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ORIGINAL PAPER

In vitro activity of voriconazole and amphotericin B against *Candida albicans, Candida krusei*, and *Cryptococcus neoformans* in human cerebrospinal fluid

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Abstract

Purpose Fungal central nervous system (CNS) infections show a high mortality rate and only a few antifungal agents are available to treat these infections. We hypothesize that the different biochemical properties of human cerebrospinal fluid (CSF) compared to the standard growth medium lead to the altered activity of antifungal agents in CSF. We investigated the in vitro activity of two of these agents, i.e., amphotericin B (AmB) and voriconazole (VOR), against three different fungi in CSF in comparison to sabouraud-dextrose broth (SDB).

Methods CSF samples from patients who did not receive any antibiotics were collected. Time-kill curves were performed in CSF and SDB using static antibiotic concentrations of AmB and VOR against ATCC strains of *Candida albicans, Candida krusei*, and *Cryptococcus neoformans*.

Results In our experiments, both AmB and VOR showed superior activity in SDB compared to CSF. Nevertheless, AmB achieved fungicidal activity in CSF after 24 h against all test strains. Voriconazole only achieved fungistatic activity against *C. albicans* and *C. neoformans* in CSF.

Conclusions In summary, our data demonstrate that growth of fungal pathogens but even more importantly activity of antifungal agents against *Candida* and *Cryptococcus* species can differ significantly in CSF compared to the standard growth medium. Both findings should be taken into consideration when applying PK/PD simulations to fungal infections of the CNS.

Keywords Antifungal agents · Antifungal activity · Meningitis · Fungal infections · PK/PD

Introduction

Central nervous system (CNS) infections are a potentially life-threatening condition and often necessitate a stay on an intensive-care unit [1]. Among neurological infections, cryptococcal meningitis is the largest single cause of death worldwide with mortality rates of 25–50% [2]. Only a few antifungal agents are available to treat CNS mycoses and development of resistance is an increasing problem. The narrow therapeutic indices of some compounds and the high potential for toxicity further complicate the treatment of CNS fungal infections [3].

We conducted this study to investigate the activity of two frequently administered antifungal agents, i.e., voriconazole (VOR) and amphotericin B (AmB), in cerebrospinal fluid (CSF) against typical fungi known to cause CNS infections.

In the literature, there is evidence that the activity of antibiotics in CSF can differ considerably from their activity in blood and standard growth media [4–6]. As a consequence, translating PK/PD indices from blood or growth medium to CSF leads to misinterpretation of antimicrobial activity. We hypothesize that this also applies to antifungal agents. To our knowledge, the present study is the first in vitro study that explores the activity of antifungal agents in CSF.

Methods

In total, 142 CSF samples were collected from patients who did not receive any antibiotics or antifungal agents. These were sterile-filtered, pooled, and stored at − 80 °C. *Candida*

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albicans (ATCC 10231 and 90028), *Candida krusei* (ATCC 6258), and *Cryptococcus neoformans* (ATCC 90112) were used as test strains. *C. albicans* strains ATCC 10231 and 90028 were used for VOR and AmB, respectively. Minimal inhibitory concentrations (MIC) were determined according to the current EUCAST guidelines in Roswell Park Memorial Institute (RPMI) medium in sixfold [7].

Time-kill curves (TKC) were performed in sabouraud-dextrose broth (SDB) and CSF using static antibiotic concentrations of AmB and VOR of 0.25- to 16-fold the MIC. In addition, time-kill experiments in SDB were performed with AmB concentrations of fourfold the MIC in combination with VOR at concentrations of 1- to 16-fold the MIC to study pharmacodynamic interaction of the substances. Each experiment was performed in triplicate and included a growth control. The respective antifungal agent was added to the baseline inoculum of $1–5 \times 10^5$ colony-forming units (CFU)/ml at predefined concentrations of 0.25-, 1-, 4-, and 16-fold the MIC. Fungal counts were determined at baseline and 3, 7, and 24 h after the addition of the antifungal agent by taking samples from the tube, diluting the samples multiple times, and pouring 20 µl of each dilution step from the sample onto sabouraud agar plates. Following an 18–24 h period of incubation at 37 °C, counting of colonies and back extrapolation were undertaken to determine the fungal count. As previously reported, fungicidal activity was defined as a $\geq 99.9\%$ or ≥ 3 log reduction in CFU/ml from the starting inoculum and fungistatic activity was defined as $< 99.9\%$ or < 3 log reduction in CFU/ml from the starting inoculum without fungal growth [8, 9].

