Combination Therapy with Isavuconazole and Micafungin for Treatment of Experimental Invasive Pulmonary Aspergillosis

Vidmantas Petraitisa,b, Ruta Petraitienea,b, Matthew W. McCarthya, Laura L. Kovandac, Myo H. Zawa, Kaiser Hussaina, Naima Shaikha, Bo Bo W. Maunga, Navjot K. Sekhona, William W. Hoped, and Thomas J. Walsha, e

Transplantation-Oncology Infectious Diseases Program, Division of Infectious Diseases, Department of Medicine, Weill Cornell Medicine of Cornell University, New York, New York, USAa;Institute of Infectious Disease and Pathogenic Microbiology, Prienai, Lithuaniab; Astellas Pharma Global Development, Inc., Northbrook, Illinois, USAc; Antimicrobial Pharmacodynamics and therapeutics, Department of Molecular and Clinical Pharmacology, University of Liverpool, Liverpool, United Kingdomd; Departments of Pediatrics and Microbiology & Immunology, Weill Cornell Medicine of Cornell University, New York, New York, USAe

**Running Title:** Isavuconazole and micafungin for treatment of IPA

Address correspondence to Thomas J. Walsh, MD, thw2003@med.cornell.edu and Vidmantas Petraitis, MD, vip2007@med.cornell.edu

Correspondence: Dr. Thomas J. Walsh, Weill Cornell Medicine, 1300 York Ave., Room A-421, New York, NY, USA, 10065, Phone number: 1 212 746 6320, Fax number: 1 212 746 8675, E-mail: thw2003@med.cornell.edu

**Keywords.** Isavuconazole, micafungin, combination therapy, *Aspergillus fumigatus*, aspergillosis, pharmacokinetics, galactomannan, (1→3)-β-D-glucan, neutropenia

**ABSTRACT**

Invasive pulmonary aspergillosis (IPA) is an important cause of morbidity and mortality in immunocompromised patients. We hypothesized that simultaneous inhibition of biosynthesis of ergosterol in the fungal cell membrane and (1→3)-β-D-glucan in the cell wall, respectively, by the antifungal triazole isavuconazole and the echinocandin micafungin, may result in improved outcome in experimental IPA in persistently neutropenic rabbits. Treatment groups included isavuconazole (ISA) at 20 (ISA20), 40 (ISA40), and 60 (ISA60) mg/kg/day, micafungin at 2 mg/kg/day (MFG2), or combinations of (ISA20+MFG2), (ISA40+MFG2), (ISA60+MFG2), and untreated rabbits (UC). Galactomannan index (GMI) and (1→3)-β-D-glucan levels were measured in serum. Residual fungal burden (CFU/g) was significantly reduced in ISA20-, ISA40-, ISA60-, ISA20+MFG2-, ISA40+MFG2-, and ISA60+MFG2-treated rabbits vs that of MFG2-treated or UC (p<0.01). Measures of organism-mediated pulmonary injury, lung weights and pulmonary infarct score, were lower in ISA40+MFG2-treated rabbits in comparison to those of ISA40 or MFG2 alone (p<0.01). Survival in ISA40+MFG2-treated rabbits was prolonged in comparison to those treated with ISA40 or MFG2 alone (p<0.01). These outcome variables correlated directly with a significant decline of GMI and serum (1→3)-β-D-glucan levels during therapy. GMI correlated with measures of organism-mediated pulmonary injury, lung weights (r=0.764; p<0.001) and pulmonary infarct score (r=0.911; p<0.001). In summary, rabbits receiving combination therapy with isavuconazole and micafungin demonstrated significant dose-dependent reduction of residual fungal burden, decreased pulmonary injury, prolonged survival, lower GMI and serum (1→3)-β-D-glucan levels in comparison to that of single agent isavuconazole or micafungin.

**INTRODUCTION**

Invasive pulmonary aspergillosis (IPA) is a life-threatening infection in immunosuppressed patients, particularly in those with severe and prolonged neutropenia as a consequence of aplastic anemia, myelotoxic chemotherapy for treatment of acute leukemia, and in those receiving immunosuppressive medication for rejection prophylaxis after organ transplantation or treatment of graft-versus-host disease in allogeneic bone marrow transplantation (1-5). Despite advances in antifungal therapy, mortality and morbidity remains unacceptably high. New therapeutic strategies for IPA are clearly needed.

Our recent *in vitro* studies of the new extended-spectrum antifungal triazole, isavuconazole, and the echinocandin, micafungin, demonstrated synergistic interaction against *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus terreus* (6). We then hypothesized that simultaneous inhibition of the biosynthesis of ergosterol in the fungal cell membrane and (1→3)-β-D-glucan in the cell wall, respectively, by the antifungal triazole isavuconazole and the echinocandin micafungin, may result in improved outcome in experimental IPA in persistently neutropenic rabbits.

We therefore studied the efficacy of isavuconazole in combination with micafungin in treatment of experimental IPA in persistently neutropenic rabbits. The data from this study will establish the foundation for further clinical evaluation.

**MATERIALS AND METHODS**

**Isolate.** NIH *Aspergillus fumigatus* isolate 4215 (ATCC No. MYA-1163), as previously described (7), obtained from a patient with a fatal case of pulmonary aspergillosis, was used in the study. The minimal inhibitory concentrations (MIC) of isavuconazole and minimum effective concentration (MEC) of micafungin against *A. fumigatus*, determined according CLSI standard (M38-A2) microdilution methods (8), were 1 µg/mL and 0.06 µg/mL, respectively. This isolate has been extensively used in previous studies of antifungal agents against invasive pulmonary aspergillosis for more than two decades.

