In vivo synergy of amphotericin B plus posaconazole in murine aspergillosis.

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ABSTRACT

Aspergillus fumigatus is the main mold causing invasive fungal infection showing high mortality rates. Therapeutic failure and the increase in drug-resistance make it necessary to explore alternative treatments for this infection. We have evaluated the efficacy of amphotericin B at 0.8 mg/kg or 0.3 mg/kg combined with 40 mg/kg of posaconazole against three *A. fumigatus* isolates in a murine model of disseminated infection. The combination of the polyene and the azole has led to a greater increase in survival and a significantly greater reduction in tissue burden than monotherapies.

INTRODUCTION

Aspergillus fumigatus is the most common mold causing invasive fungal infection (IFI) in immunocompromised patients (1), especially in those with hematological malignancies, with high mortality rates (2–4). Voriconazole (VRC) is the first-line therapy for the treatment of aspergillosis, but in patients with infections that are refractory to this drug, therapy options include other azoles such as itraconazole or posaconazole (PSC), lipid formulations of amphotericin B (LAMB) or echinocandins (5). Because of the relatively limited efficacy of the current antifungal treatments, exploring alternative strategies against this difficult-to-treat infection is crucial. Combinations of antifungal agents are not common therapies, but could be a good alternative for infections by resistant organisms or when the standard treatments fail (6–11). Synergistic interactions of two drugs with different targets on the fungal cell can be more effective than each drug working alone. In addition, combined therapies can allow lower doses to be administered, with lower toxicity, faster cure and probably lower costs. Since the efficacy of different antifungal combinations has been demonstrated by several studies in patients with aspergillosis (6, 7, 12), we considered of interest to evaluate the *in vivo* efficacy of the combination amphotericin B (AMB) plus PSC against *A. fumigatus*. This combination had already been tested in a murine model of invasive aspergillosis by *A. flavus* (13), although no improvement over the PSC monotherapy was observed. Another study demonstrated the efficacy of suboptimal doses of VRC plus anidulafungin in a murine model of *A. fumigatus* infection (14) suggesting that combined therapies could have an important role as alternative treatments against systemic aspergillosis, allowing a reduction of the doses administered. One of the

isolates tested in the present study (FMR 10528) had already been used in previous studies, showing poor *in vivo* response to VRC when administered at 25 mg/kg despite having low MIC (15, 16). The goals of this study were, i) to evaluate efficacy of the combination AMB plus PSC against isolates of *A. fumigatus* in a murine model of disseminated aspergillosis, comparing the results with those of the corresponding monotherapies and VRC, ii) to investigate the presence of *CYP51A* gene mutations that might explain the poor *in vivo* response of such isolate and iii) to perform adaptation experiments that can assess the ability of this isolate to develop azole resistance.

MATERIALS AND METHODS

Fungal isolates. Two clinical isolates (FMR 10528 and FMR 13142) and one environmental isolate (FMR 7739) of *A. fumigatus* were used in this study. Fungi were grown on potato dextrose agar (PDA). The minimum inhibitory concentrations (MICs) were previously determined in triplicate following the recommendations of CLSI guidelines (17). The MICs of AMB and PSC were 2 µg/ml and 0.5 µg/ml, respectively, for the strain FMR 7739, and 1 µg/ml and 0.25 µg/ml, respectively for the strains FMR 10528 and FMR 13142. MIC of VRC was 0.25 µg/ml for the three strains.

Inocula for both *in vitro* drugs interaction testing and *in vivo* assays, were prepared from 5-day-old cultures incubated at 37 ºC. Conidia were harvested with a sterile pipette by flooding the plates with sterile saline containing 0.025 % Tween 20. The suspensions were adjusted to the desired concentrations by hemocytometer counting and viability was assessed by placing 10-fold dilutions on PDA plates.

***In vitro* antifungal interaction testing.** The interaction testing was carried out using a two-dimensional checkerboard microdilution method with two-fold serial dilutions of AMB and PSC ranging from 0.12 to 8 µg/mL and from 0.002 to 1 µg/mL, respectively. Reading was taken 48 h after incubation at 35 ºC using an inverted mirror and MIC-0 (100 % growth inhibition) as the endpoint criterion. The fractional inhibitory concentration index (FICI) was used to classify drug interactions, which were defined as: synergistic if the FICI was ≤ 0.5, antagonistic if it was > 4, and indifferent if the FICI was > 0.5 but ≤ 4 (18). Tests were carried out in duplicate.

