C. dublinienesis* (132). Resistance to azoles is associated with mutation in, or overexpression of, the target enzyme *ERG11*, with mutations in other enzymes in the ergosterol biosynthetic pathway, with active efflux of the drug by overexpression of efflux pumps of the ATP-binding cassette transporter or major facilitator families, and with decreased membrane permeability due to alterations in membrane sterols (56, 109). Resistance to 5-FC develops frequently when it is used as the sole antifungal agent. Fungal resistance to 5-FC is associated with impaired cytosine deaminase or mutations in any of the enzymes necessary for 5-FC action, particularly phosphoribosyl transferase (132). The evolution of drug resistance requires the recruitment of such mechanisms in populations.

Studying the Evolution of Drug Resistance

The recruitment of mechanisms conferring antifungal drug resistance in pathogen populations is determined by population-genetic processes including mutation, selection, genetic drift, recombination, and migration (remembering that this includes transmission between hosts). Mutation is the source of genetic variation. The fate of a mutation in a population depends on both the fitness effect of the mutation and on the population size, which determines the relative contribution of selection and genetic drift to evolution. Adaptation may proceed by the accumulation of numerous mutations of small effect or by few mutations of large effect (16, 83). The degree of dominance of a mutation is also important in a diploid background, in which a fully recessive mutation would confer no immediate advantage. Genetic exchange and recombination shuffle mutations that occur in different individuals and combine beneficial mutations in novel combinations in a single individual. Recombination may contribute to efficient adaptation to novel environments (84, 141) or, if the sequence in which beneficial mutations occur is important, may actually retard the rate of adaptation (53). In populations with no genetic exchange among individuals, genotypes that carry different beneficial mutations compete with one another, a process that may interfere with the progression of a mutation to fixation (40). The emergence of resistance mechanisms in individuals and their proliferation in populations are studied in samples of pathogen populations on different temporal and spatial scales.

Sampling Pathogen Populations from Patient Populations

Trends in antimicrobial resistance can be monitored by sampling clinical isolates over long temporal and broad geographic scales. There are both national (91, 93) and international (90) surveillance programs to monitor the frequency of different pathogens encountered in the clinic and their susceptibilities to antifungal drugs. Surveillance data can be especially useful for spotting a trend, such as the pervasiveness of resistance to therapeutic agents, or a correlation, such as an association between treatment regimens (and patient compliance) with emergence of resistance.

The spread of certain strains can be monitored by resolving the genotypes of fungal pathogens recovered from patients (116). Of particular interest is whether strains of restricted origin have a higher degree of genetic similarity than strains of general origin. Restricted samples could include strains from the same anatomical site (72), patient population (137), hospital (92), geographic region (34, 69, 70, 138, 140), or strains with similar antifungal susceptibility profiles (25, 92, 139). For example, if strains isolated from health care workers' hands in an intensive care unit are identical to those isolated from indwelling catheters in patients, then sanitation measures can be specifically targeted to prevent this kind of transmission (107, 128). Alternatively, if isolates from these sources are as dissimilar as isolates randomly chosen from broader samples, then the emphasis remains on more general sanitation practices (126). In both cases, observations of the distribution of pathogen genotypes can improve prophylaxis, treatment, and prevention of pathogen transmission and thereby limit the spread of preexisting drug resistance.

Whether fungal genotypes based on molecular-genetic markers with no known relationship to drug resistance (neutral markers) can be predictive of drug resistance depends on the population structure of the pathogen. In highly clonal populations genomes are reproduced and transmitted intact. In this case neutral markers will predict drug resistance, provided that resistance is maintained as a stable trait in the population (2). Alternatively, in a population with a high frequency of genetic exchange and recombination, alleles that are not tightly linked are shuffled, and any association between neutral markers and drug resistance will decay with time (60). Most fungal species, including those with no known sexual stage in their life cycles, show evidence of genetic exchange and recombination (2, 116).

Inferences of genetic exchange and recombination in diploid genomes are not as clear-cut as for haploid genomes. Because *C. albicans* is diploid, recombination detected with nuclear markers does not necessarily reflect genetic exchange between individuals. The difficulty arises from the ability of a highly heterozygous individual to produce a plethora of genotypes through the intracellular processes of mitotic recombination, which include crossing over and gene conversion. Even in the complete absence of genetic exchange between individuals, mitotic recombination within diploid individuals of *C. albicans* leads to loss of heterozygosity (24) and can produce distributions of genotypes that are indistinguishable from those expected with genetic exchange between individuals (2). From the available evidence based on nuclear markers (45, 95, 119, 127), it is not possible to conclude that genetic exchange between individuals of *C. albicans* is ongoing or has ever been frequent; clonal reproduction clearly predominates in this fungus.