**Animals.** Healthy female New Zealand White rabbits (Covance Research Products, Inc., Denver, PA) weighing 2.6 to 3.5 kg at the time of endotracheal inoculation were used in replicate experiments. All rabbits were monitored under humane care and use of standards in facilities, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and according to the guidelines of the National Research Council (9) for the care and use of laboratory animals, and under the approval of the Institutional Animal Care and Use Committee. Rabbits were individually housed and maintained with water and standard rabbit feed *ad libitum*. Atraumatic vascular access was established by modified surgical placement of a Silastic tunneled central venous catheter as previously described elsewhere (10). The Silastic catheter permitted nontraumatic venous access for administration of parental agents and for repeated blood sampling for study of plasma pharmacokinetics, serum galactomannan and (1→3)-β-D-glucan levels, biochemical and hematological parameters.

**Inoculum and inoculation.** For each experiment, an inoculum of 1 × 108 to 1.25 × 108 conidia of A. fumigatus in a volume of 250 μL to 350 μL was prepared. Inoculation was performed on day 2 of the experiments while rabbits were under general anesthesia (0.5 to 0.6 mL of a 2:1 mixture (vol/vol) of ketamine 100 mg/mL and xylazine 20 mg/mL administrated intravenously), as described previously (7).

**Immunosuppression and maintenance of neutropenia.**Immunosuppression and profound persistent neutropenia (a neutrophil concentration of < 100 neutrophils/µL) was established and maintained using cytarabine (Ara-C) (Cytarabine injection; Zydus Hospira Oncology Private Ltd., Gujarat, India for Hospira, Inc., Lake Forest, IL) and methylprednisolone (Solu-Medrol®, Pfizer for Pharmacia & Upjohn Co., Division of Pfizer Inc., NY, NY), as described previously (7). Antibiotics (ceftazidime, gentamicin, vancomycin) were used for prevention of opportunistic bacterial infections during neutropenia (7).

**Antifungal compounds and treatment regimens.**Treatment groups included rabbits receiving orally administered prodrug isavuconazonium sulfate (BAL8557) equivalent to active moiety isavuconazole (BAL4815, ISA) at 20 (ISA20), 40 (ISA40), and 60 (ISA60) mg/kg/day, micafungin intravenously at 2 mg/kg/day (MFG2), or the combination of (ISA20+MFG2), (ISA40+MFG2), (ISA60+MFG2), or untreated rabbits (UC). Treatment started 24 h after endotracheal administration of the *A. fumigatus* inoculum and continued once daily for up to 12 days. The dosage of micafungin used in this study is comparable in plasma exposure to approximately 0.5 mg/kg/d in humans. The dosage of isavuconazole 20 to 40 mg/kg/d in the rabbit model approximates plasma exposure achieved with the licensed human adult dose of 200 mg/d, while the dosage of 60 mg/kg/d produces exposure approximating that achieved with 400 mg/d in patients.

**Outcome variables.**The following panel of outcome variables was used to assess antifungal efficacy: survival, pulmonary infarct score, lung weight, and residual fungal burden (log CFU/g). The outcome variable panel was applied to all study rabbits when possible.

***Pulmonary lesion scores, lung weights, and residual fungal burden.***The lungs were carefully resected at autopsy. Pulmonary lesion scores and lung weights were assessed and calculated as previously described (7). Lung tissue from each rabbit was sampled and cultured by a standard excision of tissue from each lobe as previously described (7). The number of CFU of *A. fumigatus* was counted and recorded for each lobe, and the CFU/g was calculated.

***Survival.*** The survival time in days post inoculation was recorded for each rabbit in each group. Following humane end points, rabbits were euthanized by intravenous (i.v.) administration of pentobarbital (65 mg of pentobarbital sodium/kg of body weight; Beuthanasia-D Special [euthanasia solution]; Schering-Plough Animal Health Corp., Union, NJ) on day 13 postinoculation, 24 h after the last dose of study drug (10).

**Bronchoalveolar lavage.** BAL was performed, as described previously (11), on each lung preparation by the instillation of 10 mL of sterile normal salineinto the clamped trachea with a sterile 12 mL syringe and subsequent withdrawal. The instillations repeated twice. The lavage was then centrifuged for 10 min at 400 × *g*. Part of the supernatantwas discarded, leaving 2 mL of pellet with supernatant, which was then vortexed. An aliquot of 100 µL of this fluidand 100 µL of a dilution (101) of this fluid were cultured on 5% SGAplates.

**Detection of galactomannan.** Sera samples from each rabbit were collected every other day and stored at -80°C before analysis. Galactomannan antigen levels were determined in serial serum samples and postmortem obtained BAL fluid by one-stage immunoenzymatic sandwich microplate assay method (12) (Platelia® *Aspergillus* Enzyme Immunoassay (EIA); Bio-Rad, Marnes la Coquette, France) according to manufacturer’s instructions and described elsewhere (11). Enzyme immunoassay data were expressed as a serum galactomannan index (GMI) plotted over time. The GMI for each test serum or BAL fluid sample was equal to the absorbance of a standard sample divided by the absorbance of a threshold serum provided by the manufacturer. A GMI less than 0.5 was considered negative.