Infection. For the *in vivo* studies, male OF-1 mice (Charles River, Criffa S.A., Barcelona, Spain) weighing approximately 30 g were used. Animals were housed under standard conditions with water and food *ad libitum*. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

One day prior to the infection, animals were immunosuppressed by an intraperitoneal (i.p.) injection of 200 mg/kg of cyclophosphamide (Genoxal; Laboratories Funk S.A., Barcelona, Spain) and a single intravenous (i.v.) injection of 150 mg/kg of 5-fluorouracil (Fluorouracilo; Ferrer Farma S.A., Barcelona, Spain) (19).

Groups of 16 animals, 8 for survival and 8 for fungal load study and for determining drug serum levels, randomly chosen, were challenged i.v. via the lateral vein with 1 x 10^3 CFU/animal of the strain FMR 13142 or 1 x 10^4 CFU of the strains FMR 7739 and FMR 10528. Inocula sizes were adjusted for each strain in order to obtain a similar degree of infection in all the cases, causing

100 % of the animals to die within 9 days. After challenge, mice were checked daily for 30 days.

Treatments. All treatments started one day after infection and the animals were treated daily for 7 days. Treatments consisted of AMB at 0.8 mg/kg (amphotericin B deoxycholate; Xalabarder Pharmacy, Barcelona, Spain) administered i.v. (20); PSC (Noxafil; Schering-Plough Ltd., Hertfordshire, United Kingdom) at 20 mg/kg given orally by gavage (p.o.) twice a day (BID) (21) or VRC at 25 mg/kg (Vfend; Pfizer S.A., Madrid, Spain) administered p.o. (22). Combined therapies consisted of AMB plus PSC at the given doses, with the exception of animals infected with the strain FMR 7739, who received AMB 0.3 mg/kg in the combination due to the good efficacy obtained after the monotherapy with AMB at 0.8 mg/kg. From 2 days before the infection, animals receiving VRC were given 50 % grapefruit juice instead of water. Control animals received no antifungal treatment. In order to prevent bacterial infections, mice received subcutaneous injections of 5 mg/kg/day of ceftazidime.

Tissue burden and bioassay. Mice included in the tissue burden study (n = 8) were euthanized on day 6 post infection, in order to compare fungal load with the control group. Five animals from each group were also used to determine drug serum concentration by bioassay. For the bioassay, 2 hours after the 6 th dose, animals were anesthetized by inhalation of isoflurane and approximately 1 ml of blood was extracted by cardiac puncture, the serum being obtained by blood centrifugation. The concentrations of PSC, VRC and AMB from serum samples were determined by bioassay, using *Candida parapsilosis* ATCC 22019, as previously described (23). After blood extraction animals were euthanized by cervical dislocation and kidneys and lungs were aseptically

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removed, weighed, and homogenized in 1 ml of sterile saline. Serial 10-fold dilutions of the homogenates were placed on PDA plates and incubated for 48 h at 37 °C to determine CFU/g of tissue.

Amplification and sequencing of *CYP51A.* Genomic DNA of each *A. fumigatus* isolate was extracted from 3-day-old cultures (24). PCRs were carried out in a 25 µl volume, containing 10.5 µl of water, 12.5 µl of Taq Kapa 2G Robust Ready (Kapa Biosystems Inc., Wilmington, MA, USA), 0.5 µl of each primer (10 µM) and 50 ng of genomic DNA. Table 1 lists the primers used for amplification and sequencing of the *CYP51A* gene. Amplification took place in a thermal cycler for one cycle of 5 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 58 °C, and 2 min at 72 °C, followed by one final cycle of 10 min at 72 °C. The PCR products were analyzed by electrophoresis on 1 % agarose gel.

CYP51A gene sequences of 10 known wild-type *A. fumigatus* isolates were used to generate a consensus sequence (data not shown). This sequence served as a negative control (*CYP51A* without mutations) and was used for comparison with the sequences of the three isolates tested. Lasergene SeqMan (DNAStar, Madison, WI, USA) was used for generating the consensus wild-type *CYP51A* sequence and for checking the quality of the sequences, and Mega 6 (25) for alignment and gene comparison.