Unlike in diploids, recombination in haploid genomes necessarily reflects genetic exchange between individuals. In haploids recombinant genotypes for nonduplicated loci arise only with genetic exchange between individuals and not with mitotic recombination. Even in diploid fungi such as *C. albicans* the mitochondrial genome is effectively haploid and can be exploited for determining whether genetic exchange between individuals has occurred. Analysis of mitochondrial nucleotide sequence variation in a population of *C. albicans* from HIV-infected patients

identified infrequent past events of genetic exchange and recombination followed by clonal proliferation of genotypes (3), again indicating that clonal reproduction predominates. Despite the signature of past recombination in fungal pathogens, little is known about the actual rates or times of recombination. In the host even those species showing evidence for past genetic exchange generally reproduce clonally, with little or no evidence for recombination (59, 116). Taken together, the available evidence suggests that the evolution of resistance in fungal pathogens is more likely to occur by sequential accumulation of mutations within asexual lineages than by recombination of mutations that occurred in different lineages.

Only a few studies have attempted to find an association between drug resistance and molecular-genetic markers in *C. albicans*. Despite the predominantly clonal population structure in *C. albicans*, Pfaller et al. found that DNA fingerprints of fluconazole-resistant bloodstream isolates were no more similar than would be expected from a random set of clinical isolates (92). Cowen et al. showed that multilocus genotypes of oral isolates from HIV-infected patients were not predictive of azole resistance (25). These results suggest that resistance is labile and may be gained or lost too quickly to be predicted in the overall population of *C. albicans* by association with neutral markers. In contrast, Xu et al. identified a small group of genotypically similar fluconazole-resistant strains isolated from patients infected with HIV (139). This suggests that prediction of fluconazole resistance may be possible over a short timescale.

In all studies of pathogen populations based on samples from patient populations, evolutionary processes, including the evolution of drug resistance, cannot be observed directly but must be inferred from indirect evidence. By measuring allele frequencies in the pathogen population, parameters including mutation rates, recombination rates, intensity of selection, population size, and migration rates can be estimated but cannot be known with certainty or controlled. Although this approach provides important information about the distribution of antifungal resistance among genotypes in both pathogen and patient populations, it provides limited insight into the process or the genetic mechanisms underlying the evolution of antifungal drug resistance. Even a comprehensive sample, or census, of the general pathogen population over a long time and a wide geographical scale would be unlikely to provide these insights, given the diversity of the genotypes present and the complex patterns of descent and transmission. Sampling of *C. albicans* populations from individual patients over the course of antifungal drug treatment reduces this complexity and provides the opportunity to identify the emergent molecular mechanisms responsible for the evolution of drug resistance in a clinical setting.

Serial Isolations from Individual Patients

Serial isolations of the pathogen from individual patients over the course of drug treatment can be appropriate for tracking the increase in resistance over a shorter timescale, with the associated genetic alterations. This approach relies on identifying a series of isolates with increasing levels of drug resistance that are

determined by high-resolution DNA typing methods to be identical or very similar (132). In this case, in which the isolates are assumed to be clonally related, changes in genotype and phenotype that emerge over the course of treatment can be identified.

Studies based on serial isolations from patients have shown that the emergence of azole resistance in *C. albicans* is often an incremental process that involves the accumulation of multiple mechanisms of resistance (35, 109). For example, in one series of 17 isolates of *C. albicans* from an HIV-infected patient, a gradual increase in resistance was associated with specific molecular alterations: first, overexpression of the *MDR1* multidrug efflux transporter gene; second, overexpression and mutation of the target gene, *ERG11*, and mitotic recombination rendering the mutation homozygous; and third, overexpression of the *CDR* multidrug efflux transporter genes (132). Confirming that the specific genetic changes associated with the emergence of resistance in clinical isolates are causally related to the resistance phenotype relies on in vitro study of both *C. albicans* and *S. cerevisiae* (109, 132).

While providing a biologically realistic view of in vivo changes in drug resistance, there is at least one serious limitation to the use of serial isolations of fungal pathogens from individual patients. The chronological recovery of isolates from patients may not reflect the actual sequence of evolutionary events because evolutionary relationships among the isolates are inferred rather than known with certainty. For example, genotypes may emerge from quiescent reservoirs of the pathogen during relapses of infection. Also, as with studies of populations of the pathogen isolated from populations of the host, parameters including mutation rate, recombination rate, intensity of selection, population size, and transmission of genotypes can only be inferred.