Results

MICs of AmB against both *C. albicans* strains were 0.25 mg/l, against *C. krusei* 0.5 mg/l and against *C. neoformans* 0.125 mg/l. For VOR, we determined MICs of 0.015 mg/l, 0.25 mg/l, and 0.031 mg/l against *C. albicans* (10231), *C. krusei*, and *C. neoformans*, respectively.

Figure 1 shows the TKCs of AmB and VOR in SDB and CSF against the three fungi.

When comparing fungal CFU/ml after 24 h with the respective baseline inoculum, AmB induced fungal killing > 4 log in CSF against all fungi at 16xMIC (Table 1). However, at 4xMIC, fungicidal activity was only achievable in CSF against *C. krusei* and *C. neoformans*. In SDB, AmB concentrations of 4xMIC and above led to fungal count reductions of > 4 log against *C. albicans* and *C. neoformans*, but against *C. krusei* AmB did not reduce fungal count by ≥ 3 log at concentrations of up to 16xMIC.

For VOR, the decrease in fungal count after 24 h when compared with baseline inoculum was less pronounced. In SDB, fungicidal activity was achieved against *C. krusei* at 16xMIC, whereas only negligible antifungal activity was achieved against *C. neoformans* and no antifungal activity was achieved against *C. albicans*. Remarkably, VOR did not show any fungal killing compared to baseline inoculum against *C. krusei* in CSF, but did show fungistatic activity against *C. albicans* and *C. neoformans* over the entire concentration range (Table 1).

In Table 2, the fungal count reductions are compared to growth control in SDB and CSF respectively, and differences in $\log_{10}$ CFU/ml after 24 h are presented. The difference in activity of AmB between the two matrices was most pronounced against *C. albicans* and *C. neoformans*, with differences of 4.54 log and 4.48 log CFU/ml at 4xMIC, respectively. For VOR, the difference between the matrices was less distinct against *C. albicans* and *C. neoformans*, but, against *C. krusei*, the fungal count reduction in SDB was notably bigger when compared to CSF, with a difference of 5.12 log CFU/ml at 4xMIC (Table 2).

In summary, AmB demonstrated fungicidal activity against *C. krusei* and *C. neoformans* at ≥ 4xMIC and against *C. albicans* at 16xMIC in CSF after 24 h. For VOR, reductions in fungal counts were markedly lower, and only fungistatic activity was achieved against *C. albicans* and *C. neoformans* in CSF. Furthermore, AmB and VOR showed superior activity against all the tested strains in SDB compared to CSF when matching the fungal count reductions with the respective growth control.

To investigate if the combination of AmB with VOR enhances the activity of AmB, we performed further time-kill experiments in SDB (Table 3). The addition of VOR at 16xMIC slightly increased fungal killing of AmB against *C. krusei* by 1.27 log after 24 h, but lower concentrations only marginally increased the antifungal activity. Against *C. neoformans*, the addition of VOR at ≥ 1xMIC led to an increase of killing of 2.67 log compared to AmB alone. Fungal killing of AmB against *C. albicans* was not enhanced by the combination with voriconazole, but it has to be noted that AmB alone at 4xMIC already demonstrated maximum activity against *C. albicans*.

