Aspergillus fumigatus harbouring the sole Y121F mutation shows decreased susceptibility to voriconazole but maintained susceptibility to itraconazole and posaconazole

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Objectives: Voriconazole, itraconazole and posaconazole are members of the azole family and widely used for the treatment of aspergillosis. They act by inhibiting the activity of the fungal Cyp51A enzyme. The emergence of environmental azole-resistant Aspergillus fumigatus strains raises major concerns for human health.

Methods: Recently, a new cyp51A-mediated resistance mechanism (namely TR46/Y121F/T289A) was described in clinical samples and patient-frequented environmental sites. In an azole-naive patient, we isolated an A. fumigatus strain that was not susceptible to voriconazole but was susceptible to itraconazole and posaconazole.

Results: A molecular analysis indicated a single Y121F substitution without the TR46 or T289A alterations, which to our knowledge has never been reported. Structure modelling and molecular dynamics offered an explanation for the resistance profile consistent with the structural differences between the three azoles.

Conclusions: Taken together, these observations suggest an original mechanism conferring resistance to azoles mediated by cyp51A of environmental origin. This uncommon susceptibility pattern might represent a ‘missing link’ between the wild-type A. fumigatus and the fully azole-resistant strain harbouring the TR46/Y121F/T289A mutations.

Keywords: azole drugs, antifungal resistance, Cyp51A, ergosterol

Introduction

Aspergillus fumigatus is a major mould pathogen responsible for a broad spectrum of human diseases. Aspergillosis cases are mainly treated with azole drugs that inhibit the activity of the fungal Cyp51A enzyme. Recently, due to the widespread use of azoles for agricultural purposes, azole-resistant strains of A. fumigatus have emerged, posing a threat for human health.1–4 More recently, Van der Linden et al.5 described a group of 15 patients with azole-resistant A. fumigatus strains. Their article described a new cyp51A-mediated resistance mechanism including two amino acid substitutions and a 46 bp tandem repeat in the gene promoter (TRuc/Y121F/T289A). Interestingly, this mechanism was related to a high resistance level towards voriconazole (MIC of ≥16 mg/L) and an attenuated activity of itraconazole (MIC of 1 to ≥16 mg/L) and posaconazole (MIC of 0.25 to ≥2 mg/L). TRuc/Y121F/T289A was found in 20.6% of the Van der Linden et al.5 patients harbouring azole-resistant strains and was furthermore described shortly thereafter in Belgium6 and very recently in India7 and Germany.8 However, the contributions of individual mutations in conferring resistance remain elusive. We isolated an A. fumigatus strain from a pulmonary sample of a patient with a medical history of emphysema, bronchiectasis and healed tuberculosis. This strain was susceptible to itraconazole and posaconazole but not to voriconazole, thus providing an opportunity for both molecular and structural analysis.

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Materials and methods

**Aspergillus fumigatus isolate characterization**

Identification was confirmed by sequencing of the internal transcribed spacer (ITS) regions and the β-tubulin gene. Antifungal susceptibility testing was performed first with the Etest method and then with the EUCAST method. Breakpoints for the interpretation of MICs were based on EUCAST recommendations (version 6.1) (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Antifungal_breakpoints_v_6.1.pdf). Briefly, resistance was defined as itraconazole and voriconazole MICs of >2 mg/L and a posaconazole MIC of >0.25 mg/L; susceptibility was defined as itraconazole and voriconazole MICs of ≤1 mg/L and a posaconazole MIC of ≤0.12 mg/L. The cyp51A gene was sequenced as previously described. As one of the previously used primers (namely primer AF766-F of set 3) interferes with the mutation and impairs amplification, an alternative primer was designed to replace it, i.e. 5′-TCTCAACGGCAAGCTCAA-3′.

**Modelling of Cyp51A and of the azole complexes**

To assess the azole drug–target interactions and the putative role of the Y121F substitution in the resistance mechanism, we modelled the Cyp51A complex with voriconazole, itraconazole and posaconazole, respectively (Figure 1). The Aspergillus Cyp51A structure was modelled using the human Cyp51 protein (PDB code 4K0F) and the ExPasy modelling server (http://swissmodel.expasy.org/workspace/) in a procedure similar to that described by Snelders et al. The voriconazole, itraconazole and posaconazole (PDB codes 3MDT and 2X2N) structures were docked into the active site of the A. fumigatus Cyp51A homology model using standard docking methods and were further subjected to molecular dynamics simulations using standard protocols. Simulation trajectories were visualized using VMD and figures generated using PyMOL (www.pymol.org).

**Results**

The ITS regions and the β-tubulin sequence analysis were indicative of an A. fumigatus sensu stricto isolate. Etest antifungal susceptibility testing showed an uncommon pattern with resistance to voriconazole (MIC of 4 mg/L) but susceptibility to itraconazole and posaconazole (MIC of 1 and 0.125 mg/L, respectively). EUCAST antifungal susceptibility testing performed in three independent experiments showed a similar pattern with a higher MIC of voriconazole (2 mg/L) than of itraconazole (0.5 mg/L) and posaconazole (0.125 mg/L, respectively).

