

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 ($\text{MIC}_{90} > 32 \mu\text{g} \cdot \text{ml}^{-1}$) and that azoxystrobin minimal inhibitory concentrations (MICs) showed a wide range (0.06 to $32 \mu\text{g} \cdot \text{ml}^{-1}$). MIC ranges of azoles were compound-dependent. MIC_{90} for voriconazole, itraconazole, imazalil and prochloraz were within a range of 0.13 to $1 \mu\text{g} \cdot \text{ml}^{-1}$ and similar between clinical and environmental isolates, whereas propiconazole was the least active compound (MIC_{90} : $4\text{--}8 \mu\text{g} \cdot \text{ml}^{-1}$). Ten clinical and 36 environmental isolates with high itraconazole MIC ($\geq 2 \mu\text{g} \cdot \text{ml}^{-1}$) 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 $16 \mu\text{g} \cdot \text{ml}^{-1}$) but still susceptible to itraconazole (MIC of $2 \mu\text{g} \cdot \text{ml}^{-1}$). 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 airborne 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), anilipyr-imides (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°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°C.

Preparation of conidial suspensions

Conidia were obtained by growth of isolates on Sabouraud agar at 37°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 × 10⁵ 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 to 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 µg · 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 × 10⁵ 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. MIC₅₀ or MIC₉₀ 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 (MIC₉₀ > 32 µg · 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 strobilin agent. Even though azoxystrobin MICs showed a wide distribution (0.06 to 32 µg · ml⁻¹), azoxystrobin was the least active antifungal agent with a MIC₉₀ of 32 µg · ml⁻¹. In general, azoles derivatives showed a wide distribution of MIC across similar concentration ranges (from 0.02 to 16 µg · ml⁻¹). MIC₉₀ values of the medical azole antifungals (voriconazole, itraconazole) were within a range of 0.5 to 2 µg · ml⁻¹ in both clinical and environmental isolates. With MIC₉₀ values of 1 and 0.5 µg · ml⁻¹ for clinical and agricultural isolates, respectively, voriconazole was slightly more active than itraconazole, which had MIC₉₀ values of 1 and 2 µg · ml⁻¹ for clinical and environmental isolates, respectively. Amphotericin B was the medical antifungal agent with the lowest activity: MIC₉₀ values of 4 µg · 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. MIC₉₀ of 0.5, 0.5 and 2 µg · ml⁻¹ for itraconazole, voriconazole and amphotericin B, respectively, were reported [12]. MIC₉₀ of 2 and 1 µg · 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 (MIC₉₀ value of 8 µg · ml⁻¹) in both clinical and environmental isolates; MIC₉₀ of imazalil and prochloraz were lower in environmental isolates (0.13 for imazalil and 0.25 µg · 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 (µg · 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 ($\mu\text{g} \cdot \text{ml}^{-1}$)					
	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 ($0.5 \mu\text{g} \cdot \text{ml}^{-1}$ for imazalil and $1 \mu\text{g} \cdot \text{ml}^{-1}$ for prochloraz, respectively).

Isolates with high itraconazole MIC (MIC $\geq 2 \mu\text{g} \cdot \text{ml}^{-1}$) 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 $\geq 16 \mu\text{g} \cdot \text{ml}^{-1}$; voriconazole, imazalil and prochloraz had MICs of 1, 0.06 and $1 \mu\text{g} \cdot \text{ml}^{-1}$, respectively. However, isolate AFenv119 had an itraconazole MIC of $2 \mu\text{g} \cdot \text{ml}^{-1}$; voriconazole, imazalil and prochloraz had MICs of 2, 8 and $16 \mu\text{g} \cdot \text{ml}^{-1}$, respectively. The MIC profile of isolate AFenv155 was however similar to AFenv119,

with the exception of the prochloraz MIC $\geq 16 \mu\text{g} \cdot \text{ml}^{-1}$. Clinical isolates with itraconazole MIC $\geq 2 \mu\text{g} \cdot \text{ml}^{-1}$ were less heterogeneous in their azole MIC patterns. The voriconazole MICs of these isolates remained at $0.5 \mu\text{g} \cdot \text{ml}^{-1}$, 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

