Mycopathologia
DOI 10.1007/s11046-010-9375-5

Combination Efficacy of Voriconazole and Amphotericin B in the Experimental Disease in Immunodeficient Mice Caused by Fluconazole-resistant *Cryptococcus neoformans*

Eriques Gonçalves Silva · Claudete Rodrigues Paula · Amanda Latercia Tranches Dias · Marilene Rodrigues Chang · Luciana da Silva Ruiz · Valderez Gambale · Renato Araujo Prates · Martha Simões Ribeiro

Received: 15 July 2010 / Accepted: 8 October 2010

Abstract The therapeutic efficacy of amphotericin B and voriconazole alone and in combination with one another were evaluated in immunodeficient mice (BALB/c-SCID) infected with a fluconazole-resistant strain of *Cryptococcus neoformans* var. *grubii*. The animals were infected intravenously with 3×10^5 cells and intraperitoneally treated with amphotericin B (1.5 mg/kg/day) in combination with voriconazole (40 mg/kg/days). Treatment began 1 day after inoculation and continued for 7 and 15 days post-inoculation. The treatments were evaluated by survival curves and yeast quantification (CFUs) in brain and lung tissues. Treatments for 15 days significantly promoted the survival of the animals compared to the control groups. Our results indicated that amphotericin B was effective in assuring longest-term survival of infected animals, but these animals still harbored the highest CFU of *C. neoformans* in lungs and brain at the end of the experiment. Voriconazole was not as effective alone, but in combination with amphotericin B, it prolonged survival for the second-longest time period and provided the lowest colonization of target organs by the fungus. None of the treatments were effective in complete eradication of the fungus in mice lungs and brain at the end of the experiment.

Keywords Amphotericin B · Voriconazole · Combination therapeutic · *Cryptococcus neoformans* · SCID · Immunodeficient murine model

E. G. Silva (✉) · C. R. Paula · L. d. S. Ruiz · V. Gambale
Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo (USP), Av. Professor Lineu Prestes 1374, 2° andar, sala 247, São Paulo (USP) 05508-900, Brazil
e-mail: eriques@usp.br

A. L. T. Dias
Universidade Federal de Alfenas-UNIFAL, Alfenas, Minas Gerais, Brazil

M. R. Chang
Universidade Federal de Mato Grosso do Sul, Campo Grande, Brazil

R. A. Prates · M. S. Ribeiro
IPEN/CNEN, Centro de Lasers e Aplicações (CLA)-Instituto de Pesquisas Energéticas e Nucleares, São Paulo, Brazil

Introduction

Cryptococcosis is a subacute or chronic systemic mycosis with a cosmopolitan nature [1], showing tropism for the central nervous system (CNS), [1, 2]. This disease is caused by two opportunistic yeasts: *Cryptococcus neoformans* var. *grubii* (serotype A), *Cryptococcus neoformans* var. *neoformans* (serotype D) and Cryptococcus *gattii* (serotypes B

Published online: 24 October 2010

and C) [1]. Serotype A is the most prevalent strain. Cryptococcosis of the CNS continues to be a lethal disease for immunocompromised patients [1, 3].

The model of systemic cryptococcosis in mice with severe combined immunodeficiency (SCID) is useful for immunological and therapeutic study of the disease in immunodeficient hosts [4]. BALB/c-SCID mice are more susceptible to experimental systemic cryptococcosis caused by *C. neoformans* var. *grubbii* (serotype A) [4, 5], and the use of the mouse model is possible to evaluate voriconazole treatment in a variety of systemic mycoses [6].

The treatment of choice remains amphotericin B, alone or in combination with flucytosine, though for more prolonged treatment, fluconazole is recommended [7]. The toxicity of amphotericin B and the isolation of an increasing number of fluconazole-resistant isolates both demonstrate the need for alternative treatments and novel strategies [8].

Voriconazole is a triazole with high bioavailability, large distribution volume, excellent penetration of the CNS [9], shows excellent activity in vitro against *C. neoformans* [10], achieves good concentration levels in the serum [7]. Voriconazole suppresses laccase production, which reduces the ability of *C. neoformans* to use phenolic compounds as the substrate for melanin production in the brain and other tissues, thus incapacitating the organism from causing disease [11]. The high activity of voriconazole in melanized and non-melanized cells suggests that this drug may be particularly valuable for treatment of cryptococcosis, especially in the cases of strains resistant to fluconazole [12].

In the last few years, the introduction of new classes of antifungal drugs naturally leads to the hypothesis that the use of these drugs in association could be more effective than their use alone. One of the principal reasons for the associated use of antifungal treatments is to diminish toxicity [13].