Discussion

In our time-kill experiments, fungal growth of all the tested fungi was faster and significantly more pronounced in SDB compared to CSF after 7 and 24 h (Mann–Whitney *U* test, $p = 0.029$; growth controls in Fig. 1). The diminished growth of the microorganisms in CSF might be caused by the lower nutrient content of CSF.

It must be pointed out that, obtaining human, antibiotic-free CSF for pharmacokinetic (PK) and pharmacodynamic (PD) studies is difficult and only limited amounts are available, and hence, the experiments were only performed

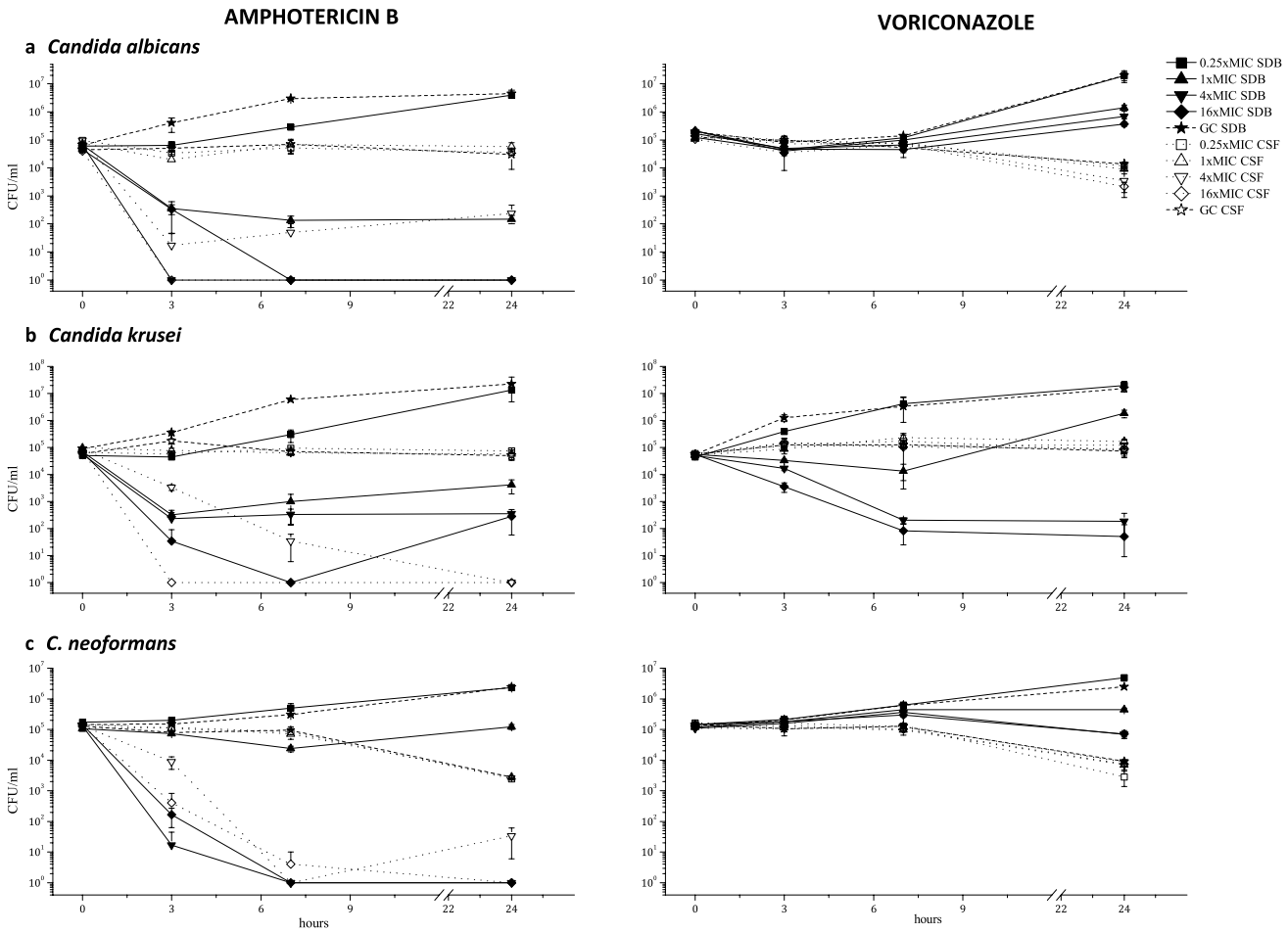

Fig. 1 Time-kill curves of amphotericin B and voriconazole against *C. albicans* (**a**), *C. krusei* (**b**), and *C. neoformans* (**c**) in SDB and CSF at concentrations of 0.25- to 16-fold the MIC. *MIC* minimal inhibitory concentration, *CSF* cerebrospinal fluid, *GC* growth control, *CFU* colony-forming unit

Table 1 Fungal killing of different concentrations of amphotericin B (AmB) and voriconazole (VOR) against *C. albicans, C. krusei*, and *C. neoformans* in SDB and CSF (shown as fungal colony count reduction vs baseline inoculum after 24 h in $\log_{10}$ CFU/ml)

	0.25xMIC SDB	1xMIC SDB	4xMIC SDB	16xMIC SDB	0.25xMIC CSF	1xMIC CSF	4xMIC CSF	16xMIC CSF
Amphotericin B								
C. albicans	1.82	−2.64	−4.81	−4.65	−0.42	−0.11	−2.61	−4.60
C. krusei	2.43	−1.23	−2.20	−2.36	0.05	−0.22	−4.93	−4.88
C. neoformans	1.12	0.07	−5.11	−5.15	−1.65	−1.71	−3.60	−5.13
Voriconazole								
C. albicans	2.19	0.82	0.60	0.24	−1.15	−1.08	−1.60	−1.68
C. krusei	2.65	1.52	−2.43	−3.03	0.12	0.52	0.34	0.22
C. neoformans	1.52	0.49	−0.20	−0.22	−1.77	−1.23	−1.10	−1.26