Experimental Evolution

The dynamics and genetic mechanisms of the evolution of antifungal resistance can be more directly observed through experimental evolution of fungal pathogens in the laboratory than in samples of existing pathogen populations or serial isolations from individual patients. The main attraction of this approach is the ability to replicate experiments and to control conditions such as ploidy (73), size of population (129, 141), strength of selection (129, 141), rate of mutation (4, 54, 112, 141, 143), and opportunity for genetic exchange and recombination (141). Experimental evolution subjects populations to natural, as opposed to artificial, selection (105). Any fungal pathogen can be evolved in the presence of any antifungal drug in the laboratory, and both genotypic and phenotypic changes in the evolved populations can be measured relative to the ancestor. An introduction to the features of experimental evolution precedes a discussion of how this approach has been used to study the evolution of drug resistance in *C. albicans*.

Experimental evolution begins with one or more known genotypes and then follows a trajectory of change over time. With experimental populations founded from a single genotype, immigration of genotypes and genetic exchange between

individuals may be prevented so that mutation during the experiment is the only source of genetic variation. This allows the specific genetic changes that underlie adaptation and the temporal sequence in which they occur to be identified relative to a known ancestral genotype. Experiments with replicate populations of different sizes address the element of chance in adaptation. Similarly, experiments with replicate populations founded with different ancestral genotypes address the contribution of history to adaptation (122). Whether genetic background constrains the evolution of drug resistance or whether all genotypes show comparable ability to evolve resistance can be determined.

Starting from an ancestral genotype, the evolutionary trajectory of an experimental population depends on the availability of mutations. The two most important parameters affecting availability are mutation rate and population size. Because the number of cell generations is known in experimental populations and the occurrence of mutations can be bracketed in time, mutation rates can be determined (71). The fitness effects of mutations and the relationship of mutation rate to the evolutionary rate can also be measured (4, 113, 136, 141, 142). Given a constant mutation rate, population size becomes the most important determinant of adaptation. In a population of infinite size, evolution should be entirely deterministic, with all possible mutations available all of the time. At any given point, the fittest genotype is favored by natural selection. In reality, populations are not infinitely large. In populations of finite size, the emergence of adaptive mutations and the sequence in which they occur is often stochastic, with only a subset of all possible mutations available at any given time.

The effect of natural selection is more apparent in large populations than in small populations (129, 141). In smaller populations genetic drift is more likely to overwhelm selection, with some genotypes increasing in frequency by chance rather than by dint of their adaptive fitness. Although large experimental population sizes may be more appropriate for studying adaptation, in the real world pathogen populations are often bottlenecked to very small sizes during transmission between hosts; only a small fraction of the population infecting one host is transmitted and successfully colonizes a new host (11). If this population bottleneck is small enough, the best-adapted genotypes are not necessarily transmitted to a new host, especially if these genotypes are at a low frequency in the population (66). In addition, because of the physical association and relatedness of nearby individuals, the effective size of structured populations found within a host will always be smaller than that of unstructured populations with comparable numbers of individuals.

In populations of sufficient size, fitness (or reproductive output over a defined period of time) is expected to improve with adaptation to an environment (61). This expectation is not always met. Evolving populations can actually decrease in fitness relative to their ancestor (85). If genotype B has a fitness advantage over the ancestral genotype A and genotype C has a fitness advantage over genotype B, this does not necessarily imply that genotype C will have a fitness advantage over genotype A. This decrease in fitness could be attributed to negative epistatic interactions, in which observed fitness is less than fitness expected if sequentially accumulated adaptive mutations are additive in their effect in one environment.

Also, adaptation of a population to one environment may result in a decrease in fitness in another environment (a trade-off) (21, 26). It is often assumed that a pathogen that evolved with a drug will be fitter than its ancestor in the presence of the drug; resistant genotypes increase in frequency relative to their sensitive counterparts when the drug is deployed. On the other hand, if drug resistance carries a fitness cost for the pathogen, once the drug is removed, resistant microbes decrease in frequency relative to microbes that are more drug-sensitive but fitter.

