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Azole and fungicide resistance in clinical and environmental *Aspergillus fumigatus* isolates

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Aspergillus fumigatus is a human pathogen but it is also a widespread filamentous fungus in the environment. *A. fumigatus* can therefore be exposed to antifungals used in medical and agricultural environments. Only the class of azoles is used in both of these environments (i.e. voriconazole and itraconazole in medicine; prochloraz, propiconazole or imazalil in agriculture). Exposure to azoles provides the potential for the development of resistance. Several clinical itraconazole-resistant isolates have been reported in *A. fumigatus* and their resistance mechanisms have been partially resolved. Since limited data exist on the susceptibility of *A. fumigatus* to both medical and agricultural antifungals, we undertook a drug susceptibility study including clinical (400) and agricultural (150) *A. fumigatus* isolates (Swiss origin). We tested azoles and also compounds of major antifungal classes used in agriculture (i.e. azoxystrobin, iprodione, benalaxyl or cyprodinil). The results showed that all *A. fumigatus* isolates were intrinsically resistant to iprodione, benalaxyl or cyprodinil ($MIC_{90} > 32\ \mu g \cdot ml^{-1}$) and that azoxystrobin minimal inhibitory concentrations (MICs) showed a wide range (0.06 to 32 $\mu g \cdot ml^{-1}$). MIC ranges of azoles were compound-dependent. MIC_{90} for voriconazole, itraconazole, imazalil and prochloraz were within a range of 0.13 to 1 $\mu g \cdot ml^{-1}$ and similar between clinical and environmental isolates, whereas propiconazole was the least active compound (MIC_{90}: 4–8 $\mu g \cdot ml^{-1}$). Ten clinical and 36 environmental isolates with high itraconazole MIC ($\geq 2\ \mu g \cdot ml^{-1}$) were detected. In clinical isolates, no cross-resistance was observed between itraconazole and all others azoles tested. Several patterns of azole MICs were, however, observed in the environmental isolates. Unexpectedly, a single environmental isolate was voriconazole-resistant (MIC of 16 $\mu g \cdot ml^{-1}$) but still susceptible to itraconazole (MIC of 2 $\mu g \cdot ml^{-1}$). Taken together, our results demonstrate the absence of susceptibility of *A. fumigatus* isolates to non-azole agricultural agents and that there is little impact of azole resistance in both clinical and environmental isolates. When detected, azole resistance was compound-specific.

Keywords antifungal, *Aspergillus fumigatus*, azole, resistance

Introduction

Aspergillus fumigatus is one of the most prevalent airbone fungal pathogens, causing severe fatal invasive aspergillosis in immunocompromised patients [1]. It is also found in the environment as a plant contaminant or participates in the degradation of organic material present in compost sites.

A few antifungal agents are available for the treatment of aspergillosis in humans. All these antifungal agents belong to one of three groups, the polyenes (amphotericin B), the azoles (itraconazole and voriconazole), and the echinocandins (caspofungin).

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Among antifungal agents used in the environment for crop protection, the class of azoles is also widely used (e.g. propiconazole, prochloraz, or imazalil). Other agents belonging to the class of dicarboximides (which affects cell division, DNA and RNA metabolism; iprodione is a specific agent of this class), phenylamides (which affects RNA synthesis; e.g. benalaxyl), anilipyrimides (which inhibits amino acid biosynthesis; e.g. cyprodinil) or axoxystrobin (which inhibits mitochondrial respiration) are also used. Given the ubiquity of *A. fumigatus* in the environment, these isolates are often exposed to the agricultural antifungals. In the environment, antifungal agents (such as imazalil, prochloraz, azoxystrobin) can be detected. Fruit and vegetables have been shown to contain substantial amount of these agents (between 0.01–5 $mg.kg^{-1}$) [2,3].