SDB sabouraud-dextrose broth, *MIC* minimal inhibitory concentration, *CSF* cerebrospinal fluid

with three fungi. Additional studies with different fungi are warranted. Furthermore, we only used test strains in our experiments and not clinical isolates, and did not account for potential changes in CSF composition caused by CNS infection. Keeping these limitations in mind, in the following, we used previously reported in vivo PK data to translate our in vitro findings to a clinical context.

Table 2 Fungal killing of different concentrations of amphotericin B (AmB) and voriconazole (VOR) against *C. albicans, C. krusei*, and *C. neoformans* in SDB and CSF (shown as fungal colony count reduction vs growth control after 24 h in $\log_{10}$ CFU/ml)

	0.25xMIC SDB	1xMIC SDB	4xMIC SDB	16xMIC SDB	0.25xMIC CSF	1xMIC CSF	4xMIC CSF	16xMIC CSF
Amphotericin B								
C. albicans	−0.05	−4.48	−6.65	−6.65	0.09	0.29	−2.11	−4.48
C. krusei	−0.23	−3.73	−4.81	−4.90	0.17	0.05	−4.70	−4.70
C. neoformans	−0.02	−1.29	−6.38	−6.38	−0.04	0.02	−1.90	−3.43
Voriconazole								
C. albicans	−0.03	−1.17	−1.48	−1.75	−0.08	−0.18	−0.60	−0.81
C. krusei	0.12	−0.91	−4.91	−5.47	0.03	0.35	0.20	0.09
C. neoformans	0.29	−0.74	−1.55	−1.54	−0.50	−0.11	−0.02	−0.08

SDB sabouraud-dextrose broth, *MIC* minimal inhibitory concentration, *CSF* cerebrospinal fluid, *CFU* colony-forming units

Table 3 Fungal killing of amphotericin B (AmB) at 4xMIC alone and in combination with different concentrations of voriconazole (VOR) against *C. albicans, C. krusei*, and *C. neoformans* in SDB (shown as fungal colony count reduction vs growth control after 24 h in $\log_{10}$ CFU/ml)