Running an evolution experiment requires conditions that are conducive to the evolution of the population and that are appropriate to the experimental question. This requires fine-tuning. Drug concentrations must be adjusted to substantially inhibit the growth of the fungus without resulting in extinction. If the drug under investigation is fungicidal, then the range of possible concentrations is more limited than if the drug in question is fungistatic. Cross-resistance can be observed by testing populations previously adapted to one drug for resistance to another drug, or by evolving populations simultaneously in the presence of different drugs. The stability of resistance can be monitored by subsequent evolution in the absence of any drug.

Setting a timescale for the evolution experiment depends on the question. Evolution experiments of a few hundred generations reflect a timescale approximating that of a pathogen evolving drug resistance in its host during a course of drug treatment. Evolution experiments over thousands of generations test whether the populations ultimately converge on one stable, adaptive optimum (62).

Finally, a choice must be made between an unstructured (Figure 1) and a structured (Figure 2) environment. The host is a spatially structured environment, not

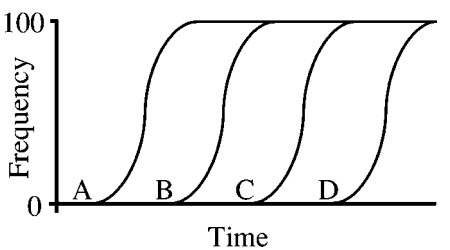

Figure 1 Evolution in an unstructured environment, such as a chemostat or batch culture with constant agitation. Spatial associations between individuals are continually disrupted. In large populations, with strong selection and rare mutation, evolution often proceeds as a series of sweeps in which an adaptive mutation (designated A, B, C, and D) rises to fixation and then serves as the background genotype for another adaptive mutation, which then also rises to fixation [see (134) for an excellent example]. The predominant genotype at the end is ABCD. Because mutations A, B, C, and D all reach fixation quickly and because these mutations accumulate within one lineage, determining their order of occurrence is easily reconstructed by regular sampling of the population over time.

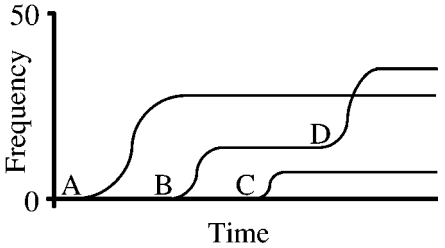

Figure 2 Evolution in a highly structured environment, such as an animal host. Physical and spatial associations between individuals are rarely disrupted. This population may also be spatially subdivided into groups between which migration is restricted. Adaptive mutations (designated A, B, C, and D) therefore tend to remain at low frequency and do not sweep to fixation. Different genotypes may predominate in different spatial sectors, and genotypes may also persist for periods of time in reservoirs without reproducing. Under these conditions an adaptive mutation only rarely (e.g., in the case of mutation B) serves as the background for subsequent mutations. The most frequent genotypes at the end are A, BD, and C. Because mutations A, B, C, and D never approach fixation and because these mutations (except B and D) occur in separate lineages (as a radiation), determining their order of occurrence would not be easily reconstructed by regular sampling of the population over time.

a flask full of liquid. Most experimental evolution studies have evolved microbial populations in liquid media either in a chemostat or in batch cultures that are serially transferred at regular intervals into fresh medium (121, 141). The chemostat affords more uniform conditions, whereas the batch culture affords a higher degree of replication. Despite spatial heterogeneities that may arise from differences between the surface of the test tube or chemostat and the agitating medium, this environment represents our closest approximation of an unstructured system. One would expect a succession of genotypes to increase in frequency in the population. In contrast, a spatially structured environment may favor radiations if different genotypes predominate in different niches (96, 97). Even the surface of a petri dish provides a structured environment in which genotypes are spatially segregated rather than in direct competition. Animal models also provide a structured environment, but with the added factors of the host physiology and immune system.

The biofilm is another important type of naturally occurring, spatial structuring. Fungal biofilms represent a structured environment with highly heterogeneous architecture. *C. albicans* can form biofilms in vivo as well as in vitro. Biofilms are important clinically because their development is associated with increased antifungal resistance (19a, 97a, 97b). Several in vitro models have been developed to study biofilm growth processes, architectural organization, and antifungal resistance. These models use either 96-well microtiter plates or bioprosthetic materials (19a, 97a, 97b). Such models could be used to determine whether the increased drug resistance associated with *C. albicans* biofilm formation is a consequence of

the production of extracellular material that physically interacts with antifungal drugs or whether it is due to genetic and biochemical alterations of the fungal cells.