**Detection of (1→3)-β-D-glucan**. Serum from each rabbit was collected every other day for determination of (1→3)-β-D-glucan levels by using a colorimetric assay (Fungitell™, Associates of Cape Cod, Inc.) read at 405 nm (with 490 nm background subtraction), based upon *para*-nitroanilide absorption at that wavelength, performed according to the manufacturer’s instructions, and described in detail elsewhere (13). The (1→3)-β-D-glucan levels were determined by taking the mean optical density of the duplicate readings and comparing with the standard curve of pre-determined concentrations. Interpretation of (1→3)-β-D-glucan values, according to manufacturer’s instructions, was as follows: < 60 pg/mL, negative; 60 to 79 pg/mL, indeterminate; ≥ 80 pg/mL, positive. The median correlation coefficient of the standard curves performed in these studies was r≥0.9992 (range from 0.9982 to 0.9998).

**Statistical analysis.**Comparisons between the groups were performed by analysis of variance (ANOVA) with Bonferroni’s correction for multiple comparisons or the Mann-Whitney U-test, as appropriate. The central hypothesis of this analysis was based upon the response of isavuconazole in comparison to that of voriconazole and of untreated controls. A two-tailed p value of ≤ 0.05 was considered to be statistically significant.Survival was plotted by Kaplan-Meier analysis.Differences in survival of treatment groups and untreated controls were analyzed by log-rank test. Values are expressed as means ± standard error of the means (SEMs). Pharmacokinetic parameters were compared using ANOVA or Student’s t-test, as appropriate.

**RESULTS**

Organism-mediated pulmonary injury was measured by total lung weights and pulmonary infarct score. Total lung weights and pulmonary infarct score were significantly lower in ISA60-, ISA20+MFG2-, ISA40+MFG2-, and ISA60+MFG2-treated rabbits in comparison to that of MFG2-treated and UC (p<0.05) (Fig1A, 1B). In addition, rabbits treated with ISA40+MFG demonstrated significantly lower lung weights and pulmonary infarct score in comparison to those of ISA40 or MFG2 alone (p<0.01). Rabbits treated with ISA20+MFG2, ISA40+MFG2, and ISA60+MFG2 significantly prolonged survival in comparison to that of UC (p<0.01) (Fig. 2A). Mortality was numerically greater in monotherapy regimens of ISA20, ISA40, and MCG2. ISA40+MFG2-treated rabbits demonstrated significantly prolonged survival in comparison to that of single therapy of ISA40 or MFG2 (p<0.01) (Fig. 2A). There was a significant reduction of residual fungal burden (CFU/g) in ISA20-, ISA40-, ISA60-, ISA20+MFG2-, ISA40+MFG2-, and ISA60+MFG2-treated rabbits vs that of MFG2-treated or UC (p<0.01) (Fig. 2B).

Serum (1→3)-β-D-glucan levels were significantly lower in all isavuconazole combination treatment groups in comparison to isavuconazole or micafungin alone treatment with exception of ISA60 (p<0.05) (Fig. 3). Serum GMI was significantly lower in all isavuconazole combination treatment groups in comparison to isavuconazole or micafungin alone treatment (p<0.05) (Fig. 4). GMI strongly correlated with measures of organism-mediated pulmonary injury (total lung weights (r=0.764, (p<0.001)) and pulmonary infarct scores (r= 0.911, (p<0.001)) (Fig. 5).

**DISCUSSION**

This study demonstrated that the combination of ISA40+MFG2 was significantly more active than either single agent isavuconazole at 40 mg/kg and micafungin in significantly reducing mortality, as well as the parameters of organism-mediated pulmonary injury (lung weights and pulmonary infarct score) in treatment of experimental pulmonary aspergillosis in persistently neutropenic rabbits. Moreover, ISA20+MFG2 and ISA40+MFG2 were more active in combination than monotherapy in significantly reducing serum GMI and circulating levels of (1→3)-β-D-glucan. GMI also correlated with measures of organism-mediated pulmonary injury (lung weights and pulmonary infarct score). Thus, rabbits receiving combination therapy of isavuconazole with micafungin demonstrated significant dose-dependent reduction of residual fungal burden, decreased pulmonary injury, prolonged survival, lower GMI, and lower serum (1→3)-β-D-glucan levels in comparison to that of single agent isavuconazole or micafungin.

The study of combination antifungal therapy in experimental model systems of invasive aspergillosis is essential for understanding the basic pharmacology, as well for designing, implementing and de-risking clinical protocols for treatment of invasive fungal infections. The model system herein investigates a series of endpoints that individually and collectively allow for a detailed understanding of the antifungal efficacy related to the distinctive properties of invasive pulmonary aspergillosis (14). The markers of organism-mediated pulmonary injury (lung weights and pulmonary infarct scores) are essential in understanding the impact of an echinocandin based regimen, where quantitative cultures may be paradoxically elevated. As patients succumb to pulmonary aspergillosis as the result of organism-mediated pulmonary injury, understanding the impact of a combination regimen yields insight in improving a key clinical outcome parameter. Figure 1 demonstrates significant reductions of lung weights and pulmonary infarct scores with all three ISA+MFG2 combinations in comparison to untreated controls and a greater numerical effect in comparison to that of monotherapy. While the effect on reducing organism-mediated tissue injury by the combination of ISA40+MFG2 versus ISA40 achieved statistical significance, a similar pattern is observed for the combination of ISA20+MFG2 vs monotherapy. The effect of monotherapy with ISA60 also is as effective as the combination therapeutic regimens. However, in order to achieve the plasma exposure conferred by ISA60, the comparable human dose would be approximately 400 mg, which is double that of the licensed daily dose of 200 mg. By comparison, the plasma exposure achieved in the human dose of 200 mg is approximated within the dosage range between ISA20 and ISA40.

