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Defining the frontiers between antifungal resistance, tolerance and the concept of persistence

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ABSTRACT

A restricted number of antifungal agents are available for the therapy of fungal diseases. With the introduction of epidemiological cut-off values for each agent in important fungal pathogens based on the distribution of minimal inhibitory concentration (MIC), the distinction between wild type and drug-resistant populations has been facilitated. Antifungal resistance has been described for all currently available antifungal agents in several pathogens and most of the associated resistance mechanisms have been deciphered at the molecular level. Clinical breakpoints for some agents have been proposed and can have predictive value for the success or failure of therapy. Tolerance to antifungals has been a much more ignored area. By definition, tolerance operates at antifungal concentrations above individual intrinsic inhibitory values. Important is that tolerance to antifungal agents favours the emergence of persister cells, which are able to survive antifungal therapy and can cause relapses. Here we will review the current knowledge on antifungal tolerance, its potential mechanisms and also evaluate the role of antifungal tolerance in the efficacy of drug treatments.

1. Introduction

Resistance of microbes to anti-infectives is an increasing threat to human health and has a significant socio-economic impact in national health systems. However, the actions undertaken to counteract drug resistance remain largely insufficient. Bacterial antibiotic resistance is a dominant research area due the high prevalence of hospital bacterial infections. Resistance to antifungal drugs has received less attention, although the occurrence of fungal diseases is far from negligible. These diseases range from superficial (oral or genito-urinary tract thrushes) to life-threatening systemic infections and cause high levels of morbidity and mortality. It has been reported that about 1.7 billion people worldwide are infected with pathogenic fungi, from which 1.5 million die every year (Brown et al., 2012). While the majority of pathogenic fungi are opportunistic pathogens, they become deadly pathogens in immunocompromised patients. The increased use of immunosuppressive therapies for cancer and organ transplants has resulted in rising incidence of invasive fungal diseases. The number of fungal sepsis has risen by 207% between 1979 and 2000 in the United States and disseminated systemic candidiasis is associated with alarmingly high rates of mortality of up to 50% (Martin et al., 2003).

Candida albicans remains a major fungal pathogen in invasive diseases and is followed by several *Candida* spp. including *C. glabrata*, *C. parapsilosis* and *C. tropicalis* (Arendrup, 2010). Among filamentous fungi, *Aspergilli* spp. are predominant and especially *A. fumigatus*. This fungal species is a major cause of death in acute leukemia patients. Approximately 30% of bone marrow or organ transplants will develop invasive aspergillosis with poor prognosis (Richardson and Lass-Flörl, 2008).

A restricted number of chemical classes are currently in clinical use as antifungal agents including polyenes, pyrimidine analogues, echinocandins and triazoles. Polyenes such as amphotericin B have the ability to bind ergosterol and act as a sterol "sponge" thus destabilizing membrane functions (Anderson et al., 2014). Amphotericin B exerts intrinsic toxic effects in human, however this negative effect can be alleviated by liposome formulations (Sanglard and Odds, 2002). Echinocandins block the catalytic subunit of the ß-1,3 glucan synthase and thus inhibit cell wall biosynthesis (Arendrup and Perlin, 2014). Triazoles are still the mostly used antifungals. These compounds target a specific step in ergosterol biosynthesis catalysed by lanosterol 14α-demethylase (Sanglard and Odds, 2002). Fluconazole is the major triazole in clinical settings, probably due to its high oral availability and tolerability by patients.

The activity of these different antifungal classes on fungal pathogens can vary and several factors are involved in this process. Variations of antifungal activities have different bases, including occurrence of intrinsic or acquired resistance. Antifungal activities

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can also depend on underlying mechanisms that are related to antifungal tolerance. Finally, antifungal activity can also be altered by persistence mechanisms. The terminology and definitions behind these different mechanisms is often not properly used in published studies. The terms "drug resistance" and "drug tolerance" are often used for reporting the same phenotypes, thus creating confusions. In this review, we will attempt to more clearly define these different terms and summarize the present knowledge on the different mechanisms that determine the activity of antifungal agents. We will also evaluate the impact of antifungal tolerance on the fate of antifungal therapy.

