Does pre-exposure of *Aspergillus fumigatus* to voriconazole or posaconazole *in vitro* affect its virulence and the *in vivo* activity of subsequent posaconazole or voriconazole, respectively?

A study in a fly model of aspergillosis

G. A. Lamaris1, R. Ben-Ami1, R. E. Lewis1,2 and D. P. Kontoyiannis1*

1Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA; 2University of Houston College of Pharmacy, Houston, TX, USA

Received 25 March 2008; returned 28 April 2008; revised 8 May 2008; accepted 12 May 2008

**Objectives:** Voriconazole and posaconazole are effective as both prophylaxis and treatment for invasive aspergillosis (IA) in immunocompromised patients. Hence, it is important to determine whether *Aspergillus* pre-exposure to voriconazole or posaconazole diminishes subsequent posaconazole or voriconazole activity, respectively.

**Methods:** We used *Aspergillus fumigatus* (AF) 293 conidia with or without prior exposure to voriconazole or posaconazole [three serial passages on plates containing regular yeast extract-glucose (YAG) media, YAG + 0.0625 mg/L voriconazole or YAG + 0.025 mg/L posaconazole]. *Toll*-deficient *Drosophila melanogaster* flies were infected by injection, and 8 day survival was monitored. Following infection, flies were fed either regular food, food containing 1000 mg/L voriconazole (posaconazole-exposed conidia) or 1000 mg/L posaconazole (voriconazole-exposed conidia). Voriconazole and posaconazole concentrations in flies were confirmed by HPLC.

**Results:** AF inoculation resulted in 71% mortality 8 days post-infection (median survival 4 days). Prior conidial exposure to voriconazole or posaconazole did not affect mortality (73%, $P = 0.8$ for voriconazole pre-exposed and 76%, $P = 0.49$ for posaconazole pre-exposed). Voriconazole treatment post-infection had a protective effect, reducing mortality to 42% ($P = 0.0002$), while prior conidial exposure to posaconazole did not alter the protective effect of voriconazole (34% 8 day mortality, $P = 0.35$). Likewise, posaconazole treatment post-infection reduced mortality to 36%, while prior conidial exposure to voriconazole did not alter the protective effect of posaconazole (39% mortality, $P = 0.92$). Median fly homogenate concentrations of voriconazole and posaconazole were 0.44 and 2.05 mg/L, respectively.

**Conclusions:** Prior exposure of AF to voriconazole or posaconazole did not affect the virulence of AF nor the subsequent activity of the alternate triazole in a *Drosophila* model of IA.

**Keywords:** fungal infections, triazoles, *Drosophila melanogaster*

**Introduction**

Invasive fungal infections have emerged as major causes of morbidity and mortality in severely immunocompromised patients such as those undergoing chemotherapy or haematopoietic stem cell transplantation. In view of the high associated mortality, patients at risk for invasive fungal infections receive antifungal prophylaxis. Voriconazole is widely used in high-risk patients because of its activity against both *Candida* and *Aspergillus* species. Furthermore, posaconazole was found to be superior to itraconazole and/or fluconazole in preventing invasive fungal infections. Despite effective prophylaxis options, invasive aspergillosis (IA) remains the most common breakthrough infection in patients receiving voriconazole or posaconazole.
However, because voriconazole and posaconazole are effective against established IA, their use in different sequences is expected either as prophylaxis or treatment of established aspergillosis in high-risk patients. Whether breakthrough aspergillosis to voriconazole or posaconazole results in a higher risk of subsequent failure of the other drug and whether there are subtle changes in virulence in triazole pre-exposed Aspergillus are important questions, for which in vivo data are lacking. Thus, we assessed whether pre-exposure of Aspergillus fumigatus (AF) to voriconazole or posaconazole diminishes the subsequent activity of posaconazole or voriconazole both in vivo using a Drosophila melanogaster model of IA and in vitro using standardized susceptibility testing. Furthermore, we used our in vivo Toll-deficient fly model to assess the effect of voriconazole and posaconazole exposure on IA virulence.

Materials and methods

AF293 conidia were exposed to voriconazole or posaconazole by three serial passages on yeast extract-glucose (YAG) plates (2% glucose, 0.5% yeast extract, 2% agar, trace elements) containing a subinhibitory concentration of either drug (0.0625 mg/L voriconazole and 0.025 mg/L posaconazole), as described previously.5,7 Toll (Tl) transheterozygotes were generated by crossing flies carrying a thermosensitive allele of Tl (Tl<sup>66-12</sup>) with flies carrying a null allele of Tl (Tl<sup>S</sup>).5 Female 2–4-day-old Tl flies (30 flies/experimental group) were used. Fly infection was performed by injection into the thorax with a thin (0.1 μm) needle previously dipped in an AF293 conidial solution [10<sup>6</sup> conidia/mL (counted using a haemocytometer)].5 Following infection, flies were kept at 29°C, and survival was assessed until day 8 after infection.5,7 All experiments were performed in triplicate at different times.

For the assessment of voriconazole and posaconazole efficacy, flies were first housed in empty vials for 8 h and then transferred to voriconazole- or posaconazole-containing fly food vials (concentration, 1 g/L).5,7 A minute amount of corn oil (1:10<sup>4</sup> dilution) was added to the posaconazole-containing vials to improve drug absorption. Flies were inoculated 24 h later, to allow enough time for drug absorption, and flies were transferred to fresh voriconazole- or posaconazole-containing vials daily for 8 days. The absorption of voriconazole and posaconazole was verified using drug-level quantification in fly homogenate using HPLC (Pharmacokinetics Lab, National Jewish Medical and Research Center, Denver, CO, USA). Briefly, two groups of 24 flies each, fed for 24 h with either voriconazole- or posaconazole-containing food, were anesthetized and placed in a 500 μL Dulbecco’s PBS solution with glass beads. After homogenization, the solution was sent for analysis in dry ice.