The Kaplan-Meier plot of survival is consistent with the findings in reduction of organism-mediated pulmonary injury, demonstrating that the greatest efficacy is observed with ISA+MFG combinations as well as with ISA60 monotherapy. The greater mortality observed with MFG2, ISA20, and ISA40, further supports the role of combination therapy for improved outcome of invasive aspergillosis in persistently neutropenic hosts. The antifungal effect is further reflected in the pulmonary fungal burden, where complete clearance to the lower limit of quantitation is achieved only by these two regimens of ISA40+MFG2 and ISA60.

The temporal patterns of serial serum biomarkers for (1→3)-β-D-glucan and galactomannan also support the efficacy of combination ISA+MFG. Consistent with other endpoint parameters, serum (1→3)-β-D-glucan levels in animals treated with ISA20, ISA40, or MFG2 monotherapy remained persistently elevated by end of therapy. By comparison, combination therapy with ISA20+MFG2 or ISA40+MFG2 resulted in resolution of serum (1→3)-β-D-glucan. As observed with other parameters, the serial (1→3)-β-D-glucan levels also resolved with either ISA60 monotherapy or with ISA60+MFG2 combination therapy. This correlation between therapeutic response to combination therapy or triazole monotherapy with the temporal pattern of (1→3)-β-D-glucan levels also has been demonstrated in experimental and clinical invasive aspergillosis (7, 15-19).

Similarly, serial GMI values in animals treated with ISA20, ISA40, or MFG2 monotherapy remained persistently elevated by end of therapy, while those treated with ISA20+MFG2 or ISA40+MFG2 combination therapy resulted in resolution of serum GMI. Recapitulating the findings of the parameters of organism-mediated injury, survival, pulmonary fungal burden, and serial (1→3)-β-D-glucan levels, animals treated with ISA60 or ISA60+MFG2 also demonstrated resolution of serum GMI. The validity of GMI in reflecting therapeutic response is buttressed by previous studies of animal models of pulmonary aspergillosis (7, 15, 19, 20) and of patients with invasive aspergillosis (21-27)

Current treatment of IPA in immunosuppressed hosts relies on the administration of antifungal triazoles, particularly voriconazole, as primary therapy (28). Unfortunately, the overall response rate of invasive aspergillosis to voriconazole remains at approximately 50% to 60% with responses as low as nearly 30% in hematopoietic stem cell transplantation recipients. Although voriconazole is an important therapeutic advance against IPA, the problems of visual hallucinations, cutaneous solar hypersensitivity, hepatotoxicity, drug interactions, variable plasma pharmacokinetics, and need for therapeutic drug monitoring warrants the need for new antifungal agents against *Aspergillus* spp. (29). Clearly new strategies are needed for the treatment of IPA.

The antifungal triazoles inhibit fungal cell membrane biosynthesis through inhibition of ergosterol formation at the level of lanosterol C14-demethylase (30). Isavuconazole is a new broad-spectrum triazole antifungal agent that has been recently approved by FDA for primary treatment of invasive aspergillosis and mucormycosis (31-33). Isavuconazole *in vitro* demonstrates superior hyphal growth inhibition and minimum inhibitory concentrations (MICs) against *A. fumigatus* in comparison to that of voriconazole (34-37). The pharmacodynamics and efficacy of single agent isavuconazole were explored in a murine neutropenic IPA model by Lepak and colleges (38), in an disseminated aspergillosis model in immunocompetent mice by Seyedmousavi et al (39), and in the persistently neutropenic rabbit model of invasive pulmonary aspergillosis (20, 40).

Micafungin is a cyclic hexapeptide echinocandin that inhibits (1→3)-β-D-glucan synthase, an enzyme complex specific to fungi and essential for fungal cell wall biosynthesis. *In vitro* studies indicate that micafungin has broad-spectrum fungicidal activity against *Candida* spp. (including azole-resistant *C. albicans*) and fungistatic activity against *Aspergillus* spp.. More recent studies demonstrate that the combination of micafungin and the triazole isavuconazole achieve significant synergy against *Aspergillus* spp..

The comparative pharmacodynamics of micafungin and isavuconazole merits discussion. Micafungin demonstrates an *in vitro* and *in vivo* concentration-dependent effect of paradoxically increasing the number of colony forming units as its mechanism of disrupting cell wall biosynthesis of *Aspergillus fumigatus* (41). The disruption of cell wall integrity results in truncated hyphal elements with dose-dependent decreased angioinvasion, reduced pulmonary infarcts, and increased survival. By comparison, isavuconazole causes an *in vitro* concentration-dependent and *in vivo* dose-dependent reduction of colony forming units of *A. fumigatus*, which correlates with reduced organism-mediated pulmonary injury and increased survival (40). Moreover, using serum GMI as the dynamic PD variable, a mean plasma isavuconazole AUC/MIC (EC50) ratio of 79.65 (95% CI 32.2, 127.1) produced a half-maximal effect in GMI suppression (20). By comparison, the serum GMI paradoxically increases during micafungin treatment of invasive aspergillosis as the result of dispersal of cell wall fragments following inhibition of (1→3)-β-D-glucan synthesis (41). The dosage of micafungin used in this study is comparable in plasma exposure to approximately 0.5 mg/kg/d in humans. The dosage of isavuconazole 20 to 40 mg/kg/d in the rabbit model approximates plasma exposure achieved by the human adult dose of 200 mg/d, while the 60 mg/kg dosage of isavuconazole in rabbits would correspond to approximately 400 mg/d in human adults.

We hypothesized that simultaneous inhibition of biosynthesis of ergosterol in the fungal cell membrane and (1→3)-β-D-glucan in the cell wall, respectively, by the antifungal triazole isavuconazole and the echinocandin micafungin, may result in improved outcome in experimental IPA in persistently neutropenic rabbits. Such findings provide a scientific foundation of this combination for the treatment of proven and probable IPA in immunocompromised patients and build upon previous combination triazole/echinocandin work suggesting that combination therapy may be more effective than triazole therapy alone.