The objective of this study was to evaluate the therapeutic efficacy of amphotericin B, used in combination with voriconazole against experimental systemic cryptococcosis caused by *Cryptococcus neoformans* var. *grubbii* (serotype A) resistant in vitro to fluconazole in an immunodeficient murine model (BALB/c-SCID).

Materials and Methods

Cryptococcus neoformans Strain

Cryptococcus neoformans, serotype A (ICB/USP 483), was isolated from the CNS of an HIV-positive patient, a 43-year-old male, treated with antiretroviral therapy (HAART). The patient had been hospitalized twice at the University Hospital. In the first hospitalization, the patient received only fluconazole; whereas in the second week, he was treated with amphotericin B for 12 days before he died. The strain was identified by Kurtzman and Fell [14] and serotyped by agglutination method (Iatron, Japan) and interpreted according to the description of the authors [15]. This isolate was sensitive to amphotericin B (MIC 0.016 μg/mL) and resistant to fluconazole (MIC 128 μg/mL), as verified in vitro using the microdilution test in accordance with CLSI (Clinical Laboratory Institute, 2008), [16]. The isolate is maintained in tubes containing Sabouraud dextrose agar (Difco Laboratories, Detroit, MI, USA) and glycerol at −20°C, in the Laboratory of Pathogenic Yeasts of the Department of Microbiology, Institute of Biomedical Sciences of São Paulo University, São Paulo, Brazil.

Experimental Cryptococcosis

The study used a total of 50 immunodeficient mice, BALB/c-SCID, with a mean weight of 20 g, obtained from the Animal Center, which was responsible for breeding isogenic animals at the Institute for Energy and Nuclear Research, São Paulo, Brazil. These mice were housed in microisolator cages, provided with sterile feed and water and randomly distributed into eight groups. The clinical isolate (ICB/USP 483) was cultivated in YPD medium (1% yeast extract (Difco), 1% Bacto Peptone (Difco) and 2% dextrose (Sigma–Aldrich, Milwaukee, WI, USA) for 18 h at 30°C; the cells were collected after centrifugation, washed twice in phosphate buffer solution (PBS) and resuspended at the inoculation concentration. Seven groups were inoculated with 100 μL of the suspension containing 3×10^5 yeast cells via the lateral tail vein. Among these seven groups, six groups of 5 mice each were treated, one group ($n = 10$) was not treated, serving as the positive control, and other group ($n = 10$) was inoculated with PBS, serving as

the negative control. Animal handling and treatment observed the Ethical Principals of the Brazilian College of Animal Experimentation (COBEA).

Treatments

Treatment began 1 day after inoculation and continued daily for 7 and 15 days. The animals were inoculated intraperitoneally at a volume of 0.1 mL of amphotericin B, (1.5 mg/kg/day) (Fungizone, Bristol-Meyers, Squibb S.p.A., Sermoneta, Italy), [17], 0.1 mL of voriconazole (40.0 mg/kg/day) (Vfend® IV) (Pfizer Inc, New York, NY, USA), [18, 19] and 0.1 mL of amphotericin B (1.5 mg/kg/day) in combination with 0.1 mL of voriconazole (40.0 mg/kg/day). At the end of the study (day 50), all mice survivors were euthanized in a CO_2 chamber. The dead and survivor mice were evaluated by survival curves and yeast quantification (CFUs) in brain and lung tissues. Mice brains and lungs were aseptically removed, weighted and homogenized in 1 mL of sterile saline solution. Ten-fold serial dilutions of the tissue homogenates were plated (100 µL) in triplicate on SDA, (Difco), incubated at 35°C and examined daily for 3 days. The numbers of CFU per gram of tissue were calculated.

Statistical Analysis

The mean survival times were estimated by the Kaplan–Meier method and compared among groups by using the log-rank test. The data obtained in relation to the CFUs in the brains and lungs underwent logarithmic transformation to achieve an approximation of a normal distribution, prior to statistical analysis using the Mann–Whitney test. All the statistical tests were performed using the software GraphPad Prisma 5 (GraphPad PrismTM, Version 5.0, and GraphPad Software Incorporated). Differences were considered significant when $P < 0.05$.

Results

Survival of the Animals

All the treatments significantly prolonged the survival of the animals compared to the control groups ($P < 0.05$). Amphotericin B significantly prolonged the survival of the mice compared to all other treatments. For the groups receiving amphotericin B in combination with voriconazole, the animals' survival increased significantly when compared to the groups treated only with voriconazole, (Fig. 1).

