Seeking Antifungal Drug Synergies

The search for synergy starts with direct comparisons of chemical pairs but extends to gene disruption strain collections

Malcolm Whiteway

Vaccines and antibiotic treatments have generated amazing successes in our battle against infectious diseases. However, diseases such as AIDS and SARS emerge, while diseases such as tuberculosis continue to be important public health problems. Thus we need to identify new means and approaches to deal with infectious diseases. One promising approach against pathogens is to use drug combinations, borrowing from a strategy that works so well against HIV, the virus that causes AIDS. While single compounds proved ineffective because HIV develops resistance so rapidly, the simultaneous use of several drugs that target different viral processes changes HIV infection from a death sentence to a chronic disease—less than an ideal outcome, but a great improvement for the patient.

The logic of combination therapy is also being applied to the fungal pathogen Candida albicans, a leading cause of opportunistic infections.

On the positive side for this approach is the potential for synergy, where two drugs hitting different targets could be effective at doses lower than either would work at separately, potentially reducing side effects and maybe lowering costs. Further, this strategy might lower the chance of generating resistance, and in cases where two approved drugs are being combined, there could be regulatory advantages when not starting the development process from scratch. However, the potential for complicated interactions with other medications is likely greater when two or more anti-infective agents are added to the mix.

Combinatorial Strategy for Combating Fungal Infections

Drugs for combating fungal pathogens are currently limited in terms of variety. The predominant antifungal drugs include the polyenes and azoles, both of which target the fungal cell membrane, and the echinochandins that target the cell wall. Because fungi are eukaryotes, other, more central physiologic processes that are just as likely to be the same in the host, do not make good drug targets.

Beyond the simple need for new targets, widely used first-generation azoles such as fluconazole are coming off-patent, making other compounds that could work in combination with azoles attractive to drug developers. Although fluconazole is effective, it is fungistatic rather than fungicidal, meaning it blocks proliferation but does not kill. However, if combined with another antifungal drug, its use might lead to efficient killing of Candida cells, which would improve matters for infected patients. Further, making an improvedazole to combine with another drug could be an easier challenge than would be

Summary

- Drug combinations are being sought to combat fungal pathogens such as Candida albicans, a leading cause of opportunistic infections.
- The predominant antifungal drugs are limited, and include the polyenes and azoles, both of which target the fungal cell membrane, and the echinochandins that target the cell wall.
- Many compounds that synergize with fluconazole against fungal pathogens in high-throughput screens prove to be antiseptics or other nonspecific membrane disrupters.
- A promising approach to screening for candidates that are synergistic with azole antifungal agents entails use of gene disruption strain collections to identify promising targets.

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identifying a new class of antifungals. In any case, this approach opens the possibility of identifying compounds that, while not effective as stand-alone drugs, have therapeutic potential when combined with other antifungal entities.

There are various ways to search for compounds that synergize with fluconazole (Fig. 1). The simplest strategy is to conduct high-throughput screening (HTS) in the presence of this azole to identify combinations that kill the pathogen. C. albicans is simple to grow in liquid media, making it well suited to HTS. For example, we tested 2,500 drug-like molecules for synergy with fluconazole against a standard strain of C. albicans. Cells treated with fluconazole alone, although blocked in growth, could recover when transferred to a fresh growth medium; cells that did not recover were considered as having been exposed to a potentially fungicidal combination. We then retested the potentially fungicidal pairs of candidates in reaction wells in which the concentrations of fluconazole and the interacting compounds were systematically varied. Many of these compounds proved synergistic with fluconazole. Those combinations inhibited growth or killed cells at concentrations at which either of the individual drugs had little or no effect (Fig. 2).

Many of these synergizing compounds were antiseptics that likely either improved uptake of fluconazole or helped it in disrupting membrane integrity. In either case, antiseptics cannot be used safely to treat systemic disease, ruling out this class of compounds. However, we also identified more promising synergies between fluconazole and various other compounds, which acted through different mechanisms. For example, some of these compounds activated G protein coupled receptors. Understanding how these and other chemical compounds, when combined with fluconazole, can kill C. albicans cells could lead to the development of novel antifungal drugs.

Other Approaches for Seeking Synergistic Antifungal Agents

While direct HTS for synergistic compounds seems simple, it has drawbacks. One limiting factor is the chemical library itself. The more extensive the library, the greater is the chance for success. However, testing large numbers of compounds can prove costly in terms of time and resources.

