reported. Our previous study showed that resistance to high doses of micafungin (MFG) (known as the paradoxical effect) is related to heat shock protein 90 (Hsp90) stress responses [11]. This therefore led us to explore the relationship between Hsp90-related stress responses and the antagonistic effect of voriconazole (VRC) against MFG.

Materials and methods

Strains and growth conditions

All strains used in this study were from our laboratory collection. A standard *C. albicans* strain, namely, SC5314, was employed in most experiments. *C. albicans* strains ATCC 10261 and ATCC 10231 and *C. glabrata* strain CG1 were also used in some experiments. All strains were grown in yeast nitrogen base with 2% dextrose (YNB) broth.

Biofilm formation

Biofilms were prepared using a slightly modified version of a previously described method [12]. Briefly, small silicone elastomer (SE) disks (diameter, 4 mm; thickness, 1 mm) were immersed in a *Candida* cell suspension for 90 min to allow attachment, and biofilms were allowed to form by further incubating the disks in YNB for 24 h. We used SE disks that were smaller than those used in previous experiments so that they could be placed in 96-well microplates.

Antifungal agents, inhibitors, and treatment

MFG and VRC were kindly provided by Astellas Pharma Inc. (Japan) and Pfizer Japan Inc. (Japan), respectively. Radicicol (Rad; an Hsp90 inhibitor), cyclosporin A (CsA; a calcineurin inhibitor), and nikkomycin Z (NZ; a chitin synthase inhibitor) were purchased from Sigma-Aldrich (USA), and cercosporamide (Cer; a PKC1 inhibitor) was purchased from BioAustralis Fine Chemicals (Australia). MFG and NZ were dissolved in distilled water, whereas VRC, Rad, CsA, and Cer were dissolved in dimethylsulfoxide (DMSO). These were stored at –30 °C as stock solutions until use at concentrations of 10 mg/ml (MFG, VRC, and NZ), 1 mM (Rad and Cer), and 10 mM (CsA). The final concentrations have been indicated in the sections describing individual experiments. The biofilms on the SE disks were soaked in the medium containing drugs and incubated at 37 °C for 24 h. The minimum inhibitory concentrations (MICs) of MFG, VRC, Rad, CsA, Cer, and NZ against the planktonic cells were determined in flat bottom, 96-well microtiter plates using a broth microdilution protocol modified from the CLSI M27-A standard [13]. Studies to assess MICs were prepared in YNB medium (a total volume of 0.2ml/well) containing each drug. Cell densities of overnight cultures were determined and dilutions were prepared such that approximately 2×10^3 cells were inoculated into each well. MICs of MFG, VRC, Rad, CsA, Cer, and NZ against the planktonic cells were 0.031 μg/ml, 0.008 μg/ml, 8 μM, >80 μM, >8 μM, and 6.25 μg/ml, respectively.

Quantification of biofilms

The metabolic activities of cells in the biofilms were quantified by performing a 3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay as previously described [14]. Briefly, after treatment, the medium was removed carefully, and 200 μl of 50 μg/ml XTT and 4 μM menadione in phosphate-buffered saline (PBS) was added to biofilms on the SE disks in each well. The plates were incubated for 1 h at 37 °C, and the absorption of each well at 490 nm (reference 630 nm) was read by using a plate reader. The results were normalized to control (untreated), and the relative metabolic activity of cells was expressed as the percent of the activity in control cells (% control).

To quantify the biomass of biofilms, dry weight measurements and crystal violet (CV) assays were performed. For dry weights, biofilms on SE disks were scraped and transferred onto the preweighed filter papers, and the filter papers with biofilms were dried and weighed. For CV assays, biofilms were briefly dried and then stained with 200 μl of 0.05% CV for 15 min. The disks were rinsed by repeated submersion in distilled water until CV was no longer observed in the rinse water. The disks were dried at 25 °C, and the remaining CV was solubilized with 200 μl of 30% ethanol with 1mM hydrochloride for 10 min at 25 °C. The remaining CV was quantified by measuring absorbance at 570 nm in a plate reader.