Studies of experimental evolution of fungi [reviewed in (141)], bacteria [reviewed in (121, 129)], and viruses (134, 135) have focused on the evolutionary dynamics of adaptation to general culture conditions. Few studies have addressed the dynamics of adaptation to antimicrobial drugs over a timescale of hundreds of generations. Experimental evolution of drug resistance recapitulates a realistic process in fungi in which mutations conferring resistance arise within a lineage. There is no conclusive evidence of horizontal transfer of antifungal drug resistance between lineages (106). In comparison, given the extensive horizontal transfer of determinants of antibiotic resistance, studies of bacteria have focused on the fate of resistance once it has evolved rather than on the emergence of resistance.

We have studied the dynamics and mechanisms of evolved resistance in both *C. albicans* and *S. cerevisiae*. In *C. albicans* (24) 12 replicate populations were established from a single drug-sensitive cell and were serially transferred each day over 330 generations. There was no genetic exchange among individuals and no migration between populations. Mutation during the experiment was the only source of genetic variability. Six populations were evolved with fluconazole adjusted to twice the minimum inhibitory concentration (MIC) measured at regular intervals, and six were evolved without the drug. All of the populations evolved with fluconazole adapted to the presence of the drug as indicated by an increase in MIC. Their evolutionary trajectories, however, were strikingly different, with each population reaching different levels of drug resistance. The populations showed distinct overexpression patterns of the four genes known to be involved in azole resistance (*CDR1*, *CDR2*, *MDR1*, and *ERG11*). In this experiment chance, in the form of the random occurrence of mutations that confer an adaptive advantage, was a major factor in the evolution of drug resistance. Because the minimum population size with each transfer was large (10^6 cells), genetic drift was less likely a factor than the randomness of mutation in explaining the divergence of trajectories.

We observed a different pattern in haploid experimental populations of *S. cerevisiae* that were 10 times as large (minimum population size was 10^7 cells) as those of *C. albicans*. Three uniquely marked populations were established from a single genotype and were evolved with increasing concentrations of fluconazole. Again, there was no genetic exchange among individuals and no migration between populations. Over 400 generations the three populations followed parallel adaptive trajectories with the same underlying molecular mechanisms of azole resistance, including overexpression of the ATP-binding cassette transporter genes *PDR5* and *SNQ2*. Genetic analysis showed that each population had recruited the same two determinants of fluconazole resistance, one of a large effect occurring first in the evolution experiment and the second of a smaller effect occurring later (J.B. Anderson, unpublished data). The more uniform trajectories in *S. cerevisiae* than in *C. albicans* may have been due to differences in population size, ploidy, or the frequency of mutations contributing to resistance.

Selection under incremental increases in drug concentration and selection under one step at a high drug concentration will likely yield different mechanisms of resistance. Under incremental steps the pathogen adapts, whereas under one-step selection the pathogen has no opportunity for compensatory adaptation. To test this hypothesis, starting with the same genetic background of *S. cerevisiae* as was used in our evolution experiment, we selected fluconazole-resistant mutants by plating cells on medium containing the highest concentration of fluconazole that was reached during the 400 generations of evolution. Each of three resistant mutants recovered from the one-step selection overexpressed the target gene of the azoles, *ERG11*, in contrast to the ones selected over 400 generations of evolution, which overexpressed the ATP-binding cassette transporters *PDR5* and *SNQ2*. Genetic analysis showed that each of the three mutants harbored one mutation and that these three mutations were allelic. Clearly, different mutations are favored in the different selective regimes using the same drug. This means the evolutionary outcome depends on how the drug is deployed.

In another one-step selection experiment Perepnikhatka et al. (87) exposed drug-sensitive clones of *C. albicans* to a high concentration of fluconazole on solid medium and recovered resistant mutants. These mutants were associated with specific chromosomal alterations. Whether these specific chromosomal alterations are directly related to drug resistance is not yet known. Barchiesi et al. (9) conducted a serial transfer experiment in which one replicate of an initially sensitive clone of *C. tropicalis* was evolved in each of three different concentrations of fluconazole; resistant mutants were recovered that showed different degrees of resistance depending on the concentration of the drug used for selection. Fluconazole resistance was associated with overexpression of the multidrug efflux transporters *MDR1* and *CDR1*.