***In vitro* and *in vivo* adaptation experiments.** An *in vitro* adaptation experiment (26, 27) was carried out to investigate the poor *in vivo* efficacy of azoles, against the strains FMR 7739 and FMR 10528, despite their low MICs. Each strain was passaged 3 times at 1-week intervals on PDA containing PSC or VRC at a concentration corresponding to half of the MIC. Susceptibility to PSC and VRC of both strains before antifungal exposure and of their 3

subcultures exposed to azoles was determined by microdilution method (17). The assay was carried out in duplicate. In addition, *A. fumigatus* strains were recovered from the lung and kidneys of those animals infected and treated for 6 days with the azole monotherapies and the MICs were determined.

Statistical analysis. The mean survival time (MST) was estimated by the Kaplan-Meier method and compared among groups using the log rank test. Tissue burden from control and treated groups was compared using the Mann-Whitney U test. All statistical analyses used GraphPad Prism 6.0 for Windows. *P* values ≤ 0.05 were considered statistically significant.

RESULTS

***In vitro* interaction testing.** The effect of the *in vitro* interaction between AMB and PSC was indifferent for the three strains with FICIs ranging from 0.56 to 0.73 (data not shown).

***In vivo* studies.** Untreated animals began to die on day 4 - 5 post infection, and on day 10 no animals were alive. For the strain FMR 7739, AMB at 0.8 mg/kg significantly increased the survival of the animals compared to the controls ($p < 0.0001$) and the other monotherapy groups ($p \leq 0.0085$), all animals being alive at the end of the experiment (day 30 post infection). Therefore, in the combined therapy, a suboptimal dose of AMB was tested, i.e. AMB at 0.3 mg/kg, which also prolonged significantly the survival with respect to control group ($p = 0.0384$). By contrast, neither PSC nor VRC on their own were able to increase survival ($p = 0.3926$ and $p= 0.227$, respectively). The combination significantly increased survival compared to animals treated with AMB 0.3, PSC or VRC ($p \leq 0.0313$).

With the strain FMR 10528, an increase in survival was only observed with AMB 0.8 mg/kg and the combination ($p \leq 0.029$). In addition, the combined therapy worked better than the monotherapies ($p \leq 0.0418$). In the case of the strain FMR 13142, all the therapies, including the combination, significantly increased survival of animals ($p \leq 0.0269$).

In the fungal load study, the combination of AMB plus PSC showed efficacy in reducing the number of CFUs in both organs and in all strains studied, which was even better than monotherapies with either azole in all strains, with the exception of lung from animals infected with the strain FMR 13142, where the combination equaled the efficacy of VRC. The combination also improved the efficacy of AMB 0.8 in lung of the animals infected with the strain FMR 7739 and in kidney from those infected with FMR 10528.

AMB 0.8 and VRC were able to reduce tissue burden in both organs from animals infected with each of the three strains ($p \leq 0.0002$, and $p \leq 0.0298$, respectively), with the only exception of VRC against the strain FMR 10528 in lungs ($p = 0.1044$). PSC reduced fungal burden of kidneys of animals infected with each of the three strains ($p \leq 0.0463$), and in lungs of animals infected with the strain FMR 10528 ($p = 0.0002$).

Serum concentrations of AMB 0.8, PSC 40 and VRC 25 were higher than the MICs, with values of 4.28 ± 0.31, 6.34 ± 0.90, and 9.99 ± 0.71 μg/ml, respectively.

Amplification and sequencing of *CYP51A*. No mutations were found in the *CYP51A* gene sequences of the three strains tested, such sequences being identical to that of the wild-type consensus sequence.

Accession numbers. The *CYP51* sequences from FMR 7739, FMR 10528 and FMR 13142 have been deposited in the NCBI database and are available under accession numbers KT070084, KT070085 and KT070086, respectively.

***In vitro* and *in vivo* adaptation experiments.** With two of the strains grown on PDA plates containing PSC or VRC, no increase in MICs was observed. Only the strain FMR 10528 showed VRC MIC two dilutions higher than before drug exposure. However, important morphological changes were observed. The colony growth rate decreased in the three strains tested. There was also a noticeable reduction of sporulation and change in the pigmentation of the colonies of the strain FMR 10528, from green to pale green.

MICs of VRC and PSC against the isolates recovered from treated-animals were the same or one dilution higher to that obtained originally. These isolates also showed a reduction in the growth rate and sporulation, and also a change in the colony color.