Observation of the Yeasts in Brain and Lung

There were significant decreases ($P < 0.05$) in the isolation of *C. neoformans* in the brains of animals treated with amphotericin B combined with voriconazole, when compared to untreated animals and those treated only with amphotericin B and voriconazole (Table 1). In the lung, reduction in the burden of yeast was significant ($P < 0.05$) in the animals groups treated with amphotericin B combined with voriconazole, compared to groups of animals not treated and or treated only with amphotericin B. In the group of animals that received only voriconazole, reduction was significant ($P < 0.05$) compared with the group that received amphotericin B (Table 1).

Discussion

This study is the first report evaluating the treatment of cryptococcosis by amphotericin B (1.5 mg/kg/day)

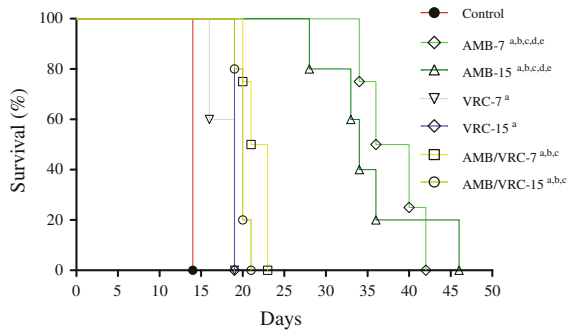

Fig. 1 Cumulative mortality of mice treated with amphotericin B(AMB-7) at 7 days, (AMB-15) at 15 days, voriconazole (VRC-7) at 7 days, (VRC-15) at 15 days, combination amphotericin B with voriconazole (AMB/VRC-7) at 7 days, (AMB/VRC-15) at 15 days. Mean survival times was estimated by the Kaplan–Meier method and compared among groups by using the log-rank test, and the survival curve was plotted using software GraphPad Prism™, Version 5.0. Differences were considered significant when $P < 0.05$. [a] $P < 0.05$ *versus* control; [b] $P < 0.05$ *versus* VRC-7; [c] $P < 0.05$ *versus* VRC-15;[d] $P < 0.05$ *versus* AMB/VRC-7; [e] $P < 0.05$ *versus* AMB/VRC-15

Table 1 Effect of cryptococcosis treatment with amphotericin B (1.5 mg/kg/day), voriconazole (40.0 mg/kg/day) and combination of both antifungal agents in immunodeficient murine model (BALB/c-SCID)

	Log CFU/g of tissue (Mean ± SD)			
	Treatments	Survival (days)	Lung	Brain
Seven days	Control	14	7.5 ± 0.2	7.5 ± 0.1
	AMB	34	8.1 ± 0.4	8.1 ± 0.6
	VRC	19	5.9 ± 1.3[a, b]	7.6 ± 1.2
	AMB/VRC	22	6.4 ± 1.1	7.6 ± 0.3
Fifteen days	AMB	34	8.0 ± 0.4	8.1 ± 0.5
	VRC	19	7.4 ± 0.7[b]	7.1 ± 1.2
	AMB/VRC	20	6.1 ± 0.9[a, b]	6.6 ± 0.5[a, b, c]

AMB, amphotericin B; VRC, voriconazole; control, without treatments. Statistics performed using the Mann–Whitney

[a] $P < 0.05$ *versus* control; [b]$P < 0.05$ *versus* AMB 7 days and 15days; [c]$P < 0.05$ *versus* VRC 7 days

used in combination with voriconazole (40 mg/kg/day) in an immunodeficient animal model, utilizing the *C. neoformans* var. *grubbii* (serotype A) strain, and resistant in vitro to fluconazole. A study of in vitro resistance may be able to identify isolates that are less likely to respond to a specific antifungal regimen [20]. In this study, we observed that all treatments prolonged the survival of animals compared to the control group. The survival of the groups only treated with amphotericin B was significantly higher compared to the other treatments. We point out that the efficacy of amphotericin B may depend on the isolate [18].

In the present study, animal survival was significantly higher in groups treated with amphotericin B (1.5 mg/kg/day) combined with voriconazole (40.0 mg/kg/day) than in those treated only with voriconazole (40.0 mg/kg/day). In other studies using a murine model, it was demonstrated that the treatment of cryptococcosis with voriconazole (60.0 mg/kg/day) over the course of 15 days was effective at increasing the survival of the mice treated [18, 19]. We point out that these studies were conducted in immunocompetent mice and the treatment consisted of monotherapy with voriconazole. The treatment of experimental cryptococcosis in a murine model with voriconazole diminished the isolation of *C. neoformans* in the brains and lungs of the treated mice [18, 19].