Relative Quantification by Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNAs were respectively isolated using the hot phenol method as described elsewhere [15]. To synthesize cDNA, approximately 800 ng of total RNA was used as the template with normalization to actin (*ACT1*) mRNA levels. Gene expression levels of *UTR2* were measured using Applied Biosystems 7000 Real Time PCR System (TaqMan). RT-PCR was performed in 96-well optical reaction plates in quadruplicate (each reaction containing 1xSYBR Green PCR Master mix (invitrogen, USA), 0.15 pmol/ml forward primer and reverse primer and 1 μl template cDNA in a final volume of 10 μl). *ACT1* served as the internal control. Cycling profile included 40 cycles of 95 °C for 5 s, 55 °C for 30 s and 72 °C for 30 s. Data acquisition and the analysis of the real-time PCR assay were performed using the 7000 System SDS Software Version 1.0 (Applied Biosystems). Primers ACT1-for (5′- TTGGTGATGAAGCCCAATCC -3′) and ACT1-rev

(5′-CATATCGTCCCAGTTGGAAAC-3′) were used for amplification of *ACT1*, and primers UTR2-for (5′-GATTCT GGTAGTAGTGGAAGCAGTTCT-3′) and UTR2-rev (5′-ATGGAAGCAAATATACCACTGATAACAC-3′) were used for amplification of *UTR2*.

Statistical Analysis

The data were analyzed by using Mann-Whitney *U* tests. Unless otherwise indicated, the data are presented as the mean ± standard deviation (SD) of 4 or more replicates. The error bars represent the SD. The data are representative of 2 or more individual experiments.

Results

VRC attenuated the effect of MFG against Candida biofilms

We examined whether VRC attenuated the effect of MFG against biofilms in our model. VRC alone decreased XTT activity of biofilms, but the decrease was never below 50% (Fig. 1a). MFG alone greatly reduced biofilm XTT activity