DISCUSSION

Due to the important increase in azole resistance of *Aspergillus* and the associated therapeutic failure, finding alternatives to the current therapies is crucial. In the present study, we tested the combination AMB plus PSC in a neutropenic model of disseminated aspergillosis, using three *A. fumigatus* strains. In previous studies conducted in animal models, VRC at 25 mg/kg demonstrated poor efficacy against systemic aspergillosis by one of the strains included in the present study i. e., strain FMR 10528. (15, 16). Now, we can corroborate the lack of efficacy of VRC administered at 25 mg/kg and, in addition, we have also found therapeutic failure of PSC administered at 40

mg/kg against two of the three strains assayed. It is worth mentioning that no correlation was found between the two parameters of efficacy used, i.e. survival and fungal burden. Azoles were not able to improve survival in those animals infected with the strains FMR 7739 or FMR 10528, but there was a reduction in fungal burden in at least one organ.

PSC is known to show good efficacy against *A. fumigatus* infections, which decreases when the fungus harbors mutations in the *CYP51A* gene (28, 29) however, in the present study, no mutations in the *CYP51A* gene were found. In order to explain the lack of efficacy, azole adaptation assays were carried out to determine the ability of the strains to develop resistance to the drugs, as continuous contact with a compound is known to be able to result in tolerance and development of resistance to it, as seems to occur with *A. fumigatus* and azoles (30–32).

In a previous study, Salas et al., (15) demonstrated that *A. fumigatus* strains varied greatly in their *in vivo* responses to VRC, particularly for those isolates with MIC values of ≥ 0.25 µg/ml. This has been observed in the present study for both VRC and PSC, suggesting a strain-to-strain variability. In the present study, all three strains show MIC of VRC of 0.25 µg/ml and of 0.25 to 0.5 µg/ml for PSC, all values below the epidemiological cutoff values (ECVs) (33). Different studies suggest that MICs and ECVs are useful predictors of azole efficacy *in vivo* (15, 29). However, other studies suggest that pharmacokinetics (PK) and particularly the determination of AUC/MIC correlate better with efficacy than MICs, the AUC/MIC values being > 25 and > 100 for VRC and PSC, respectively, predictors of successful outcome (34, 35). Although the PK parameters have not been determined in the present study, previous studies

have demonstrated the efficacy of VRC and PSC administered at 25 mg/kg and 40 mg/kg, respectively, against *Aspergillus* spp. in murine models (14, 15, 22, 28, 29, 36) and that multiple dosing of VRC at 20 mg/kg in mice resulted in AUC_{0-24} of 58.1 h·mg/L, leading to efficacy against invasive aspergillosis by a strain with a MIC of 0.25 µg/ml (14). Other studies testing similar PSC and VRC doses as those used in our experiment have shown AUC values indicative of correct exposure (28, 37–39).

Little information is available on the *in vitro* interaction of AMB and PSC against *A. fumigatus*. Perkhofer et al. (40) reported *in vitro* indifference of this combination against 88 % of the *Aspergillus* isolates studied. Although we found indifferences in the *in vitro* studies, such a combination has shown significant *in vivo* efficacy. *In vitro* results are not always found to be predictive of synergistic effects in animal models or in the clinical setting, which is why other authors have reviewed the categorization of drug interactions i.e., synergy, indifference or antagonism, based on the FICI. Meletiadis et al. found a better correlation between the FICI and the outcome if FICI < 1 was considered indicative of synergy (41) instead of ≤ 0.5. Our results show a good correlation between the *in vitro* and the *in vivo* data when considering synergistic effects at FICI <1, however more studies are necessary to extrapolate the meaning of FICI to the outcome.

This study has some limitations. In the combined therapy AMB was administered at 0.8 mg/kg except against the infection by one strain (FMR 7739), against which AMB was used at 0.3 mg/kg due to the good efficacy it showed as a monotherapy at 0.8 mg/kg. Moreover, we used a different inocula size for each strain in order to obtain the same degree of infection.

Overall, our results demonstrate that the combination AMB plus PSC show efficacy against *A. fumigatus*, improving the efficacy of the monotherapies with azoles and AMB in some cases, and might represent an alternative when the recommended treatment fails, with a possible reduction of the dose and, consequently, the toxicity and the cost. More studies testing more strains are needed to determine more accurately the role of such combination in the treatment of IA.

REFERENCES

1. **Mayr A, Lass-Flörl C**. 2011. Epidemiology and antifungal resistance in invasive aspergillosis according to primary disease: review of the literature. Eur J Med Res **16**:153–157.