The MIC of voriconazole and posaconazole against AF293 after each serial passage was determined using Clinical and Laboratory Standards Institute guidelines (M38-A document).8 Survival curves were plotted using the Kaplan–Meier analysis, and differences in survival were assessed using the log-rank test. Statistical analyses were performed using the GraphPad Prism software program (version 4.0; GraphPad Software, San Diego, CA, USA). P values less than 0.05 were considered statistically significant. The quantification of AF293 conidia in the injected inoculum was performed by transferring cells from the tip of a needle previously dipped in a conidial solution into 1 mL of 0.85% normal saline. Serial dilutions of that solution (100 μL each) were plated on YAG plates and incubated at 37°C, and cfu were counted after 72 h. Using a 10<sup>6</sup> conidia/mL solution resulted in inoculation with ~5 × 10<sup>5</sup> conidia.

Results

AF293 was virulent in Tl flies, resulting in a mortality rate of 71% 8 days after infection (median survival 4 days). Infection with AF293 conidia pre-exposed to voriconazole resulted in a comparable 8 day mortality rate (73%; median survival 4 days; P = 0.8) (Figure 1a). Similarly, infection with AF293 conidia pre-exposed to posaconazole resulted in a mortality rate of 76% (median survival 4 days; P = 0.49) (Figure 1b).

Both voriconazole and posaconazole were absorbed in Tl flies. The median homogenate voriconazole and posaconazole concentrations were 0.44 and 2.05 mg/L, respectively. Treatment with posaconazole had a protective effect compared with untreated controls, reducing the mortality rate to 36% (P < 0.0001). Prior conidial exposure to voriconazole did not alter posaconazole efficacy (8 day mortality, 39%; P < 0.92) (Figure 1c). Treatment with voriconazole also protected Tl flies, reducing the mortality rate to 42% (P = 0.0002). Similarly, prior conidial exposure to posaconazole did not alter voriconazole efficacy (8 day mortality, 34%; P = 0.35) (Figure 1d).

The MIC of both voriconazole and posaconazole was 0.5 mg/L in AF293 at baseline. No MIC change was observed following each passage through voriconazole- or posaconazole-containing plates (total of four passages).

Discussion

Whether breakthrough aspergillosis to voriconazole or posaconazole results in higher probability of subsequent failure of the other drug is an important question for which no in vivo data are available.4 Using a validated model of IA,5 we found that pre-exposure of AF293 to voriconazole did not affect the efficacy of posaconazole in protecting infected Tl-deficient flies. Similarly, pre-exposure to posaconazole did not affect subsequent voriconazole antifungal activity. Additionally, in contrast to what we recently observed regarding Zygomycetes species,8 exposure of AF to voriconazole or posaconazole does not affect its virulence in fruit flies.

We also assessed the effect of AF exposure to voriconazole and posaconazole on in vitro MICs. Serial passages through voriconazole- or posaconazole-containing media did not attenuate posaconazole or voriconazole inhibitory activity in vitro in accordance with our previous study in which AF exposure to fluconazole did not affect the MIC of voriconazole or itraconazole.10 The effect of staggered exposure of AF to different triazoles is neutral in vitro and appears to differ from the enhanced effects of caspofungin and itraconazole or the antagonistic effect of amphotericin B when administered after itraconazole.11,12

The present study has certain limitations. First of all, the assessment of cross-tolerance between only two triazoles as well as the use of only one Aspergillus isolate precludes generalization of our findings. A significant heterogeneity in susceptibility among AF isolates has been described, suggesting different mechanisms of resistance even within the same species.6 Furthermore, our findings in phylogenetically disparate models of aspergillosis (e.g. mice) need to be validated. Additional studies investigating whether our findings are applicable to non-fumigatus Aspergillus spp. and non-Aspergillus moulds and assessing the cross-tolerance between other azoles...
and non-azole antifungals are subjects of ongoing efforts from our group.

In conclusion, this is the first in vivo study assessing the effect of pre-exposure of Aspergillus to voriconazole or posaconazole on its virulence as well as the efficacy of subsequent treatment of aspergillosis with posaconazole and voriconazole, respectively. Our findings, although preliminary, show that treatment of aspergillosis with voriconazole or posaconazole following posaconazole or voriconazole protection, respectively, is effective.

Acknowledgements

We thank Nathaniel D. Albert for excellent technical assistance. This work was presented in part at the 47th Interscience Conference on Antimicrobial Agents and Chemotherapy, 17–20 September 2007, Chicago, IL, USA (Abstract B-1451).

Funding

This work was supported in part by an E. N. Cobb Scholar Award Research Endowment (to D. P. K.).

Transparency declarations

D. P. K. has received research support and honoraria from Merck&Co., Inc., Fujisawa, Enzon, Pfizer and Schering-Plough. R. E. L. has received research support from Merck&Co., Inc., Fujisawa, Pfizer, Enzon and Schering-Plough. All other authors have none to declare.

References

5. Lionakis MS, Lewis RE, May GS et al. Toll-deficient Drosophila flies as a fast, high-throughput model for the study of antifungal drug