This study has several limitations. Although this study used only one isolate of *A. fumigatus*, *in vitro* assays demonstrated similar properties of synergy between isavuconazole and micafungin for other isolates, suggesting the applicability of findings from this study. Further *in vivo* studies using additional strains of *A. fumigatus* with varying MICs would provide valuable insight into potential clinical utility of the combination of isavuconazole and micafungin. While additional dosages of echinocandin may have bee used, our earlier data demonstrated an optimal effect with 2 mg/kg/d of micafungin, allowing a more focused investigation of the range of dosages of isavuconazole.

Given the observations in this study that combination therapy with ISA40+MFG2 and monotherapy with ISA 60 are comparable, one must consider two options for clinical trials. The first option would be combining isavuconazole 200 mg/d plus micafungin versus isavuconazole 400 mg/d as monotherapy. As the licensed dose of isavuconazole for adults, 200 mg/d has been well studied and found to have a significantly more favorable toxicity profile than that of voriconazole (42), patient safety could be better assured in lieu of administering 400 mg/d. A doubling of the dose of isavuconazole from 200 mg/d to 400 mg/d may incur dose-dependent intolerance and end-organ toxicity. Thus, a study of combination therapy with isavuconazole plus micafungin would be the next logical step in harnessing these data for improved treatment of invasive aspergillosis.

Previously, the combination of the echinocandin anidulafungin and voriconazole was studied against *A. fumigatus* with an *in vitro* broth microdilution checkerboard assay based on the CLSI M-38A method (19). To quantify the concentration-effect relationships of anidulafungin and voriconazole alone, a sigmoid Emax model was fitted to the % growth inhibition obtained at each concentration of the drugs alone for each replicate to allow for Bliss independence-based drug interaction analysis. In parallel with these *in vitro* studies, anidulafungin and voriconazole were studied in treatment of experimental invasive pulmonary aspergillosis in persistently neutropenic rabbits (7). These experiments demonstrated *in vitro* and *in vivo* concentration and dose-dependent synergistic interactions between the echinocandin and triazole by Bliss independence drug interaction analysis of microbiological, radiological, and antigenic endpoints. There was a significant decrease of pulmonary infarct score, lung weight, residual fungal burden, and galactomannan antigenemia in combination with anidulafungin at 5 mg/kg/day and voriconazole when compared to monotherapies. Importantly, the magnitude of these interactions was similar for the *in vitro* and *in vivo* combination studies when analyzed by the Bliss independence drug interaction analysis. These results, as well as subsequent animal and retrospective human studies showing a trend toward improved survival with combination therapy, laid the groundwork for prospective echinocandin-triazole clinical trials in the treatment of invasive aspergillosis in humans (43).

This randomized, double-blind, placebo-controlled, multicenter trial was performed to assess the safety and efficacy of voriconazole and anidulafungin compared with voriconazole monotherapy for treatment of invasive aspergillosis (44). Among 277 patients with hematologic malignancy or hematopoietic stem cell transplantation in whom invasive aspergillosis was confirmed, mortality rates at 6 weeks were 19.3% for combination therapy and 27.5% for monotherapy [95% CI, −19.0 to 1.5]; *P*  = 0.087). However, as most patients (n = 218) with invasive aspergillosis were diagnosed by radiographic findings and maximum galactomannan positivity, a *post hoc* analysis of this subgroup was conducted. Six-week mortality was significantly lower in those patients receiving combination therapy than in those receiving monotherapy (15.7% vs. 27.3%; [95%CI, −22.7 to −0.4]; *P* = 0.037). Although there were limitations to the study design, this trial demonstrated the potential utility of combination antifungal therapy for the treatment of invasive pulmonary aspergillosis in patients with hematologic malignancy or hematopoietic stem cell transplantation.

Further clinical trials of echinocandin/triazole combinations in primary treatment of invasive aspergillosis are warranted. Our data show that rabbits treated with combination therapy with isavuconazole and micafungin demonstrate significant dose-dependent reduction of residual fungal burden, decreased pulmonary injury, prolonged survival, lower GMI, and lower serum (1→3)-β-D-glucan levels in comparison to that of single agent isavuconazole or micafungin. These encouraging results in conjunction with the favorable pharmacokinetic and pharmacodynamic profiles of these compounds make them attractive agents for use in patients with invasive pulmonary aspergillosis. These data may help guide the design and interpretation of isavuconazole and micafungin in prospective clinical trials in treatment of invasive aspergillosis.

**ACKNOWLEDGMENTS**

This research was supported by an Investigator-Initiated grant from Astellas. T.J.W. is a Scholar of the Henry Schueler Foundation and a Scholar of Pediatric Infectious Diseases of the Sharp Family Foundation and receives support from the Save our Sick Kids Foundation, as well as research grants for experimental and clinical antimicrobial pharmacotherapeutics from Novartis, Merck, ContraFect, Pfizer, and Cubist. He has served as a consultant to Astellas, ContraFect, Drais, iCo, Novartis, Pfizer, Methylgene, SigmaTau, and Trius. W.W.H. has acted as a consultant and/or received research support from Gilead, Astellas, Pfizer, and F2G. L.L.K. is an employee of Astellas Pharma Global Development, Inc.. V.P., R.P., M.W.M., M.H.Z., K.H, N.S., B.B.W.M., and N.K.S.: none to declare.