In this research, we verified that treatment with amphotericin B only significantly increased the animals' survival when compared with other studied treatments. We could suggest that this same treatment was not very satisfactory because we verified that it didn't significantly reduce the number of cells, as in the lung and brain tissues of these animals. The contradiction that we found in this study between increased survival and no reduction in the number of *C. neoformans* in these organs has been reported by Schwarz et al., [21]. According to these authors, this could be because of the severity of the studied model and the short treatment period. In our study, we worked with an animal model with severe combined immunodeficiency (SCID), and these animals were inoculated with a clinical isolate resistant to fluconazole. The animal model used in our study as well as the inoculated strain in these animals may have led us to this results. According to Serena et al. [18], the efficacy of amphotericin B could be dependent on the serotype of *C. neoformans* or that whether it is strain-dependent.

The yeast quantification of the brain is an important marker utilized to verify the fungicide activity of the drugs in patients with cryptococcosis of the CNS [22]. We found a significant reduction in the *C. neoformans* isolation in the groups treated with voriconazole. The voriconazole is an effective treatment in vivo against the *C. neoformans* animal model [19] and potentially useful in the treatment of CNS infections [23], the principal factor in the death of mice during experimental cryptococcosis. Voriconazole demonstrated excellent potency against various serotypes of *C. neoformans*, including isolates resistant to fluconazole [24]. The reduction in the fungal burden of lung and brain tissues in the groups treated with amphotericin B, combined with voriconazole,

was significant when compared with groups treated only with amphotericin B or voriconazole. The combination of the antifungal drugs of different classes appears to increase their therapeutic effect [25]. Actually, with the increased incidence of isolates resistant to the antifungal drugs, conventionally used in the treatment of cryptococcosis, as fluconazole, voriconazole has assumed an important role in the treatment of the systemic mycoses [26].

The results obtained in this study, based on a significant increase in survival and significant reduction in the burden of yeasts in the lung and brain tissues, found in the groups treated with amphotericin B combined with voriconazole versus the groups treated only with amphotericin B or voriconazole, suggest that this therapy—using amphotericin B (1.5 mg/kg/day) in association with voriconazole (40.0 mg/kg/day)—could be a promising alternative for the treatment of cryptococcosis, especially when fluconazole proves to be unsatisfactory. During the maintenance of fluconazole treatment, isolates resistant to this drug can appear; however, these isolates can be sensible in vitro to voriconazole [27]. It was observed, when both drugs were used following the treatments of murine systemic cryptococcosis, that mutual potentiation of these drugs can occur [17].

Our under experimental conditions, and utilizing fluconazole resistant strains, our results indicated that amphotericin B was effective in supporting the longest survival of infected animals, but they still harbored the highest numbers of CFU of *C. neoformans* in lungs and brain at the end of the experiment. Voriconazole was not as effective alone, but in combination with amphotericin B, it prolonged survival for the second-longest duration and effected lowest colonization of target organs. None of the treatments were effective in complete eradication of the fungus in mice lungs and brain at the end of the experiment. Further studies are required to determine the best concentrations of these drugs in combination as well as the most effective treatment period.

Acknowledgments The authors are grateful to Pfizer for providing pure voriconazole, to Mr. Carlos Augusto da Silva, the Animal Center Technician, for his valuable assistance and FAPESP and CNPq/for financial support.

Conflict of interest No conflict of interest with the present study.