Informatics-based strategies to predict synergies can help to focus the testing of compounds, but this approach requires access to extensive databases. Another alternative is to screen synergistic

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**FIGURE 1**

(A) Overview of some drug screening approaches discussed in the text. In high-throughput screening (HTS), a drug of choice (in this example fluconazole, FCZ) is combined with drugs from chemical libraries. The ideal outcome here would that a particular FCZ-drug combination kills WT cells whereas each drug alone is not sufficient to cause cell death. (B) Combining clinically approved drugs with drugs that act against known targets involved in drug resistance (e.g. drug efflux pumps, ARF cycling, HSP90 etc.), might potentiate the action of the starting drug. In our hypothetical example, FCZ is combined with a drug that blocks drug efflux pumps (red circle), which results in reduced efflux of FCZ and eventually cell death. (C) In chemical genetics we are initially interested in finding mutants that cannot survive in the presence of FCZ, a drug that normally does not kill WT cells (left). Once we have identified a mutant that dies when treated with FCZ (middle), we identify the deleted gene and use HTS to find an inhibitor against this gene (right). If successful, we can either combine this inhibitor with FCZ and test for synergy, i.e. if WT cells survive this drug combination. Alternatively, we can start over again and search the mutant library for mutants that cannot survive in the presence of the newly identified inhibitor.
targets. These targets can be identified by analyzing the biology of the pathogen. In the case of *C. albicans*, an analysis of chaperonin functions led investigators to recognize that the Hsp90 protein plays an important role in the development of drug resistance. Further studies suggest that inhibitors of Hsp90 could enhance fluconazole action. More specifically, the Hsp90 inhibitor geldanamycin displays potent antifungal synergy with fluconazole.

Yet another approach entails screening genetic targets systematically (Fig. 1). For instance, we can combine fungal strains with specifically disrupted genes directly with drugs of interest to identify synergistic interactions. The most comprehensive collection of disruption fungal strains is of *Saccharomyces cerevisiae*, which, although not a pathogen, is susceptible to fluconazole. Experiments follow the same logic as in the synthetic lethal strategy, where pairs of disrupted functions are combined to establish whether double mutants show a greater growth defect than do individual

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**Whiteway: from Ridiculous Theories to Facts, Fungal Pathogens, and Old-Timers Hockey**

Malcolm Whiteway’s father was always challenging his sons with “apparently ridiculous theories that were remarkably difficult to shoot down,” Whiteway recalls. For example, that the best way to get more cars across a busy bridge was to have the vehicles drive more slowly, putting them closer together and enabling more to cross at one time. “We learned as kids that you had to look at the facts, you had to be able to defend your positions, and that it was fine to challenge authority at an intellectual level.”

Whiteway, who is a principal research officer for the National Research Council in Montreal, Quebec, Canada, plans to move across town soon to join the new genomics institute at Concordia University. Once there, he plans to continue studying the pathogenic fungus *Candida albicans*, which can be deadly, he says. “Finding out how it works can have direct implications in drug discovery.”

Whiteway, 57, grew up in Dartmouth, Nova Scotia, across the harbor from the provincial capital Halifax, in a suburban neighborhood on the edge of a woods, with a stream and swamp at the end of his street. He spent long hours in the swamp catching frogs and snakes, and chasing butterflies. “Dad was an avid fisherman and hunter, so I also did a lot of trout fishing and a bit of partridge hunting,” he says. “My parents had a camp, and I was a Boy Scout as well, so I was immersed in the natural world.”

Like his chemist father, Whiteway was drawn to science when he began college. “I was probably heading towards chemistry when I was introduced to genetics,” he says. “Genetics had two appeals. There were simple rules you could apply to lots of problems, whereas chemistry seemed to be memorizing reaction mechanisms with little logic. To a naive undergrad, chemistry seemed essentially completed, while genetics and biology seemed full of unanswered questions.”

He earned his B.Sc. in 1977 from Dalhousie University in Halifax, and his Ph.D. in 1983 from the University of Alberta in Edmonton. He then moved to the Dana-Farber Cancer Institute and later to the Massachusetts General Hospital. “I have had the privilege of interacting with some amazing researchers and individuals over the years, but my time in Jack Szostak’s lab in Boston was a defining experience,” he says. “The lab was doing amazing things with telomeres and recombination. While . . . guys like Jack were in a class by themselves, as the recent Nobel Prize attests, I did not feel out of place. Coming from a modest graduate career in Edmonton, it was nice to look around a powerhouse Harvard lab and not be totally overwhelmed.”