	4xMIC AmB	4xMIC AmB plus 1xMIC VOR	4xMIC AmB plus 4xMIC VOR	4xMIC AmB plus 16xMIC VOR
Amphotericin B plus voriconazole in SDB				
C. albicans	6.24	6.24	6.24	6.24
C. krusei	4.10	4.43	4.78	5.37
C. neoformans	3.96	6.63	6.63	6.63

SDB sabouraud-dextrose broth, *MIC* minimal inhibitory concentration, *CFU* colony-forming units

Conducting PK studies on AmB in CSF is complicated by the very low concentrations of AmB. The available literature suggests a median penetration ratio of 0.13% for liposomal AmB into CSF of pediatric patients; however, the penetration ratio seems to increase with an increasing time interval between drug administration and CSF sampling, indicating delayed tissue distribution [10]. Despite limited penetration of AmB into CSF in PK studies, it is frequently used to treat CNS mycoses and shows good clinical outcomes [11]. The popular theory explaining this discrepancy is that, in CNS infections, the permeability of the blood–brain barrier (BBB) is increased, and therefore, concentrations of AmB in patients are higher than the concentrations measured in PK studies with uninfected humans or animals [3, 12–15].

In our time-kill experiments with AmB, at least 4xMIC was necessary to achieve fungicidal activity in CSF. This would equate to a concentration of 4 mg/l assuming a MIC at which 90% of the tested isolates are inhibited (MIC_{90}) of 1 mg/l [16–19]. Considering the poor penetration of AmB into CSF, this concentration will probably not be reached with the current dosing schemes where the blood–brain barriers are intact (reported concentration range in CSF of 10–120 ng/ml). Even keeping in mind the assumption that AmB concentrations will be higher in patients with neurological infections due to the increased permeability of the blood–brain barrier, a rise in the AmB concentrations in CSF by 33- to 400-fold is unlikely [10].

However, it must be emphasized that CSF is not the only relevant target site for antimicrobial agents in CNS infections and an investigation of concentrations of liposomal AmB post-mortem brain tissue of patients treated with AmB demonstrated that concentrations in total brain tissue are much higher than in CSF [20]. In accordance with these observations, an animal study found approximately 60-fold higher liposomal AmB concentrations in brain tissue than in CSF of rabbits infected with *C. albicans* [13]. Further clinical studies concomitantly investigating the concentrations of AMB in CSF as well as the extracellular free fraction in tissue (i.e., with the means of microdialysis) are warranted to better describe target site PK profiles.

Voriconazole is routinely used in clinical practice to treat fungal CNS infections, and, in contrast to AmB several PK studies, demonstrated good penetration of VOR into CSF and brain tissue with median penetration ratios ranging from 0.46 to 0.57 [21–26]. Based on the reported median concentration levels in CSF of 0.65–2.47 µg/ml and assuming MIC_{90} values of 0.03–0.5 mg/l, the reported concentrations equate to 1.3- to 82-fold the MIC [16, 19, 24–27]. Despite this, our in vitro results in CSF indicate that VOR shows merely fungistatic activity against *C. albicans* and *C. neoformans* and no antifungal activity against *C. krusei* at concentrations of up to 16xMIC.

In our combination experiments in SDB, the only marked effect of the combined administration of VOR and AmB was achieved against *C. neoformans*. The combination of the two agents distinctly increased antifungal activity after 24 h compared to AmB alone.

The current treatment guidelines recommend AmB in combination with flucytosine for the treatment of

cryptococcal meningitis as the first-line therapy for induction and consolidation [11, 28]. Similarly, for central nervous system candidiasis, the recommended initial therapy is liposomal AmB with or without flucytosine [29, 30]. Therefore, further experiments in CSF with the combination of AmB and flucytosine would be of great interest, and might explain the gap between our data and clinical outcomes. Unfortunately, the limited amount of CFS did not allow for pharmacodynamic investigations of the combinations in CSF.