**REFERENCES**

1. **Kontoyiannis DP, Marr KA, Park BJ, Alexander BD, Anaissie EJ, Walsh TJ, Ito J, Andes DR, Baddley JW, Brown JM, Brumble LM, Freifeld AG, Hadley S, Herwaldt LA, Kauffman CA, Knapp K, Lyon GM, Morrison VA, Papanicolaou G, Patterson TF, Perl TM, Schuster MG, Walker R, Wannemuehler KA, Wingard JR, Chiller TM, Pappas PG.** 2010. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. Clin Infect Dis **50:**1091-1100.

2. **Kosmidis C, Denning DW.** 2015. The clinical spectrum of pulmonary aspergillosis. Thorax **70:**270-277.

3. **Patterson TF.** 2009. Risk stratification for invasive aspergillosis: early assessment of host susceptibility. Med Mycol **47 (**Suppl 1)**:**S255-260.

4. **Rubio PM, Sevilla J, Gonzalez-Vicent M, Lassaletta A, Cuenca-Estrella M, Diaz MA, Riesco S, Madero L.** 2009. Increasing incidence of invasive aspergillosis in pediatric hematology oncology patients over the last decade: a retrospective single centre study. J Pediatr Hematol Oncol **31:**642-646.

5. **Valdez JM, Scheinberg P, Young NS, Walsh TJ.** 2009. Infections in patients with aplastic anemia. Semin Hematol **46:**269-276.

6. **Katragkou A, McCarthy M, Meletiadis J, Petraitis V, Moradi PW, Strauss GE, Fouant MM, Kovanda LL, Petraitiene R, Roilides E, Walsh TJ.** 2014. *In vitro* combination of isavuconazole with micafungin or amphotericin B deoxycholate against medically important molds. Antimicrob Agents Chemother **58:**6934-6937.

7. **Petraitis V, Petraitiene R, Sarafandi AA, Kelaher AM, Lyman CA, Casler HE, Sein T, Groll AH, Bacher J, Avila NA, Walsh TJ.** 2003. Combination therapy in treatment of experimental pulmonary aspergillosis: synergistic interaction between an antifungal triazole and an echinocandin. J Infect Dis **187:**1834-1843.

8. **National Committee for Clinical Laboratory Standards.** 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; Approved Standard M38-A2 2nd ed. Clinical and Laboratory Standards Institute, Wayne, PA.

9. **National Research Council.** 2011. Guide for the Care and Use of Laboratory Animals: 8th ed. National Academy Press. Washington, DC.

10. **Walsh TJ, Bacher J, Pizzo PA.** 1988. Chronic Silastic central venous catheterization for induction, maintenance and support of persistent granulocytopenia in rabbits. Lab Anim Sci **38:**467-471.

11. **Petraitiene R, Petraitis V, Groll AH, 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.

12. **Stynen D, Sarfati J, Goris A, Prevost MC, Lesourd M, Kamphuis H, Darras V, Latge JP.** 1992. Rat monoclonal antibodies against *Aspergillus* galactomannan. Infect Immun **60:**2237-2245.

13. **Petraitiene R, Petraitis V, Hope WW, Mickiene D, Kelaher AM, Murray HA, Mya-San C, Hughes JE, Cotton MP, Bacher J, Walsh TJ.** 2008. Cerebrospinal fluid and plasma (1-->3)-beta-D-glucan as surrogate markers for detection and monitoring of therapeutic response in experimental hematogenous *Candida* meningoencephalitis. Antimicrob Agents Chemother **52:**4121-4129.

14. **Petraitis V, Petraitiene R, Hope WW, Walsh TJ.** 2017. Endpoint assessment in rabbit models of invasive pulmonary aspergillosis and mucormycosis. Methods Mol Biol **1625:**259-277.

15. **Hope WW, Petraitis V, Petraitiene R, Aghamolla T, Bacher J, Walsh TJ.** 2010. The initial 96 hours of invasive pulmonary aspergillosis: histopathology, comparative kinetics of galactomannan and (1->3) beta-d-glucan and consequences of delayed antifungal therapy. Antimicrob Agents Chemother **54:**4879-4886.

16. **McCarthy MW, Petraitiene R, Walsh TJ.** 2017. Translational development and application of (1-->3)-beta-D-glucan for diagnosis and therapeutic monitoring of invasive mycoses. Int J Mol Sci **18**.

17. **Neofytos D, Railkar R, Mullane KM, Fredricks DN, Granwehr B, Marr KA, Almyroudis NG, Kontoyiannis DP, Maertens J, Fox R, Douglas C, Iannone R, Kauh E, Shire N.** 2015. Correlation between circulating fungal biomarkers and clinical outcome in invasive aspergillosis. PLoS One **10:**e0129022.

18. **Petraitiene R, Petraitis V, Bacher JD, Finkelman MA, Walsh TJ.** 2015. Effects of host response and antifungal therapy on serum and BAL levels of galactomannan and (1-->3)-beta-D-glucan in experimental invasive pulmonary aspergillosis. Med Mycol **53:**558-568.

19. **Petraitis V, Petraitiene R, Hope WW, Meletiadis J, Mickiene D, Hughes JE, Cotton MP, Stergiopoulou T, Kasai M, Francesconi A, Schaufele RL, Sein T, Avila NA, Bacher J, Walsh TJ.** 2009. Combination therapy in treatment of experimental pulmonary aspergillosis: *in vitro* and *in vivo* correlations of the concentration- and dose- dependent interactions between anidulafungin and voriconazole by Bliss independence drug interaction analysis. Antimicrob Agents Chemother **53:**2382-2391.