The changes in candidate genes and their expression are not sufficient to explain the molecular basis for adaptation (111). We used DNA microarrays to monitor genome-wide changes in gene expression that became established during adaptation to fluconazole in four populations of *C. albicans* and persisted in the absence of the drug (23a). Although specific resistance determinants in *C. albicans* are well characterized, we did not anticipate the magnitude and extent of changes in genome-wide gene expression occurring with the evolution of drug resistance. Gene-expression profiling identified 301 open reading frames with expression levels that were significantly changed relative to the ancestor. Among the genes of altered expression, there were three distinct patterns underlying adaptation to the drug. One pattern was unique to one population and included overexpression of *CDR2*. A second pattern occurred at a late stage of adaptation in three populations. For two of these populations profiled earlier, as well as later, in their evolution, a third pattern was observed at an early stage of adaptation. Both the early- and late-stage patterns of gene expression included overexpression of *MDR1*. The succession of the early- and late-stage patterns of gene expression must represent a common program of adaptation to fluconazole. The same three

patterns of gene expression were also identified in fluconazole-resistant clinical isolates of *C. albicans*.

Bearing in mind that mutations and persistent changes in genome-wide gene-expression patterns are recruited through natural selection, the question is whether a drug-resistant genotype will persist or go extinct. This depends on the relative fitness of resistant and sensitive genotypes. In bacteria and viruses investigations of the fitness cost of drug resistance have focused on the relative rates of growth, survival, and competitive performance of sensitive and resistant genotypes by means of pairwise competition experiments in vitro or in animal models. In most cases resistance confers a fitness cost in the absence of the drug (13). During subsequent evolution, however, the cost of resistance tends to decline as the organisms adapt to the environment (with or without the drug), compensating for the fitness cost of their drug resistance determinants (66).

Evidence from our experimental populations of *C. albicans* indicates that adaptations that reduce the fitness cost of drug resistance may also be common in fungi (23). The populations that were evolved with fluconazole diverged in fitness, measured by direct competition with a genetically marked version of the ancestor, and any cost of resistance was eliminated with further evolution. Two drug-resistant populations were fitter than the ancestor even in the absence of the drug. Compensatory evolution mitigating the cost of resistance is expected, rather than the reversion to sensitivity, when there are more possible mutations that improve fitness of the organism than back mutation alone (64). Compensatory evolution would be thwarted by frequent recombination that separates the compensatory mutation from the resistance mutation provided that the loci involved are not tightly linked. If fungal pathogens do not regularly undergo genetic exchange and recombination, for example when they are growing in the host, then compensatory evolution should proceed.

Pathogens showing a higher MIC of a drug are assumed fitter in the presence of the drug than those with a lower MIC. Despite the logic of this expectation, in the populations of *C. albicans* evolved with fluconazole we found cases of high fitness in the presence of the drug with low MIC, as well as of low fitness with high MIC [see Figure 1 in (22)]. Three of the six populations evolved with fluconazole decreased in MIC during continued evolution at the high drug concentration (24). In no case was this reduction in MIC accompanied by a reduction in fitness measured at the high drug concentration (23); in fact, in one case the drop in MIC was accompanied by a significant increase in fitness. The population with the largest fitness advantage in the presence of the highest concentration of drug used in this experiment had only an intermediate MIC. Finally, two of the three populations with the highest MIC did not show a fitness advantage in the presence of the drug. Because azoles are fungistatic, not fungicidal, the drug-sensitive ancestor grows well at concentrations above its MIC. We have also observed this phenomenon in *S. cerevisiae* (Figures 3 and 4). In *C. albicans* competitive fitness is not predicted by MIC or by growth parameters, such as exponential growth rate and stationary phase cell density, of either competitor in isolation. The discordance between MIC

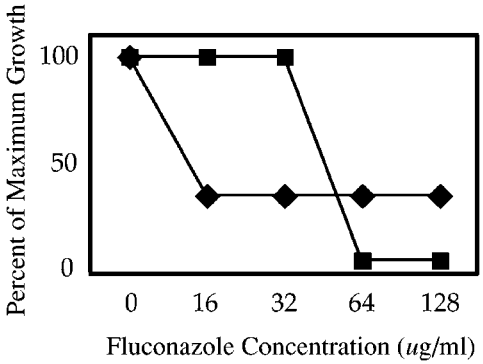

Figure 3 Minimum inhibitory concentration (MIC) is not always predictive of growth at high drug concentration. One hypothetical strain (*diamonds*) has a low MIC (16 μg/ml fluconazole) according the National Committee for Clinical Laboratory Standards protocol but continues to grow at the highest drug concentration. Another hypothetical strain (*squares*) has a high MIC (64 μg/ml) according the National Committee for Clinical Laboratory Standards protocol but grows poorly at the highest drug concentration.