Itraconazole or voriconazole are widely used to treat aspergillosis in patients, with moderate success [4]. Emergence of resistance to these antifungals might be expected among clinical *A. fumigatus* isolates owing to their increasing use, if the source of patient isolates is directly or indirectly from other patient isolates. Indeed, a selection pressure due to the presence of antifungal agents can lead to a genetic adaptation resulting in a resistant strain. However, only a limited number of clinical isolates showing itraconazole resistance have been described [5]. Whereas the mechanisms of resistance to azoles have been well investigated in *Candida albicans* isolates [6–8], few detailed studies have been performed on antifungal resistance in *A. fumigatus*. Up to now, mutations in the gene encoding the azole target, *Cyp51A*, have been detected in itraconazole-resistant isolates [9]. In our laboratory, several experiments have been performed that suggest that the expression of ATP-Binding Cassette transporter (ABC transporters) and Major Facilitator genes also contributes to itraconazole resistance [D. Sanglard, unpublished data]. Since almost no data exist in Switzerland on the status of antifungal susceptibility of *A. fumigatus*, we undertook evaluation of the *in vitro* activity of several antifungal agents on isolates using both medical (itraconazole, voriconazole and amphotericin B) and agricultural (prochloraz, propiconazole, imazalil, azoxystrobin, iprodione, benalaxyl and cyprodinil) agents. Minimal inhibitory concentrations (MIC) results were determined by a microdilution standard protocol according to the National Committee for Clinical Laboratory Standards (NCCLS MP38-A) on 400 clinical isolates from patients hospitalized in several major Swiss hospitals and on 150 isolates from several environmental sites (compost sites, vineyards, crop areas) around Switzerland. This method has proven to be useful for *in vitro* susceptibility testing in *A. fumigatus* [10].

Material and methods

Organisms

All clinical strains of *A. fumigatus* were evaluated from our current collection of 400 strains. They were all isolated from hospitalized patients with various forms of *A. fumigatus* infection from major Swiss hospitals (80 isolates from University Hospital of Zurich; 250 from University Hospital of Lausanne; 45 from University Hospital of Geneva and 25 from Istituto Cantonale di Microbiologia). Moreover, 150 environmental *A. fumigatus* isolates were obtained from different sites in Switzerland (crop areas, vineyards, compost sites). All *Aspergillus* isolates were plated to obtain individual colonies. Individual colonies were propagated on slopes of Sabouraud agar for a permanent culture collection in our laboratory and stored at $+4^{\circ}C$.

Antifungal agents

In order to carry out the *in vitro* susceptibility tests, pure compounds were obtained from their respective manufacturers. Agricultural antifungal agents (i.e. axoxystrobin, imazalil, prochloraz and propiconazole, iprodione, benalaxyl, or cyprodinil) were provided by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Amphotericin B was from Bristol-Myers Squibb (Baar, Switzerland). Itraconazole and voriconazole were from CilagAG (Shaffhausen, Switzerland) and Pfizer (Sandwich, UK), respectively. Itraconazole, voriconazole, amphotericin B, azoxystrobin, and propiconazole were dissolved in dimethyl sulfoxide (DMSO, Fluka), prochloraz in acetone, and imazalil in ethanol, at a concentration of 10 $mg.ml^{-1}$, except for itraconazole which was dissolved in DMSO in order to obtain a 1.6 $mg.ml^{-1}$ stock solution. All drugs were stored at $-20^{\circ}C$.

Preparation of conidial suspensions

Conidia were obtained by growth of isolates on Sabouraud agar at $37^{\circ}C$ for 3–4 days. Conidia were collected by flooding the agar surfaces with phosphate-buffered saline and 0.05% Tween80 (Fluka) and viability was determined for each isolate. The number of CFU per ml was determined by plating different volumes of an appropriate diluted cell suspension (10^{-6}) on Sabouraud agar. Inoculum of 7×10^5 viable spores per ml were prepared.

Media for antifungal susceptibility testing

The standard RPMI 1640 (Diagnostic Medical Distribution, Zurich, Switzerland) contained 0.2% glucose, and 0.165 M MOPS (morpholine propane sulfonic acid) buffer to a pH of 7.0. The Antibiotic Medium 3 (AM3) broth was prepared according to the instructions of the manufacturer (Difco, Detroit, Michigan). Amphotericin B MICs were determined using AM3 medium; RPMI 1640 was used for all other antifungal agents.

Antifungal susceptibility testing

We used the microdilution broth method according NCCLS (M38-A) guidelines for *in vitro* susceptibility testing. The stock conidial suspension was diluted in RPMI 1640 or in AM3 medium according to the antifungal agent used. A fixed amount of *A. fumigatus* conidia was also used [11]. First, 150 µl of drug free medium was dispensed into each well of the 96 well plates (Costar). Serial two-fold dilutions of each antifungal agent were prepared with the appropriate medium (RPMI 1640 or AM3) (dilutions ranged from 0.02 to 16 $\mu g \cdot ml^{-1}$). To prepare a drug dilution series, starting solutions of the drug were dispensed in 50 µl of medium into the first well of the plate. Two-fold dilutions were then performed to yield expected drug concentrations. Aliquots of 50 µl of the stock conidial suspension were then added to the wells. The final volume in each well was to 200 µl and the final concentration of the inoculum was 1.8×10^5 conidia per ml. The plates were incubated for two days at 37°C after which visual readings were performed. The MIC was defined as the lowest concentration of drug that completely inhibited fungal growth. MIC_{50} or MIC_{90} values correspond to antifungal concentrations that inhibit 50% or 90% of the isolates from a tested collection.