References

1. Casadevall A, Perfect JR. *Cryptococcus neoformans*. Washington, DC: American Society for Microbiology; 1998.
2. Silva E, Baroni FA, Viani F, Ruiz LS, Gandra R, Auler M, Dias A, Gambale W, Paula C. Virulence profile of strains of *cryptococcus neoformans* var. grubii evaluated by experimental infection in balb/c mice and correlation with exoenzyme activity. J Med Microbiol. 2006;55:139–42.
3. Pappalardo MC, Paschoal RC, Melhem MS. Aids-associated central nervous system cryptococcosis: a brazilian case study. AIDS. 2007;21:1971–2.
4. Clemons KV, Azzi R, Stevens DA. Experimental systemic cryptococcosis in scid mice. J Med Vet Mycol. 1996;34: 331–5.
5. Huffnagle GB, Yates JL, Lipscomb MF. T cell-mediated immunity in the lung: a *cryptococcus neoformans* pulmonary infection model using scid and athymic nude mice. Infect Immun. 1991;59:1423–33.
6. Graybill JR, Najvar LK, Gonzalez GM, Hernandez S, Bocanegra R. Improving the mouse model for studying the efficacy of voriconazole. J Antimicrob Chemother. 2003;51: 1373–6.
7. Powderly WG. Cryptococcal meningitis in HIV-infected patients. Curr Infect Dis Rep. 2000;2:352–7.
8. Sar B, Monchy D, Vann M, Keo C, Sarthou JL, Buisson Y. Increasing in vitro resistance to fluconazole in *cryptococcus neoformans* cambodian isolates: April 2000–March 2002. J Antimicrob Chemother. 2004;54:563–5.
9. Peman J, Salavert M, Canton E, Jarque I, Roma E, Zaragoza R et al. Voriconazole in the management of nosocomial invasive fungal infections. Ther Clin Risk Manag. 2006;2:129–58.
10. Sabatelli F, Patel R, Mann PA, Mendrick CA, Norris CC, Hare R et al. In vitro activities of posaconazole, fluconazole, itraconazole, voriconazole, and amphotericin b against a large collection of clinically important molds and yeasts. Antimicrob Agents Chemother. 2006;50:2009–15.
11. Martinez LR, Ntiamoah P, Gacser A, Casadevall A, Nosanchuk JD. Voriconazole inhibits melanization in *cryptococcus neoformans*. Antimicrob Agents Chemother. 2007;51: 4396–400.
12. van Duin D, Cleare W, Zaragoza O, Casadevall A, Nosanchuk JD. Effects of voriconazole on *cryptococcus neoformans*. Antimicrob Agents Chemother. 2004;48: 2014–20.
13. Powers JH. Considerations in clinical trials of combination antifungal therapy. Clin Infect Dis. 2004;39(Suppl 4): S228–35.
14. Kurtzman CP, Fell JW. The yeasts: a taxonomic study. 4th ed. Amsterdam: Elsevier Science; 1998. 1055 p.
15. Ikeda R, Shinoda T, Fukazawa Y, Kaufman L. Antigenic characterization of *cryptococcus neoformans* serotypes and its application to serotyping of clinical isolates. J Clin Microbiol. 1982;16:22–9.
16. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility of yeasts. Approved standard M27-A3. 3rd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2008.

17. Barchiesi F, Schimizzi AM, Caselli F, Novelli A, Fallani S, Giannini D et al. Interactions between triazoles and amphotericin B against *cryptococcus neoformans*. Antimicrob Agents Chemother. 2000;44:2435–41.
18. Serena C, Pastor FJ, Marine M, Rodriguez MM, Guarro J. Efficacy of voriconazole in a murine model of cryptococcal central nervous system infection. J Antimicrob Chemother. 2007;60:162–5.
19. Mavrogiorgos N, Zaragoza O, Casadevall A, Nosanchuk JD. Efficacy of voriconazole in experimental *cryptococcus neoformans* infection. Mycopathologia. 2006;162:111–4.
20. Espinel-Ingroff A. Clinical relevance of antifungal resistance. Infect Dis Clin North Am. 1997;11:929–44.
21. Schwarz P, Dromer F, Lortholary O, Dannaoui E. Efficacy of amphotericin b in combination with flucytosine against flucytosine-susceptible or flucytosine-resistant isolates of *cryptococcus neoformans* during disseminated murine cryptococcosis. Antimicrob Agents Chemother. 2006;50:113–20.
22. Brouwer A, Rajanuwong A, Chierakul W, Griffin G, Larsen R, White N et al. Combination antifungal therapies for hiv-associated cryptococcal meningitis: a randomised trial. Lancet. 2004;363:1764–7.
23. Pastor FJ, Guarro J. The role of voriconazole in the treatment of emerging mycoses. Rev Iberoam Micol. 2007;24:228–32.
24. Thompson GR 3rd, Wiederhold NP, Fothergill AW, Vallor AC, Wickes BL, Patterson TF. Antifungal susceptibilities among different serotypes of *cryptococcus gattii* and *cryptococcus neoformans*. Antimicrob Agents Chemother. 2009;53:309–11.
25. Stevens DA, Kullberg BJ, Brummer E, Casadevall A, Netea MG, Sugar AM. Combined treatment: antifungal drugs with antibodies, cytokines or drugs. Med Mycol. 2000;38(Suppl 1):305–15.
26. Hsueh PR, Lau YJ, Chuang YC, Wan JH, Huang WK, Shyr JM et al. Antifungal susceptibilities of clinical isolates of *candida species*, *cryptococcus neoformans*, and *aspergillus species* from taiwan: Surveillance of multicenter antimicrobial resistance in taiwan program data from 2003. Antimicrob Agents Chemother. 2005;49:512–7.
27. Friese G, Discher T, Fussle R, Schmalreck A, Lohmeyer J. Development of azole resistance during fluconazole maintenance therapy for aids-associated cryptococcal disease. AIDS. 2001;15:2344–5.