20. **Kovanda LL, Petraitiene R, Petraitis V, Walsh TJ, Desai A, Bonate P, Hope WW.** 2016. Pharmacodynamics of isavuconazole in experimental invasive pulmonary aspergillosis: implications for clinical breakpoints. J Antimicrob Chemother **71:**1885-1891.

21. **Chen TK, Groncy PK, Javahery R, Chai RY, Nagpala P, Finkelman M, Petraitiene R, Walsh TJ.** 2017. Successful treatment of *Aspergillus* ventriculitis through voriconazole adaptive pharmacotherapy, immunomodulation, and therapeutic monitoring of cerebrospinal fluid (1-->3)-beta-D-glucan. Med Mycol **55:**109-117.

22. **Kovanda LL, Desai AV, Hope WW.** 2017. Prognostic value of galactomannan: current evidence for monitoring response to antifungal therapy in patients with invasive aspergillosis. J Pharmacokinet Pharmacodyn **44:**143-151.

23. **Kovanda LL, Kolamunnage-Dona R, Neely M, Maertens J, Lee M, Hope WW.** 2017. Pharmacodynamics of isavuconazole for invasive mold disease: Role of galactomannan for real-time monitoring of therapeutic Response. Clin Infect Dis **64:**1557-1563.

24. **Marr KA.** 2008. *Aspergillus* galactomannan index: a surrogate end point to assess outcome of therapy? Clin Infect Dis **46:**1423-1425.

25. **Miceli MH, Grazziutti ML, Woods G, Zhao W, Kocoglu MH, Barlogie B, Anaissie E.** 2008. Strong correlation between serum *Aspergillus* galactomannan index and outcome of aspergillosis in patients with hematological cancer: clinical and research implications. Clin Infect Dis **46:**1412-1422.

26. **Nouer SA, Nucci M, Kumar NS, Grazziutti M, Barlogie B, Anaissie E.** 2011. Earlier response assessment in invasive aspergillosis based on the kinetics of serum *Aspergillus* galactomannan: proposal for a new definition. Clin Infect Dis **53:**671-676.

27. **Woods G, Miceli MH, Grazziutti ML, Zhao W, Barlogie B, Anaissie E.** 2007. Serum *Aspergillus* galactomannan antigen values strongly correlate with outcome of invasive aspergillosis: a study of 56 patients with hematologic cancer. Cancer **110:**830-834.

28. **Patterson TF, Thompson GR, 3rd, Denning DW, Fishman JA, Hadley S, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Nguyen MH, Segal BH, Steinbach WJ, Stevens DA, Walsh TJ, Wingard JR, Young JA, Bennett JE.** 2016. Practice guidelines for the diagnosis and management of aspergillosis: 2016 Update by the Infectious Diseases Society of America. Clin Infect Dis **63:**e1-e60.

29. **Schafer-Korting M.** 2003. New systemic antifungal drugs: mechanisms of action, drug interactions and side effects. Mycoses **46** (Suppl 1)**:**28-31.

30. **Podust LM, Poulos TL, Waterman MR.** 2001. Crystal structure of cytochrome P450 14alpha -sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors. Proc Natl Acad Sci U S A **98:**3068-3073.

31. **Ananda-Rajah MR, Kontoyiannis D.** 2015. Isavuconazole: a new extended spectrum triazole for invasive mold diseases. Future Microbiol **10:**693-708.

32. **Livermore J, Hope W.** 2012. Evaluation of the pharmacokinetics and clinical utility of isavuconazole for treatment of invasive fungal infections. Expert Opin Drug Metab Toxicol **8:**759-765.

33. **Miceli MH, Kauffman CA.** 2015. Isavuconazole: A new broad-spectrum triazole antifungal agent. Clin Infect Dis **61:**1558-1565.

34. **Espinel-Ingroff A, Chowdhary A, Gonzalez GM, Guinea J, Hagen F, Meis JF, Thompson GR, 3rd, Turnidge J.** 2015. Multicenter study of isavuconazole MIC distributions and epidemiological cutoff values for the *Cryptococcus neoformans*-*Cryptococcus gattii* species complex using the CLSI M27-A3 broth microdilution method. Antimicrob Agents Chemother **59:**666-668.

35. **Espinel-Ingroff A, Chowdhary A, Gonzalez GM, Lass-Florl C, Martin-Mazuelos E, Meis J, Pelaez T, Pfaller MA, Turnidge J.** 2013. Multicenter study of isavuconazole MIC distributions and epidemiological cutoff values for *Aspergillus* spp. for the CLSI M38-A2 broth microdilution method. Antimicrob Agents Chemother **57:**3823-3828.

36. **Pfaller MA, Messer SA, Rhomberg PR, Jones RN, Castanheira M.** 2013. *In vitro* activities of isavuconazole and comparator antifungal agents tested against a global collection of opportunistic yeasts and molds. J Clin Microbiol **51:**2608-2616.

37. **Pfaller MA, Rhomberg PR, Messer SA, Jones RN, Castanheira M.** 2015. Isavuconazole, micafungin, and 8 comparator antifungal agents' susceptibility profiles for common and uncommon opportunistic fungi collected in 2013: temporal analysis of antifungal drug resistance using CLSI species-specific clinical breakpoints and proposed epidemiological cutoff values. Diagn Microbiol Infect Dis **82:**303-313.

38. **Lepak AJ, Marchillo K, Vanhecker J, Andes DR.** 2013. Isavuconazole (BAL4815) pharmacodynamic target determination in an *in vivo* murine model of invasive pulmonary aspergillosis against wild-type and cyp51 mutant isolates of *Aspergillus* *fumigatus*. Antimicrob Agents Chemother **57:**6284-6289.