Results and discussion

Preliminary experiments on a subset of clinical and environmental *A. fumigatus* isolates showed that they were intrinsically resistant to iprodione, benalaxyl or cyprodinil ($MIC_{90} > 32\ \mu g \cdot ml^{-1}$); therefore, further testing of these antifungal agents with the remaining collection was abandoned. Table 1 summarizes the *in vitro* susceptibilities of 400 *A. fumigatus* clinical isolates and of 150 agricultural isolates against all the remaining antifungal agents tested except for azoxystrobin, a strobulin agent. Even though azoxystrobin MICs showed a wide distribution (0.06 to 32 $\mu g \cdot ml^{-1}$), azoxytrobin was the least active antifungal agent with a MIC_{90} of 32 $\mu g \cdot ml^{-1}$. In general, azoles derivatives showed a wide distribution of MIC across similar concentration ranges (from 0.02 to 16 $\mu g \cdot ml^{-1}$). MIC_{90} values of the medical azole antifungals (voriconazole, itraconazole) were within a range of 0.5 to 2 $\mu g \cdot ml^{-1}$ in both clinical and environmental isolates. With MIC_{90} values of 1 and 0.5 $\mu g \cdot ml^{-1}$ for clinical and agricultural isolates, respectively, voriconazole was slightly more active than itraconazole, which had MIC_{90} values of 1 and 2 $\mu g \cdot ml^{-1}$ for clinical and environmental isolates, respectively. Amphotericin B was the medical antifungal agent with the lowest activity: MIC_{90} values of 4 $\mu g \cdot ml^{-1}$ were measured in both clinical and environmental isolates. Several studies performed on clinical isolates have reported *in vitro* activities of medical antifungal agents close to those measured in this study. MIC_{90} of 0.5, 0.5 and 2 $\mu g \cdot ml^{-1}$ for itraconazole, voriconazole and amphotericin B, respectively, were reported [12]. MIC_{90} of 2 and 1 $\mu g \cdot ml^{-1}$ for itraconazole and amphotericin B were measured in a separate study [13]. Among the agricultural azoles tested, propiconazole was the least active compound (MIC_{90} value of 8 $\mu g \cdot ml^{-1}$) in both clinical and environmental isolates; MIC_{90} of imazalil and prochloraz were lower in environmental isolates (0.13 for imazalil and 0.25 $\mu g \cdot ml^{-1}$ for prochloraz,

Table 1 *In vitro* susceptibility to six antifungal agents of *Aspergillus fumigatus* clinical (400) and environmental (150) isolates.

Drug	MIC range ($\mu g \cdot /ml^{-1}$)[(a)]		MIC distribution			
	Clinical isolates	Environmental isolates	Clinical isolates		Environmental isolates	
			MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Itraconazole	0.03–16	0.06–16	0.5	1	0.5	2
Voriconazole	0.03–4	0.06–16	0.25	1	0.25	0.5
Amphotericin B	0.06–8	0.5–8	1	4	2	4
Imazalil	0.08–2	0.02–0.5	0.13	0.5	0.06	0.13
Prochloraz	0.02–8	0.02–16	0.13	1	0.06	0.25
Propiconazole	0.03–16	0.5–32	2	8	4	8

[(a)] MIC range and MIC distribution are given for both clinical and environmental isolates and for each antifungal agent tested.

Table 2 Antifungal MIC patterns of environmental and clinical *Aspergillus fumigatus* isolates with high itraconazole MIC.

	MIC ($\mu g \cdot ml^{-1}$)					
	Itraconazole	Voriconazole	Imazalil	Prochloraz	Propiconazole	Amphotericin B
AFenv51 [a)]	≥16	1	0.06	1	≥16	1
AFenv110	4	0.13	0.13	0.13	8	8
AFenv115	4	0.13	0.06	0.13	4	4
AFenv135	8	0.25	0.13	0.13	8	8
AFenv149	4	0.5	0.13	0.25	16	4
AFenv119	2	2	8	16	8	4
"typical isolate"	0.13	0.25	0.03	0.06	2	2
AFenv155	2	16	8	>16	16	4
AFL16.1 [b)]	2	0.5	0.25	0.13	8	2
AFL12.1	2	0.5	0.13	1	4	1
AFL50.7	2	0.5	0.5	1	4	4
AFL30.1	4	0.5	0.13	0.13	4	2
AFL35.9	16	0.5	0.13	0.06	4	1
"typical isolate"	0.25	0.5	0.25	0.5	8	2

[a)] AFenvxx, *A. fumigatus* Environment isolate xx in our nomenclature collection.
[b)] AFLxx.y, *A. fumigatus* from Lausanne, patient xx, isolate y.

respectively) than in clinical isolates (0.5 $\mu g \cdot ml^{-1}$ for imazalil and 1 $\mu g \cdot ml^{-1}$ for prochloraz, respectively).

