Supplementary text. Approaches to modulate resistance and tolerance

The obvious approach to addressing anti-infective resistance is to develop new, mechanistically and structurally distinct molecules. New drugs APX001[1] and Olorofim,[2] now in early clinical evaluation, represent the first two new antifungals with novel mechanisms of action to enter the clinic in over 40 years.[3] Given the challenges of antifungal drug development, multiple, non-traditional approaches should be undertaken to maximize the odds of success.

One solution to the antibiotic resistance problem is development of an adjuvant molecule that directly interferes with the resistance mechanism; for example, the use of beta-lactamase inhibitors to inhibit enzymes that degrade beta lactam antibiotics.[4] The closest analogy for antifungals would be a molecule that interfered either with efflux pump synthesis or activity; see a review of similar approaches for treating cancer or infections due to drug-resistant Gram negative bacteria.[5] Although a variety of non-specific efflux pump inhibitors have activity against fungal efflux pumps, many of these molecules are broadly active against many different types of membrane localized pumps and thus are toxic to human cells. A very promising and innovative development in this area is the recent identification of iKIX1.[6] Preliminary data indicate that iKIX1 interferes with protein-protein interactions between the Pdr1 transcription factor and the Mediator transcriptional regulator to reduce efflux pump gene expression in *C. glabrata.* This provides an important proof-of-concept for a direct approach to overcoming antifungal drug resistance through adjuvant small molecules that target the transcriptional regulator of efflux pumps, not the pumps themselves.

To overcome drug target-based resistance mutations, the chemical structure of the drug must be modified so that it regains the ability to bind to the target. Unfortunately, mutations in *ERG11* that lead to resistance to one azole are typically sufficient to enable resistance to other azoles as well.[7] For some echinocandin-resistant FKS hotspot mutants, the structurally distinct, orally-active 1,3-β-glucan

synthase inhibitor SCY-087 appears to be effective. However, other hotspot mutations are cross-resistant to both inhibitors. We suggest that systematic screens designed to identify structurally novel ergosterol or glucan synthase inhibitors with activity against resistant strains could be a fruitful approach, since the targets are validated and a relatively narrow quadrant of chemical space appears to have been screened to date.

A different approach is to find adjuvants that modulate the antifungal susceptibility of resistant strains; conceptually, such adjuvants must alter susceptibility via one or more indirect mechanisms. Operationally, screens have identified adjuvant drugs that synergize with a traditional antifungal to inhibit a strain resistant to a currently used antifungal. Potential treatment options require that the adjuvant (used at safe and achievable concentrations) lowers the MIC below the clinical breakpoint. Given the lack of successful precedents, this represents a novel, albeit unproven, approach to restoring the utility of current antifungals in the face of drug-resistance. Indeed, targeting tolerance mechanisms with an adjuvant drug may be an approach to improve clinical efficacy or to reduce the emergence of resistance.

The most widely tested set of potential adjuvants are identified in collections of approved drugs or molecules with biological activity in other systems. This "repurposing" approach provides an important strategic advantage: successful adjuvants could be expediently advanced to clinical study because the adjuvant toxicity and pharmacology are already known. Several of these have much stronger effects on tolerance than resistance (Box 2). By contrast, the only agent reported to modulate echinocandin resistance is the metal chelator, DTPA.[8] For a full discussion of the advantages of repurposing drugs for anti-infective indications see the recent review by Farha and Brown. Despite these advantages, no repurposed antifungal adjuvant that targets resistance mechanisms has progressed to studies in clinical trials.

In contrast to repurposing efforts, screening campaigns to identify novel chemical adjuvants that modulate antifungal drug resistance have been less common. An illustrative example is the high-throughput screen for molecules that re-sensitize *C. albicans* to fluconazole performed as part of the NIH Molecular Libraries program.[9] Three scaffolds with no intrinsic antifungal activity that re-sensitized fluconazole resistant strains to that drug were identified[10]. For example, Inz-1 reduced the trailing growth/tolerance displayed by *C. albicans* strain CaCi-2, which has high tolerance[11], and increased efflux pump expression. [12] Interestingly, like many of the adjuvants that inhibit tolerance (Box 2), the combination of Inz-1 and fluconazole was fungicidal while the adjuvant had little, if any, effect on fungal growth, suggesting that Inz-1 interferes with pathways required for fluconazole tolerance in *C. albicans*. Detailed mechanistic studies indicated that Inz-1 inhibits Cytochrome *bc*1,[12] is consistent with the role of altered mitochondrial function on fluconazole susceptibility.[13] AR-12, an inhibitor of fungal acetyl CoA synthetase is a small molecule that increases the susceptibility of both azole- and echincocandin-resistant *C. albicans* and *C. glabrata*, likely by a mechanism related to lipid and sterol biosynthesis.[14]

Citations for Supplementary text

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