2. **Neofytos D, Horn D, Anaissie E, Steinbach W, Olyaei A, Fishman J, Pfaller M, Chang C, Webster K, Marr K**. 2009. Epidemiology and outcome of invasive fungal infection in adult hematopoietic stem cell transplant recipients: analysis of Multicenter Prospective Antifungal Therapy (PATH) Alliance registry. Clin Infect Dis **48**:265–273.

3. **Parody R, Martino R, Sánchez F, Subirá M, Hidalgo A, Sierra J**. 2009. Predicting survival in adults with invasive aspergillosis during therapy for hematological malignancies or after hematopoietic stem cell transplantation: Single-center analysis and validation of the Seattle, French, and Strasbourg prognostic indexes. Am J Hematol **84**:571–578.

4. **Ramos ER, Jiang Y, Hachem R, Kassis C, Kontoyiannis DP, Raad I**. 2011. Outcome analysis of invasive aspergillosis in hematologic malignancy and hematopoietic stem cell transplant patients: the role of novel antimold azoles. Oncologist **16**:1049–60.

5. **Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Segal BH, Steinbach WJ, Stevens D a, van Burik J-A, Wingard JR, Patterson TF**. 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis **46**:327–360.

6. **Maertens J, Glasmacher A, Herbrecht R, Thiebaut A, Cordonnier C, Segal BH, Killar J, Taylor A, Kartsonis N, Patterson TF**. 2006.

Multicenter, noncomparative study of caspofungin in combination with other antifungals as salvage therapy in adults with invasive aspergillosis. Cancer **107**:2888–2897.

7. **Singh N, Limaye AP, Forrest G, Safdar N, Muñoz P, Pursell K, Houston S, Rosso F, Montoya JG, Patton P, Del Busto R, Aguado JM, Fisher RA, Klintmalm GB, Miller R, Wagener MM, Lewis RE, Kontoyiannis DP, Husain S**. 2006. Combination of voriconazole and caspofungin as primary therapy for invasive aspergillosis in solid organ transplant recipients: a prospective, multicenter, observational study. Transplantation **81**:320–326.

8. **Liu P, Mould DR**. 2014. Population pharmacokinetic-pharmacodynamic analysis of voriconazole and anidulafungin in adult patients with invasive aspergillosis. Antimicrob Agents Chemother **58**:4727–36.

9. **Raad II, Zakhem A El, Helou G El, Jiang Y, Kontoyiannis DP, Hachem R**. 2015. Clinical experience of the use of voriconazole, caspofungin or the combination in primary and salvage therapy of invasive aspergillosis in haematological malignancies. Int J Antimicrob Agents **45**:283–288.

10. **Marr KA, Schlamm HT, Herbrecht R, Rottinghaus ST, Bow EJ**. 2015. Combination antifungal therapy for invasive aspergillosis. Ann Intern Med **162**:81–89.

11. **Ogawa T, Matsumoto K, Tsujimoto K, Hishiya N, Yamada Y, Uno K, Kasahara K, Maeda K, Nario K, Mikasa K, Morita K**. 2015. Chronic invasive sinus and intracerebral aspergillosis controlled by combination therapy with micafungin and a daily dose of 400 mg itraconazole oral solution. J Infect Chemother **21**:134–137.

12. **Marr KA, Boeckh M, Carter RA, Kim HW, Corey L**. 2004. Combination antifungal therapy for invasive aspergillosis. Clin Infect Dis **39**:797–802.

13. **Najvar LK, Cacciapuoti A, Hernandez S, Halpern J, Bocanegra R, Gurnani M, Menzel F, Loebenberg D, Graybill JR**. 2004. Activity of posaconazole combined with amphotericin B against *Aspergillus flavus* infection in mice: Comparative studies in two laboratories. Microbiology **48**:758–764.

14. **Seyedmousavi S, Brüggemann RJM, Melchers WJG, Rijs AJMM, Verweij PE, Mouton JW**. 2013. Efficacy and pharmacodynamics of voriconazole combined with anidulafungin in azole-resistant invasive aspergillosis. J Antimicrob Chemother **68**:385–393.

15. **Salas V, Javier Pastor F, Calvo E, Sutton DA, Fothergill AW, Guarro J**. 2013. Evaluation of the in vitro activity of voriconazole as predictive of in vivo outcome in a murine *Aspergillus fumigatus* infection model. Antimicrob Agents Chemother **57**:1404–1408.