In summary, our data indicate that the antifungal activity of AmB and VOR is markedly reduced in CSF, and therefore, translating breakpoints for PK/PD indices from blood to CSF without adaption might lead to overestimation of the antifungal activity of the two agents in CSF. Further studies are needed to identify concrete PK/PD targets for the two antifungal agents in CSF. This would allow for the assessment of the currently established treatment schemes with the means of repeated pharmacokinetic measurements in CSF.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

1. Economides MP, Ballester LY, Kumar VA, Jiang Y, Tarrand J, Prieto V, Torres HA, Kontoyiannis DP. Invasive mold infections of the central nervous system in patients with hematologic cancer or stem cell transplantation (2000–2016): uncommon, with improved survival but still deadly often. J Infect. 2017. https://doi.org/10.1016/j.jinf.2017.09.011.
2. Williamson PR, Nash TE, Williamson KC, Nath A. CNS infections in 2015: emerging catastrophic infections and new insights into neuroimmunological host damage. Lancet Neurol. 2016;15:17–9.
3. Scully EP, Baden LR, Katz JT. Fungal brain infections. Curr Opin Neurol. 2008;21:347–52.
4. Karlowsky JA, Zhanel GG, Davidson RJ, Zieroth SR, Hoban DJ. In vitro postantibiotic effects following multiple exposures of cefotaxime, ciprofloxacin, and gentamicin against *Escherichia coli* in pooled human cerebrospinal fluid and Mueller–Hinton broth. Antimicrob Agents Chemother. 1993;37:1154–7.
5. Sauermann R, Schwameis R, Fille M, Camuz Ligios ML, Zeitlinger M. Antimicrobial activity of cefepime and rifampicin in cerebrospinal fluid in vitro. J Antimicrob Chemother. 2008;62:1057–60.
6. Sauermann R, Schwameis R, Fille M, Camuz ligios ML, Zeitlinger M. Cerebrospinal fluid impairs antimicrobial activity of fosfomycin in vitro. J Antimicrob Chemother. 2009;64:821–3.
7. Rodriguez-Tudela JL. EUCAST definitive document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. Clin Microbiol Infect. 2008;14:398–405.
8. Klepser ME, Ernst EJ, Lewis RE, Ernst ME, Pfaller MA. Influence of test conditions on antifungal time-kill curve results: proposal for standardized methods. Antimicrob Agents Chemother. 1998;42:1207–12.
9. Pfaller MA, Sheehan DJ, Rex JH. Determination of fungicidal activities against yeasts and molds: lessons learned from bactericidal testing and the need for standardization. Clin Microbiol Rev. 2004;17:268–80.
10. Strenger V, Meinitzer A, Donnerer J, et al. Amphotericin B transfer to CSF following intravenous administration of liposomal amphotericin B. J Antimicrob Chemother. 2014;69:2522–6.
11. Molloy SF, Kanyama C, Heyderman RS, et al. Antifungal combinations for treatment of cryptococcal meningitis in Africa. N Engl J Med. 2018;378:1004–17.
12. Redmond A, Dancer C, Woods ML. Fungal infections of the central nervous system: a review of fungal pathogens and treatment. Neurol India. 2007;55:251–9.
13. Groll AH, Giri N, Petraitis V, Petraitiene R, Candelario M, Bacher JS, Piscitelli SC, Walsh TJ. Comparative efficacy and distribution of lipid formulations of amphotericin B in experimental *Candida albicans* infection of the central nervous system. J Infect Dis. 2000;182:274–82.
14. Eltoukhy NS, Crank CW. Antifungal distribution into cerebrospinal fluid, vitreous humor, bone, and other difficult sites. Curr Fungal Infect Rep. 2010;4:111–9.
15. Polak A. Amphotericin flucytosine. Postgrad Med J. 1979;55:667–70.
16. Pfaller MA, Diekema DJ, Jones RN, Messer SA, Hollis RJ. Trends in antifungal susceptibility of *Candida* spp. isolated from pediatric and adult patients with bloodstream infections: SENTRY Antimicrobial Surveillance Program, 1997 to 2000. J Clin Microbiol. 2002;40:852–6.
17. González GM, Elizondo M, Ayala J. Trends in species distribution and susceptibility of bloodstream isolates of Candida collected in Monterrey, Mexico, to seven antifungal agents: results of a 3-year (2004 to 2007) surveillance study. J Clin Microbiol. 2008;46:2902–5.
18. Murat JB, Lebeau B, Chumpitazi B, Cornet M, Maubon D, Faure O, Quesada JL, Thiebaut-Bertrand A, Timsit JF, Pelloux H. Minimum inhibitory concentrations of amphotericin B against *Candida krusei* isolates from a French teaching hospital laboratory: a retrospective study over 8 years. Mycoses. 2013;56:56–60.
19. Maxwell MJ, Messer SA, Hollis RJ, Diekema DJ, Pfaller MA. Evaluation of Etest method for determining voriconazole and amphotericin B MICs for 162 clinical isolates of *Cryptococcus neoformans*. J Clin Microbiol. 2003;41:97–9.
20. Vogelsinger H, Weiler S, Djanani A, Kountchev J, Bellmann-Weiler R, Wiedermann CJ, Bellmann R. Amphotericin B tissue distribution in autopsy material after treatment with liposomal amphotericin B and amphotericin B colloidal dispersion. J Antimicrob Chemother. 2006;57:1153–60.
21. Poza G, Montoya J, Redondo C, Ruiz J, Vila N, Rodriguez-Tudela JL, Cerón A, Simarro E. Meningitis caused by *Pseudallescheria boydii* treated with voriconazole. Clin Infect Dis. 2000;30:981–2.
22. Verweij PE, Brinkman K, Kremer HP, Kullberg BJ, Meis JF. Aspergillus meningitis: diagnosis by non-culture-based