2. What is understood by antifungal activity?

There are two basic antifungal susceptibility patterns *in vitro* among fungal pathogens. Either the presence of the antifungal drug results in a decrease or absence of growth capacity at a given concentration as compared to untreated control or the antifungal drug does not affect fungal growth of specific species at any concentrations. The absence of drug activity in a species that was not pre-exposed to the tested agent is also known as intrinsic resistance. For example, it is known that wild type *C. albicans* is susceptible to fluconazole, whereas *A. fumigatus* is intrinsically resistant to this azole (Pfaller, 2012). Several other examples of intrinsic resistance are given in Table 1. Antifungal activity is usually measured with standard broth dilution protocols enabling inter-laboratory comparisons. Two major protocols are currently used, either originating from two major antifungal susceptibility testing subcommittees (CLSI: Clinical Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing). The protocols yield so-called minimal inhibition concentration (MIC) values (given in μg/ml) as measures of antifungal activity. These protocols use microtitre 96 well plates, albeit with well formats that are specific for each method. While these protocols differ in several technical aspects, the agreement between the two methods in terms of antifungal activities is generally high. For example, MIC values for isavuconazole in *C. albicans* range from ≤ 0.008 to 0.03 μg/ml for CLSI while these values range from ≤0.008 to 0.015 μg/ml for EUCAST (Pfaller et al., 2013). Variability in agreements depends more on the tested fungal species (Arendrup et al., 2013; Rambach et al., 2011). One of the major differences between the two methods is their incubation time (24- and 48 h for CLSI; 24 h for EUCAST) before measurement of MIC values. Second, the MIC values are determined either by optical density (OD) measurements with EUCAST (antifungal concentration that results in ≤50% of decrease in OD as compared to control) or by visual inspection with CLSI.

3. What is antifungal resistance?

Now that antifungal activity can be measured by specific protocols, it is possible to determine how these activities are distributed within a same fungal species and how these activities can be compared with other species. When taking the distribution of fluconazole MICs in *C. albicans* clinical isolates, a bell-shaped Gaussian distribution can be observed (Fig. 1). Such distributions can be obtained with other antifungal agents and other fungal species (Arendrup et al., 2013). MIC distributions can identify isolates that are different from wild type isolates. The EUCAST defines the limit above which non-wild type isolates can be detected as the epidemiological cut off (ECOFF) value. The ECOFF value is defined as the upper limit of the wild type population (Fig. 1) and can be selected based on visual inspection (the eye ball method). Taking Fig. 1 as an example, the ECOFF value for fluconazole in *C. albicans* is 1 μg/ml. Above this value, isolates differ from the wild type population by the potential occurrence of resistance mechanism. Thus, ECOFF values help to identity non-wild type isolates exhibiting potential resistance mechanisms to a given drug within a given population. Table 2 summarizes these values for several agents and major fungal pathogens.

Considering that antifungal resistance occurs *in vitro* above the ECOFF values, it should be possible to identify the resistance mechanisms prevailing in non-wild type isolates. With the help of antifungal resistance mechanisms known for *C. albicans* and azoles

Table 1
Overview on the activity of antifungal agents in several fungal pathogens[a].

Organism	Antifungal agent[b]								
	Amphotericin B	Fluconazole	Itraconazole	Voriconazole	Posaconazole	Anidulafungin	Caspofungin	Micafungin	Flucytosine
Aspergillus species									
A. flavus	±	–	+	+	+	+	+	+	–
A. fumigatus	+	–	+	+	+	+	+	+	–
A. niger	+	–	+	+	+	+	+	+	–
A. terreus	–	–	+	+	+	+	+	+	–
Candida species									
C. albicans	+	+	+	+	+	+	+	+	±
C. glabrata	+	±	±	±	±	+	+	+	±
C. krusei	+	–	±	+	+	+	+	+	–
C. lusitaniae	–	+	+	+	+	+	+	+	+
C. parapsilosis	+	+	+	+	+	±	±	±	+
C. tropicalis	+	+	+	+	+	+	+	+	+
Other species									
Cryptococcus neoformans	+	+	+	+	+	–	–	–	+
Coccidioides species	+	+	+	+	+	±	±	±	–
Blastomyces	+	+	+	+	+	±	±	±	–
Histoplasma species	+	+	+	+	+	±	±	±	–
Fusarium species	±	–	–	±	±	–	–	–	–
Scedosporium apiospermum	±	–	±	±	+	±[c]	±[c]	±[c]	–
Scedosporium prolificans	–	–	–	±	±	–	–	–	–
Zygomycetes	±	–	–	–	±	–	–	–	–

[a] Adapted from Ashley et al. (2006), Espinel-Ingroff (2003) and Denning and Hope (2010).
[b] Plus signs (+) indicate that the antifungal agent has activity against the specified organism. Minus signs (–) indicate that the antifungal agent does not have activity against the specified organism. Plus-minus signs (±) indicate that the agent has different activities (+ and –) against the specified organism.
[c] Adapted from data provided by Lackner et al. (2012) and Castanheira et al. (2012).