16. **Sandoval-Denis M, Pastor FJ, Capilla J, Guarro J**. 2013. Efficacy of amphotericin B at suboptimal dose combined with voriconazole in a murine model of *Aspergillus fumigatus* infection with poor in vivo response to the azole. Antimicrob Agents Chemother **57**:4540–4542.

17. **Clinical and Laboratory Standards Institute**. 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; Approved standard - Second edition. Document M38-A2.

18. **Odds FC**. 2003. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother.

19. **Ortoneda M, Capilla J, Pastor FJ, Serena C, Guarro J**. 2004. Interaction of granulocyte colony-stimulating factor and high doses of liposomal amphotericin B in the treatment of systemic murine scedosporiosis. Diagn Microbiol Infect Dis **50**:247–51.

20. **Graybill JR**. 2000. The role of murine models in the development of antifungal therapy for systemic mycoses. Drug Resist Updat **3**:364–383.

21. **Salas V, Pastor FJ, Rodríguez MM, Calvo E, Mayayo E, Guarro J**. 2011. In vitro activity and in vivo efficacy of posaconazole in treatment of murine infections by different isolates of the *Aspergillus terreus* complex. Antimicrob Agents Chemother **55**:676–679.

22. **Warn PA, Sharp A, Mosquera J, Spickermann J, Schmitt-Hoffmann A, Heep M, Denning DW**. 2006. Comparative in vivo activity of BAL4815, the active component of the prodrug BAL8557, in a neutropenic murine model of disseminated *Aspergillus flavus*. J Antimicrob Chemother **58**:1198–1207.

23. **Sugar a. M, Liu XP**. 2001. Efficacy of voriconazole in treatment of murine pulmonary blastomycosis. Antimicrob Agents Chemother **45**:601–604.

24. **Lackner M, Najafzadeh MJ, Sun J, Lu Q, de Hoog GS**. 2012. Rapid identification of Pseudallescheria and Scedosporium strains by using rolling circle amplification. Appl Environ Microbiol **78**:126–133.

25. **Tamura K, Stecher G, Peterson D, Filipski A, Kumar S**. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol **30**:2725–2729.

26. **Faria-Ramos I, Farinha S, Neves-Maia J, Tavares P, Miranda IM, Estevinho LM, Pina-Vaz C, Rodrigues AG**. 2014. Development of cross-resistance by *Aspergillus fumigatus* to clinical azoles following exposure to prochloraz, an agricultural azole. BMC Microbiol **14**:155.

27. **Hryncewicz-Gwóźdź A, Kalinowska K, Plomer-Niezgoda E, Bielecki J, Jagielski T**. 2013. Increase in resistance to fluconazole and

itraconazole in *Trichophyton rubrum* clinical isolates by sequential passages in vitro under drug pressure. Mycopathologia **176**:49–55.

28. **Mavridou E, Brüggemann RJM, Melchers WJG, Mouton JW, Verweij PE**. 2010. Efficacy of posaconazole against three clinical *Aspergillus fumigatus* isolates with mutations in the cyp51A gene. Antimicrob Agents Chemother **54**:860–865.

29. **Lepak AJ, Marchillo K, VanHecker J, Andes DR**. 2012. Posaconazole pharmacodynamic target determination against wild-type and Cyp51 mutant isolates of *Aspergillus fumigatus* in an in vivo model of invasive pulmonary aspergillosis. Antimicrob Agents Chemother **57**:579–585.

30. **Snelders E, Huis In't Veld RAG, Rijs AJMM, Kema GHJ, Melchers WJG, Verweij PE**. 2009. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. Appl Environ Microbiol **75**:4053–4057.

31. **Snelders E, Camps SMT, Karawajczyk A, Schaftenaar G, Kema GHJ, van der Lee HA, Klaassen CH, Melchers WJG, Verweij PE**. 2012. Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. PLoS One **7**.

32. **Verweij PE, Snelders E, Kema GH, Mellado E, Melchers WJ**. 2009. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? Lancet Infect Dis **9**:789–795.

33. **Espinel-Ingroff A, Diekema DJ, Fothergill A, Johnson E, Pelaez T, Pfaller MA, Rinaldi MG, Canton E, Turnidge J**. 2010. Wild-Type MIC Distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI Broth Microdilution Method (M38-A2 Document). J Clin Microbiol **48**:3251–3257.