Whiteway is married to Laura Glew, and they have two children, a daughter at the State University of New York in Buffalo and a son at McGill University in Montreal. In his spare time, Whiteway plays old-timers hockey year-round—twice per week in the winter, and once per week in the summer. In the evenings, he and his wife play Scrabble, or he reads, he says. “As a kid, I read a lot, mostly sci-fi, but now reading is more nonfiction. I loved *The Eighth Day of Creation, Invisible Frontiers, A Feeling for the Organism, Natural Obsession, Wonderful Life*—all great books on the process of doing science and the joys of discovery.”

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mutations. Synthetic lethal screens can be made more systematic by applying synthetic genetic array technology, which yields an almost comprehensive picture of genetic interactions in this organism.

Once a drug-gene combination is identified in S. cerevisiae, it becomes possible to test the equivalent combination in the fungal pathogen C. albicans. Unfortunately, however, gene-drug combinations identified in yeast do not regularly reflect the situation in this or other pathogenic fungi. For example, we identified more than 20 disruption strains in yeast, which, when combined with fluconazole, generated a lethal phenotype. However, this group of strains contained only a single gene for which the equivalent mutation in the C. albicans showed comparable killing.

Challenges in Working with Gene Disruption Collections of C. albicans

Although it is preferable to screen pathogens directly for drug-gene interactions, C. albicans collections are more of a challenge to prepare than is making the equivalent collection in S. cerevisiae. The diploid nature of C. albicans requires each of two alleles to be disrupted to produce a single homozygous disruption strain.

The most extensive such collection of C. albicans is known as the GRACE strains, which consists of more than 2,350 strains where individual genes can be conditionally inactivated through repression of a tet-regulated promoter. Other collections contain homozygous disruptions of a variety of nonessential genes. Altogether, these collections allow the systematic analysis of about 3,000 genes, accounting for about 50% of the C. albicans genome.

In theory, a comprehensive collection of disruption mutants would allow one to examine all the single targets available in the organism, as disrupting a gene leads to a loss of function that would be replicated only by an inactivating chemical that was totally effective in blocking the function of its target.

In practice, however, access to even a complete disruption collection does not allow one to assess every possible target. Some functions are duplicated and thus not eliminated by single mutants, other targets such as the plasma membrane are not the product of a single gene, and essential genes are not available for this particular assay. While the products of essential genes should make excellent targets for single-compound antifungal drugs, it is also possible that less than complete inhibition of an essential function could synergize when another function is also being blocked, and thus partially inactivated essential genes would be a useful addition to any systematic screen.

Overall, identifying the set of genes whose absence synergizes with a drug of choice will provide an extensive collection of potential targets. Compounds that inactivate the protein product of the disrupted gene would be expected to synergize with the selected drug in the same way as the gene disruption.

Experiences Screening Disruption Mutants

Screening collections of disruption mutants for synergy with a drug of choice is relatively straight-
forward and can prove productive. For example, we recently identified several potential targets after screening fewer than 1,000 strains of the noncommercial disruption libraries of *C. albicans* for fluconazole synergy (Fig. 3). The real challenge is to find compounds that act on the targets that we identified from these knockout screens. When we looked for gene-fluconazole interactions in disruption strains of *S. cerevisiae* model, we identified only AGE3 as a candidate target.

However, we know that Age3p is a component of the ARF (ATP ribosylation factor) GTPase cycle. This fact suggested to us that brefeldin A, which also inhibits this cycle and blocks the transport of proteins across membranes, might synergize with fluconazole. Subsequent testing showed brefeldin A interacts with fluconazole to effectively kill even drug-resistant strains of *C. albicans*. Thus, a sensible approach is to give high priority to targets for which there is an already known inhibitor. In these cases, the genetically established evidence for the validity of the target allows for a more sophisticated follow-up of candidate drugs than is likely from a direct HTS screen. If a known inhibitor of the target did not synergize as expected, it could be due to problems with getting the drug to the target or keeping the drug stable—problems with which medicinal chemistry can deal.

One major challenge is to convert other genetically identified targets into candidate compounds. One way to look for such compounds is to combine genomic disruption libraries with HTS screening of compounds. After identifying a candidate disruption gene as synergizing with a drug of choice, that disruption strain would be screened systematically against a compound library to find other synergizing compounds, preferably those with similar chemogenomic profiles.

This approach can be run iteratively, looking at each stage for patterns in the genes or compounds that may provide functional insights (Fig. 1). Once chemical-genetic evidence links two different chemical compounds, they could be combined to be tested directly for synergies.

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**SUGGESTED READING**