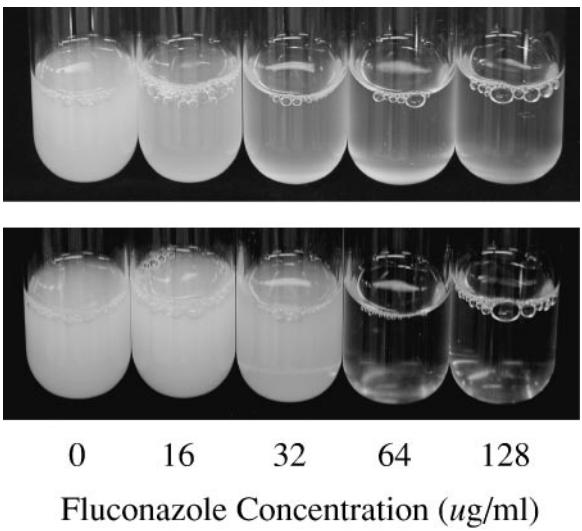

Figure 4 Growth of two strains of *S. cerevisiae* in separate cultures with various concentrations of fluconazole. One strain (*top panel*) with a low MIC (16 μg/ml fluconazole) continues to reproduce even at the highest concentration of the drug. Another strain (*bottom panel*) with a higher MIC (64 μg/ml) shows less reproduction at the highest concentration of the drug.

and fitness may be due to conditions in the tests. Our MIC tests use one starting genotype, static conditions, and small populations. Our tests of relative fitness use more than one starting genotype, constant agitation, and large populations. In the end, we suspect that interactions between mixed genotypes may also affect fitness in competition, but these interactions are not a factor in MIC tests.

The next step will be to study the evolution of drug resistance, with emphasis on fitness, in animal models. This has not yet been done with fungi, for which work with animal models has focused on measuring and comparing the virulence of mutants. Goldstein & McCusker used *S. cerevisiae* as a model pathogen to identify highly conserved signal transduction and metabolic pathways critical for survival in the host environment (44). Dominant drug-resistance markers were used to delete selected genes involved in metabolism and dimorphism in a clinically derived *S. cerevisiae* strain. Competitions were conducted in mice between a genetically marked control strain and two mutants, each homozygous for a deletion of the same gene but with different dominant drug-resistance markers. In addition to determining the importance of the selected genes for survival in the host, the fitness effects of a mutation conferring resistance to 5-FC were determined. Resistance to 5-FC was conferred by deletion of cytosine deaminase, encoded by *FCY1*. Deletion of *FCY1* was not deleterious but was actually advantageous in the mouse (44). This model could be applied to measure the fitness of *S. cerevisiae* mutants resistant to other antifungal drugs. Treating mice with the drug during the course of the competition experiment can test the relationship between MIC and fitness in vivo.

A system is also being developed to study genomic changes in *C. albicans* in a mouse model. Starting with *C. albicans* strains in which both copies of *GAL1* were deleted, this same gene was placed at various locations in the genome as a selectable marker. Loss of the introduced *GAL1* allele is expected with several different mechanisms of mitotic recombination, including gene conversion, crossing over, and other kinds of genome rearrangements. *GAL1* can be used for in vivo studies because it has no effect on virulence. The marked strains of *C. albicans* can now be passaged in mice, or in laboratory medium, in order to measure rates of mitotic recombination both in vivo and in vitro (P.T. Magee, personal communication).

PREDICTING THE EVOLUTION OF ANTIFUNGAL DRUG RESISTANCE

Mathematical models are usually the first step toward quantitative description of a complex system. If the assumptions of the model are realistic, the model will have predictive power. Most of the models of resistance to antifungal agents have been developed for fungicides in plant disease epidemics (epiphytotics). These models share common features with models of resistance to antibacterial (5, 14, 63) and antiviral (15) drugs in pathogens of humans and are useful in thinking about models of antifungal drug resistance. We consider two models of fungicide resistance

based on different assumptions. The first model applies both deterministic and stochastic formulations to analyze the dynamics of competition between resistant and sensitive strains, the supply of susceptible host tissue, the rate of application of the fungicide, and the effects of the fungicide (46). Two strains of the pathogen are considered, one sensitive and one resistant. The model assumes that there is spatially homogeneous mixing of the pathogen in the crop and no mutation or recombination. The model derives thresholds for the invasion of fungicide resistance in a pathogen population within a single season. The threshold depends on both the relative fitness of the sensitive and resistant strains and on the fungicide efficacy; fungicide efficacy is affected by the fungicide decay rate, the amount of fungicide applied, and the period between applications. This model predicts a lower probability of resistance and a longer lag time before resistance appears with the application of lower amounts of fungicide.