39. **Seyedmousavi S, Verweij PE, Mouton JW.** 2015. Isavuconazole, a broad-spectrum triazole for the treatment of systemic fungal diseases. Expert review of anti-infective therapy **13:**9-27.

40. **Petraitis V, Petraitiene R, Moradi PW, Strauss GE, Katragkou A, Kovanda LL, Hope WW, Walsh TJ.** 2016. Pharmacokinetics and concentration-dependent efficacy of isavuconazole for treatment of experimental invasive pulmonary aspergillosis. Antimicrob Agents Chemother **60:**2718-2726.

41. **Petraitis V, Petraitiene R, Groll AH, Roussillon K, Hemmings M, Lyman CA, Sein T, Bacher J, Bekersky I, Walsh TJ.** 2002. Comparative antifungal activities and plasma pharmacokinetics of micafungin (FK463) against disseminated candidiasis and invasive pulmonary aspergillosis in persistently neutropenic rabbits. Antimicrob Agents Chemother **46:**1857-1869.

42. **Maertens JA, Raad, II, Marr KA, Patterson TF, Kontoyiannis DP, Cornely OA, Bow EJ, Rahav G, Neofytos D, Aoun M, Baddley JW, Giladi M, Heinz WJ, Herbrecht R, Hope W, Karthaus M, Lee DG, Lortholary O, Morrison VA, Oren I, Selleslag D, Shoham S, Thompson GR, 3rd, Lee M, Maher RM, Schmitt-Hoffmann AH, Zeiher B, Ullmann AJ.** 2016. Isavuconazole versus voriconazole for primary treatment of invasive mould disease caused by *Aspergillus* and other filamentous fungi (SECURE): a phase 3, randomised-controlled, non-inferiority trial. Lancet **387:**760-769.

43. **Zhang M, Sun WK, Wu T, Chen F, Xu XY, Su X, Shi Y.** 2014. Efficacy of combination therapy of triazole and echinocandin in treatment of invasive aspergillosis: a systematic review of animal and human studies. J Thorac Dis **6:**99-108.

44. **Marr KA, Schlamm HT, Herbrecht R, Rottinghaus ST, Bow EJ, Cornely OA, Heinz WJ, Jagannatha S, Koh LP, Kontoyiannis DP, Lee DG, Nucci M, Pappas PG, Slavin MA, Queiroz-Telles F, Selleslag D, Walsh TJ, Wingard JR, Maertens JA.** 2015. Combination antifungal therapy for invasive aspergillosis: a randomized trial. Ann Intern Med **162:**81-89.



**Figure 1.** Response of primary pulmonary aspergillosis in persistently neutropenic rabbits to antifungal therapy measured by mean lung weight (A panel) and mean pulmonary infarct score (B panel) in untreated controls (UC) and rabbits receiving oral isavuconazole (BAL4815). Values are given as means ± SEMs. *P* values are indicated as follows: \*, *P*<0.05, †, *P*<0.01, decreased lung weights and pulmonary infarct scores, in ISA40+MFG2-treated rabbits in comparison to that of single therapy of ISA40 or MFG2.

 ****

**Figure 2.** Response of primary pulmonary aspergillosis in persistently neutropenic rabbits to antifungal therapy measured by survival (A panel) and mean pulmonary tissue residual fungal burden (log CFU/g) (B panel) in untreated controls (UC) and rabbits receiving oral isavuconazole (BAL4815). Values are given as means ± SEMs. For the measure of survival, the values on the y-axis are probability of survival. Survival was plotted by Kaplan-Meier analysis.Differences in survival of treatment groups and untreated controls were analyzed by log-rank test. *P* values are indicated as follows: †, *P*<0.01, decreased residual fungal burden in ISA20-, ISA40-, ISA60-, ISA20+MFG2-, ISA40+MFG2-, and ISA60+MFG2-treated rabbits vs that of MFG2-treated or UC; ¶, *P*<0.01, prolonged survival in ISA40+MFG2-treated rabbits in comparison to that of single therapy of ISA40 or MFG2; £, *P*<0.01, prolonged survival of rabbits treated with ISA20+MFG2, ISA40+MFG2, and ISA60+MFG2 in comparison to that of UC.

****

**Figure 3.** Serum (1→3)-β-D-glucan levels in persistently neutropenic rabbits of the experimental pulmonary aspergillosis model in groups of untreated controls (UC), and rabbits receiving oral dose of isavuconazole (BAL4815). Values are given as (1→3)-β-D-glucan concentrations pg/mL. *P* values are indicated as follows: \*, *P*<0.05, decrease of plasma (1→3)-β-D-glucan concentrations in ISA20+MFG2-, ISA40+MFG2-, and ISA60+MFG2-treated rabbits vs that of MFG2-treated or UC;

****

**Figure 4.** Expression of galactomannan antigenemia in persistently neutropenic rabbits with pulmonary aspergillosis in untreated controls (UC) and rabbits receiving oral dose of isavuconazole (BAL4815). Values are given as GMI means ± SEMs. *P* values are indicated as follows: \*, *P*<0.05, lower GMI in ISA20+MFG2-, ISA40+MFG2-, and ISA60+MFG2-treated rabbits vs that of MFG2-treated or UC;†, *P*<0.01, lower GMI in ISA20+MFG2 -treated rabbits vs that of MFG2-treated.

****

**Figure 5.** Strong correlation between galactomannan and outcome variables: (**A) Panel** Total lung weight (r = 0.764 (p<0.001). (**B) Panel** Infarct score (r= 0.911 (p<0.001)).