34. **Andes D, Marchillo K, Stamstad T, Conklin R**. 2003. In vivo pharmacokinetics and pharmacodynamics of a new triazole, voriconazole, in a murine candidiasis model. Antimicrob Agents Chemother **47**:3165–3169.

35. **Andes D, Marchillo K, Conklin R, Krishna G, Ezzet F, Cacciapuoti A, Loebenberg D**. 2004. Pharmacodynamics of a new triazole, posaconazole, in a murine model of disseminated candidiasis. Antimicrob Agents Chemother **48**:137–142.

36. **Oakley KL, Morrissey G, Denning DW**. 1997. Efficacy of SCH-56592 in a temporarily neutropenic murine model of invasive aspergillosis with an itraconazole-susceptible and an itraconazole- resistant isolate of *Aspergillus fumigatus*. Antimicrob Agents Chemother **41**:1504–1507.

37. **Seyedmousavi S, Bruggemann RJM, Melchers WJG, Verweij PE, Mouton JW**. 2014. Intrapulmonary posaconazole penetration at the

infection site in an immunosuppressed murine model of invasive pulmonary aspergillosis receiving oral prophylactic regimens. Antimicrob Agents Chemother **58**:2964–2967.

38. **Petraitiene R, Petraitis V, Groll a. H, Sein T, Piscitelli S, Candelario M, Field-Ridley a., Avila N, Bacher J, Walsh TJ**. 2001. Antifungal activity and pharmacokinetics of posaconazole (SCH 56592) in treatment and prevention of experimental invasive pulmonary aspergillosis: Correlation with galactomannan antigenemia. Antimicrob Agents Chemother **45**:857–869.

39. **Howard SJ, Lestner JM, Sharp a., Gregson L, Goodwin J, Slater J, Majithiya JB, Warn P a., Hope WW**. 2011. Pharmacokinetics and pharmacodynamics of posaconazole for invasive pulmonary aspergillosis: clinical implications for antifungal therapy. J Infect Dis **203**:1324–1332.

40. **Perkhofer S, Lugger H, Dierich MP, Lass-Flörl C**. 2007. Posaconazole enhances the activity of amphotericin B against *Aspergillus* hyphae in vitro. Antimicrob Agents Chemother **51**:791–793.

41. **Meletiadis J, Pournaras S, Roilides E, Walsh TJ**. 2010. Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations, Monte Carlo simulation analysis, and in vitro-in vivo correlation data for antifungal drug combinations against Aspergillus fumi. Antimicrob Agents Chemother **54**:602–609.

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462 **TABLES AND FIGURES**

463 **TABLE 1** Primers used for the amplification of *CYP51A* of *A. fumigatus*.

Primer	Sequence
Cyp51 AF F1	5'-CACCCTCCCTGTGTCTCCT–3'
Cyp51 AF R1	5'-CCGATCACACCAAATCCTTT–3'
Cyp51 AF S1	5'-CTCAGCCGTGAGTTTGGAAC-3'
Cyp51 AF S2	5'-CCTCACAGCCAAAAGTCCTC-3'
Cyp51 AF S3	5'-ATTGTCCCAATTCCAAGCTG-3'
Cyp51 AF S4	5'-TCTCTGCACGCAAAGAA-3'

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FIG 1 Cumulative mortality of immunosuppressed mice infected with *A. fumigatus* FMR 7739 (A), FMR 10528 (B) and FMR 13142 (C). AMB 0.8, amphotericin B at 0.8 mg/kg QD; PSC 40, posaconazole at 20 mg/kg BID; VRC 25, voriconazole at 25 mg/kg QD. [a] $P \leq 0.05$ versus control, [b] $P < 0.05$ versus PSC 40; [c] $P < 0.05$ versus VRC 25; [d] $P < 0.05$ versus AMB 0.8, [e] $P < 0.05$ versus AMB 0.3.

FIG 2 Effect of antifungal treatments on colony counts of *A. fumigatus* FMR 7739 (A), FMR 10528 (B) or FMR 13142 (C) in lungs and kidneys of immunosuppressed mice. AMB 0.8, amphotericin B at 0.8 mg/kg QD; PSC 40, posaconazole at 20 mg/kg BID; VRC 25, voriconazole at 25 mg/kg QD. Horizontal lines indicate median values. [a] $P < 0.05$ versus control; [b] $P < 0.05$ versus PSC 40; [c] $P < 0.05$ versus VRC 25; [d] $P < 0.05$ versus AMB 0.8; [e] $P < 0.05$ versus AMB 0.3.