The second model applies stochastic formulations to relate pathogen population size, the probability of fungicide-resistant individuals occurring, and the strength of selection for resistance, or fungicide efficacy (77). Again, there are only two genotypes, one resistant and one sensitive. Unlike the first model, pathogen growth is assumed to be exponential (availability of susceptible host tissue is not considered), and single mutations to resistance are allowed to occur (mutations to resistance in multiple individuals are ignored). The model also assumes that resistant and sensitive genotypes have equal fitness in the absence of the fungicide and that the fungicide has no inhibitory effect on resistant mutants. With small pathogen population size and slow pathogen growth rate, the model predicts that the probability of resistant individuals occurring is low. With a low mutation rate, resistant individuals are unlikely to appear until the population reaches a large size later in an epidemic. When the first resistant mutants occur late in the epidemic, there is little time for selection to act, and thus resistance remains at a low frequency. This model also predicts that when a population is kept small by an effective fungicide, the probability of resistance mutations occurring is low. In contrast to the first model, the second model predicts that given an initially small pathogen population size at the beginning of an outbreak of infection, the probability of developing resistance is lower when fungicide is applied intensively (implying both high fungicide concentration and frequency of application).

Models are necessarily simplifications of complex systems. While the effect of simplifying assumptions on predicted outcomes can be tested (67), the predictive power of any model also depends on accurate estimates of parameters. These parameters are often estimated from epidemiological studies, ideally on long temporal and broad geographic scales (6). Studies of experimental evolution are also especially useful here: population size, generation time, rates of different mutations conferring resistance, and the relative fitness of resistant and sensitive genotypes can be accurately measured under a variety of controlled laboratory conditions.

In addition to providing an empirical basis for setting parameters in modeling, experimental evolution can actually measure evolutionary potential. The potential for evolution of antifungal resistance can be measured at both the level of the

gene and of the genome by evolving DNA sequences or microbial populations (22). Evolutionary potential at the level of the gene—of fine-scale interactions of primary resistance mutations within a gene—depends on the DNA sequence space (33) defined by the fitness effects of all possible mutations in candidate resistance genes (i.e., drug targets) in all possible combinations. This sequence space can be explored by evolving candidate resistance genes in vitro with sexual polymerase chain reaction (PCR), or DNA shuffling (47, 114), and then measuring the fitness effects of single and various combinations of multiple mutations. Sexual PCR explores sequence space far surpassing the range of mutational possibilities likely to be explored in natural populations. It is the mutational neighborhood (17) a small number of mutational steps away from the ancestral sequence that is key to predicting the resistance mutations that might arise in natural populations.

Evolutionary potential at the level of the genome—of large-scale interactions with many genes—depends on the composite of genome-wide changes in genotype and gene expression that accompany the evolution of resistance, as well as the impact of these changes on the fitness of the pathogen. Both rare and frequent adaptive changes can be identified through experimental evolution of fungal populations with inhibitory concentrations of any antifungal drug. Genes that are always associated with resistance to particular drugs, either through altered expression or through mutation, may represent keystones in the evolution of drug resistance. Whether or not these candidate keystone genes are critical to the development of drug resistance can be tested by knocking them out individually and measuring the ability of the resulting genotype to evolve resistance. Genes whose deletion results in impaired evolvability are potential targets for companion drugs designed to minimize the evolution of resistance to existing antifungals.

The most compelling rationale for investigating the evolution of drug resistance is to find ways to prolong the efficacy of existing drugs and to strategically develop and deploy novel drugs. Managing the evolution of resistance in the course of drug deployment will require a convergence of traditionally disparate disciplines, which must include evolutionary biology and experimental evolution. Mathematical modeling is the instrument for evaluating the effect of intervention strategies, including reducing pathogen transmission, increasing prophylactic use of drugs to keep pathogen populations small, cycling drugs with different targets, and minimizing the use of antimicrobial drugs by finding minimum effective dosages. In tandem with modeling, experimental evolution provides biologically realistic values for parameters used in models, bearing in mind that experimental populations can be reared in test tubes or in animals. These parameters will only be approximated from epidemiological sampling of patient populations, although such samples can survey long periods of time and large geographical areas. Adaptive changes in genes and genomes can be directly observed only in experimental populations. The challenge in interpreting genome-wide changes will be teasing out those changes that are directly related to drug resistance from those that are collateral. The ultimate challenge will be in developing models that are predictive in treating patients.

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