microbiological methods and management. J Clin Microbiol. 1999;37:1186–9.
23. Elter T, Sieniawski M, Gossmann A, et al. Voriconazole brain tissue levels in rhinocerebral aspergillosis in a successfully treated young woman. Int J Antimicrob Agents. 2006;28:262–5.
24. Lutsar I, Roffey S, Troke P. Voriconazole concentrations in the cerebrospinal fluid and brain tissue of guinea pigs and immunocompromised patients. Clin Infect Dis. 2003;37:728–32.
25. Kobayashi R, Sano H, Kishimoto K, Suzuki D, Yasuda K, Kobayashi K. Voriconazole concentrations in cerebrospinal fluid during prophylactic use in children with acute myelogenous leukemia. Pediatr Infect Dis J. 2016;35:297–8.
26. Wiederhold NP, Pennick GJ, Dorsey SA, Furmaga W, Lewis JS, Patterson TF, Sutton DA, Fothergill AW. A reference laboratory experience of clinically achievable voriconazole, posaconazole, and itraconazole concentrations withinthe bloodstream and cerebral spinal fluid. Antimicrob Agents Chemother. 2014;58:424–31.
27. Drago M, Scaltrito MM, Morace G. In vitro activity of voriconazole and other antifungal agents against clinical isolates of *Candida glabrata* and *Candida krusei*. Eur J Clin Microbiol Infect Dis. 2004;23:619–24.
28. Zhou Y, Li G. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. Chin J Infect Chemother. 2010;10:161–6.
29. Cornely OA, Bassetti M, Calandra T, et al. ESCMID guideline for the diagnosis and management of Candida diseases 2012: non-neutropenic adult patients. Clin Microbiol Infect. 2012;18:19–37.
30. Pappas PG, Kauffman CA, Andes DR, et al. Clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. Clin Infect Dis. 2015;62:e1–50.