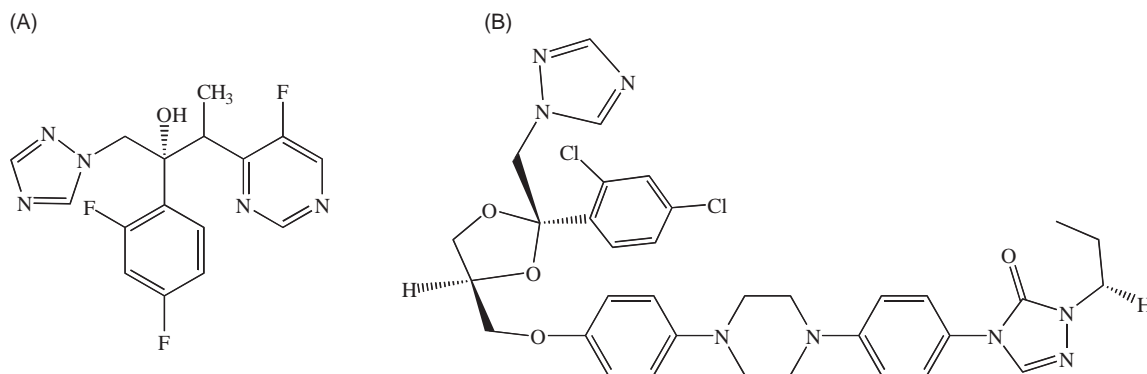


Fig. 1 Chemical structures of voriconazole (A) and itraconazole (B).

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 heterogeneous. 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, our 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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References

- 1 Bodey G, Bueltmann B, Duguid W, *et al.* Fungal infections in cancer patients: an international autopsy survey. *Eur J Clin Microbiol Infect Dis* 1992; **11**: 99–109.
- 2 Lopez ML, Riba M. Residue levels of ethoxyquin, imazalil, and iprodione in pears under cold-storage conditions. *J Agric Food Chem* 1999; **47**: 3228–3236.
- 3 Christensen HB, Granby K. Method validation for strobilurin fungicides in cereals and fruit. *Food Addit Contam* 2001; **18**: 866–874.
- 4 Latgé JP. *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* 1999; **12**: 310–350.
- 5 Denning DW, Venkateswarlu K, Oakley KL, *et al.* Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother* 1997; **41**: 1364–1368.
- 6 Sanglard D, Kuchler K, Ischer F, *et al.* Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob Agents Chemother* 1995; **39**: 2378–2386.
- 7 Sanglard D, Ischer F, Monod M, Bille J. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of *w*, a new multidrug ABC transporter gene. *Microbiology* 1997; **143**: 405–416.
- 8 Sanglard D, Ischer F, Koymans L, Bille J. Amino acid substitutions in the cytochrome P-450 lanosterol 14 α -demethylase (*CYP51A1*) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrob Agents Chemother* 1998; **42**: 241–253.
- 9 Diaz-Guerra TM, Mellado E, Cuenca-Estrella M, Rodriguez-Tudela JL. A point mutation in the 14 α -sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother* 2003; **47**: 1120–1124.
- 10 Espinel-Ingroff A, Bartlett M, Chaturvedi V, *et al.* Optimal susceptibility testing conditions for detection of azole resistance in *Aspergillus* spp.: NCCLS collaborative evaluation. National Committee for Clinical Laboratory Standards. *Antimicrob Agents Chemother* 2001; **45**: 1828–1835.
- 11 Arikan S, Lozano-Chiu M, Paetznick V, Nangia S, Rex JH. Microdilution susceptibility testing of amphotericin B, itraconazole, and voriconazole against clinical isolates of *Aspergillus* and *Fusarium* species. *J Clin Microbiol* 1999; **37**: 3946–3951.
- 12 Oakley KL, Moore CB, Denning DW. *In vitro* activity of voriconazole against *Aspergillus* spp. and comparison with itraconazole and amphotericin B. *J Antimicrob Chemother* 1998; **42**: 91–94.
- 13 Dannaoui E, Persat F, Monier MF, *et al.* *In vitro* susceptibility of *Aspergillus* spp. isolates to amphotericin B and itraconazole. *J Antimicrob Chemother* 1999; **44**: 553–555.
- 14 Mann PA, Parmegiani RM, Wei SQ, *et al.* Mutations in *Aspergillus fumigatus* resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14 α -demethylase. *Antimicrob Agents Chemother* 2003; **47**: 577–581.
- 15 Xiao L, Madison V, Chau AS, *et al.* Three-dimensional models of wild-type and mutated forms of cytochrome P450 14 α -sterol demethylases from *Aspergillus fumigatus* and *Candida albicans* provide insights into posaconazole binding. *Antimicrob Agents Chemother* 2004; **48**: 568–574.