Figure 1. Snapshots of voriconazole (a and b), itraconazole (c) and posaconazole (d) in complex with wild-type (a, c and d) and Y121F single mutant Cyp51A (b, c and d) of A. fumigatus. The ligand (yellow carbon), haem (brown carbon) and important residues in the active site are displayed in stick representation. The iron atom in the haem is shown as a sphere coloured in cyan. In (b) the π–π stacking interaction stabilized by the halogen bond in voriconazole bound to the wild-type enzyme (a) is now replaced by edge–face interactions between Y107 and Y121 and the halogen bond is disrupted.
posaconazole (0.12 mg/L). It should be noted that when using the EUCAST method and breakpoint, the MIC values did not reach the ‘resistance’ level (in contrast to the Etest method) but rather the ‘intermediate’ level, which is nonetheless above the MIC indicative of susceptibility. Unexpectedly, the Cyp51A sequence analysis uncovered an isolated Y121F substitution without the TR46 or T289A alterations. This single alteration has, to our knowledge, never been reported.

The molecular dynamics of Cyp51A–azole complexes shows that the location of the haem–voriconazole group with respect to the protein is maintained by a network of interactions including the following: (i) the carboxylic group of the haem that creates a hydrogen bond with R369; and (ii) the hydroxyl group of Y121 that forms a halogen bond with the fluorine of the pyrimidine ring of voriconazole (Figure 1a). In addition, Y121 is engaged in a π–π stacking interaction with Y107 (Figure 1a). In turn, the positioning of the azole drug is determined by the polar interaction established between the iron of the haem and theazole group of the antifungal drug. Moreover, the pyrimidine ring of voriconazole is engaged in packing interactions with Y107 and Y121 (Figure 1a). The Y121F substitution (Figure 1b) leads to the loss of this key halogen bond and consequently to the disruption of the π–π stacking, the loss of packing interactions of the drug and a slight displacement of the haem–azole group in the binding cavity lined by residues T111, F115, F214 and Y107 (Figure 1a and b). Hence, suboptimal interaction between the halogen-bearing ring of the azole and Y107/F121 (Figure 1b) is likely to lead to the observed resistance to voriconazole. In contrast, the Y121F mutation has little impact on itraconazole (Figure 1c) and posaconazole (Figure 1d) binding. These latter azoles do not contain an equivalent pyrimidine ring, resulting in binding modes to Cyp51A that are very similar between them, but different from the binding mode adopted by voriconazole. Importantly, an additional favourable factor for itraconazole and posaconazole is that their extended tails (Figure 1c and d) are able to establish additional complementarily contacts with the protein compared with the more compact voriconazole, which lacks such an extended tail.

**Discussion**

Invasive aspergillosis and chronic pulmonary aspergillosis result in high morbidity and mortality in predisposed patients. Azole drugs are the basis of medical treatment. For years, azole-resistant strains have been described in case reports or small cohorts in which the patients had received long-term therapy; thus the resistance mechanism was thought to be acquired in the patient due to the medical treatment. In 2007, a new mechanism associating a Cyp51A amino acid substitution at codon 98 with a tandem repeat in the promoter gene (TR34/L98H) was described in patients who had not been previously treated with azoles, suggesting a potential role for agricultural azoles in selecting these resistant isolates. This hypothesis has become widely accepted and is associated with a high level of resistance to voriconazole and an attenuated activity for itraconazole and posaconazole.

The resistance profile of our *A. fumigatus* isolate was similar to (higher MIC of voriconazole than of itraconazole/posaconazole) but less extensive than that of isolates harbouring the TR46/Y121F/T289A alterations. Interestingly, Cyp51A sequencing indicated a sole Y121F mutation, suggesting that the two other genetic alterations may enhance the resistance mechanism. Complementary recombinant studies should be undertaken rapidly to compare the wild-type parent with mutants carrying one, any combination of two or all three of the genetic alterations, so as to assess how each alteration donates to resistance.

Currently, no Cyp51A crystal structure of *Aspergillus* origin is available and only a model based on the human Cyp51A orthologue has been described. We also used human Cyp51 structures reported in the PDB as templates to produce atomic models for *Aspergillus* Cyp51A bound to voriconazole, itraconazole and posaconazole. We thus could unambiguously map the location of the Y121F mutation next to the azole moiety, but not in direct contact with it, indicating an indirect effect of this mutation through the loss of a single bond and a reorientation of the phenyl group in the binding pocket (compare Figure 1a and b).

Importantly, the patient had never been exposed to azole drugs, which strongly suggests an environmental resistant strain. Therefore, the mutation described here could represent a ‘missing link’ between the wild-type strain and the fully azole-resistant strain harbouring the TR46/Y121F/T289A mutations. However, this hypothesis should be assessed by performing genetic comparisons between our isolate and those harbouring the triple alteration. Whether the sole Y121F substitution leads to different outcomes for voriconazole versus itraconazole/posaconazole treatments needs to be explored in an animal model. The slight discrepancy between the Etest and EUCAST results for voriconazole should also be clarified using this approach. Our observation supports the use of both itraconazole and voriconazole for testing azole susceptibility in *Aspergillus* strains because they are likely to show different levels of inhibition. Finally, high-resolution experimental structures of the various azoles bound to the fungal enzyme are now required to understand the patterns of resistance to various azoles at the atomic level and also to design new compounds that may overcome resistance.

**Nucleotide sequence accession number**

The cyp51A sequence has been submitted to the NCBI database and is available under accession number KJ210331.

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**Transparency declarations**

J. L. is a consultant for the Novartis Institute for Tropical Diseases. A. D. has been a consultant for Merck, Schering, Gilead, Pfizer and Astellas. A. F. has received funds for speaking from Merck, for consultancy from Pfizer and for travel from Astellas, Merck and Pfizer. All other authors: none to declare.
Aspergillus fumigatus harbouring Y121F mutation

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