Isolates with high itraconazole MIC (MIC ≥2 $\mu g \cdot ml^{-1}$) were detected in both clinical (total of 10 isolates) and environmental isolates (total of 36). Antifungals MICs of only a selection of these isolates are shown in Table 2. These high itraconazole MICs were not strictly accompanied with a higher MIC to the all other azole antifungals listed in Table 2. For example, the environmental isolate AFenv51 showed an itraconazole MIC value of ≥16 $\mu g \cdot ml^{-1}$; voriconazole, imazalil and prochloraz had MICs of 1, 0.06 and 1 $\mu g \cdot ml^{-1}$, respectively. However, isolate AFenv119 had an itraconazole MIC of 2 $\mu g \cdot ml^{-1}$; voriconazole, imazalil and prochloraz had MICs of 2, 8 and 16 $\mu g \cdot ml^{-1}$, respectively. The MIC profile of isolate AFenv155 was however similar to AFenv119, with the exception of the prochloraz MIC ≥16 $\mu g \cdot ml^{-1}$. Clinical isolates with itraconazole MIC ≥2 $\mu g \cdot ml^{-1}$ were less heterogeneous in their azole MIC patterns. The voriconazole MICs of these isolates remained at 0.5 $\mu g \cdot ml^{-1}$, which is the value observed for most of the itraconazole-susceptible isolates. The MIC profiles shown in Table 2 for selected clinical isolates are in agreement with reports documenting no cross-resistance with voriconazole [9,14]. This absence of cross-resistance mainly between itraconazole and voriconazole can be attributed to their differences in chemical structure (Fig. 1). Indeed, azoles block ergosterol synthesis in *A. fumigatus* by binding to their target enzyme, the 14α-demethylase *Cyp51A*. Since voriconazole is a relatively compact molecule as compared to itraconazole with its extended side chain, binding does not involve the same residues. A study

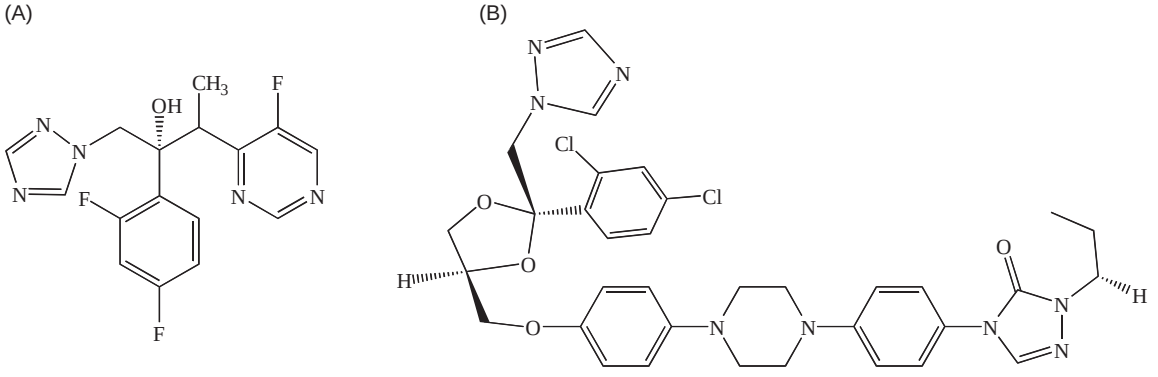

Fig. 1 Chemical structures of voriconazole (A) and itraconazole (B).

suggests that the long side chain of itraconazole occupy a specific channel within *Cyp51* and that, this additional interaction should stabilize its binding to the mutated CYP51 proteins [15] mutations known to confer itraconazole resistance [9]. Therefore, the different binding sites of azoles in the target enzyme could explain the absence of cross-resistance between itraconazole and voriconazole.

More surprising are results obtained with isolates from the environment, where cross-resistance between itraconazole and voriconazole could be measured and where the MIC patterns were more heterogenous. These data suggest that the mechanisms responsible for the MIC increase in specific azole might have different origins and will be the focus of future studies in our laboratory.

Taken together, ours results show:

1. Absence of susceptibility of *Aspergillus fumigatus* isolates to non-azole agricultural agents with an intrinsic resistance to agents such as benalaxyl, cyprodinil and iprodione, and
2. Very little impact of azole resistance in both clinical and environmental isolates.

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