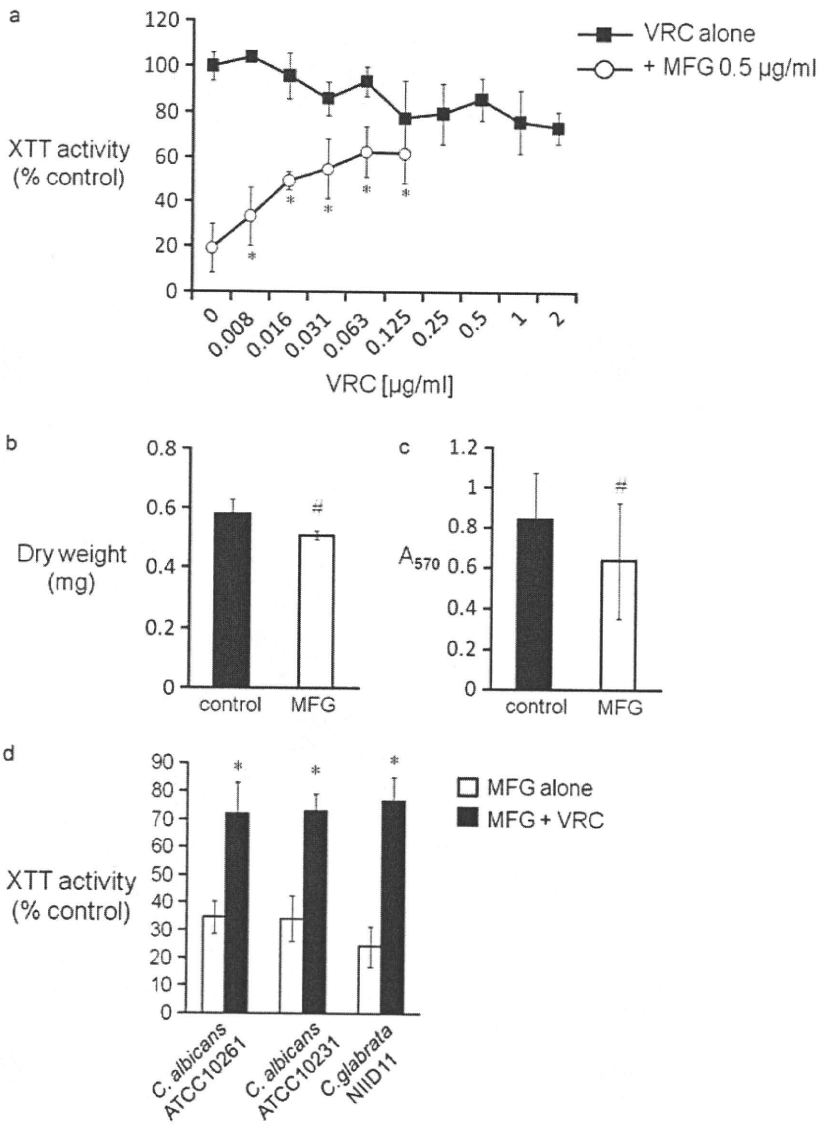

Fig. 1 VRC attenuates the effect of MFG against *Candida* biofilms. (a) 0.5 μg/ml of MFG alone reduces the relative metabolic activity of cells in biofilms by approximately 80% to 90%. The effect of MFG is attenuated by VRC, and the metabolic activity gradually increased up to 60% to 70% dose-dependently. VRC alone decreased the metabolic activity of the cells in the biofilms, but the decrease was never below 50%. Data are representative of 3 independent experiments. (b)(c) MFG alone reduces biomasses when compared with measurements of dry weights (b) and CV assay (c). (d) VRC also attenuates the effect of MFG against the biofilms of 3 strains other than SC5314 that form biofilms as well. VRC, voriconazole; MFG, micafungin; Rad, radicicol; CsA, cyclosporine A; Cer, cercosporamide; NZ, nikkomycin Z; CV, crystal violet. $*P < 0.05$ compared to MFG alone. $\#P < 0.05$ compared to control.

by 80% to 90% at a concentration of 0.5 μg/ml (Fig. 1a). However, addition of VRC attenuated the effect of MFG against biofilms and gradually increased the XTT activity dose-dependently up to 60% to 70% (Fig. 1a). In contrast, this combination showed additive effects against planktonic cells (data not shown) which is consistent with previous reports [7]. Thus, VRC, in cooperation with MFG, inhibits the growth of planktonic cells, but attenuates the effect of MFG against cells in biofilms. We also assessed the effects of MFG by measuring biomass. In both weight measurements and CV assays, MFG reduced biomass, but the effect was less than what was observed in XTT assay (Fig. 1b, c). These results imply that biomass could include living as well as dead cells, along with extracellular matrices. In order to examine whether this antagonistic effect was restricted to only SC5314 strain, we studied several other strains. While some of them did not form biofilms well, VRC attenuated the effect of MFG against biofilms of all 3 strains noted in the methods section (Fig. 1d).

A very high concentration of MFG was required to reverse the antagonistic effect of VRC

To determine the concentration of MFG that could reverse the antagonistic effect of VRC, we increased the doses of MFG added to biofilms in the presence of VRC (0.125 μg/ml). Surprisingly, even a high concentration of 64 μg/ml of MFG in the presence of VRC was not sufficient to exceed the effect of 0.5 μg/ml of MFG alone (Fig. 2). This shows that biofilms become highly resistant to MFG in the presence of VRC, and even an increased dose of MFG could not disrupt biofilms effectively.

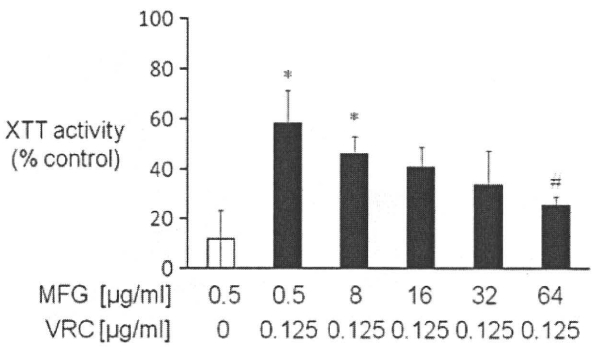

Fig. 2 A very high concentration of MFG is required to reverse the VRC-induced antagonism of MFG against biofilms. MFG reduces the metabolic activity dose-dependently in the presence of VRC, but very high doses of MFG are required to restore the effect of a low dose of MFG alone. VRC, voriconazole; MFG, micafungin. $*P < 0.05$ compared to 0.5 μg/ml of MFG alone. $\#P < 0.05$ compared to 0.5 μg/ml of MFG + 0.125 μg/ml of VRC.

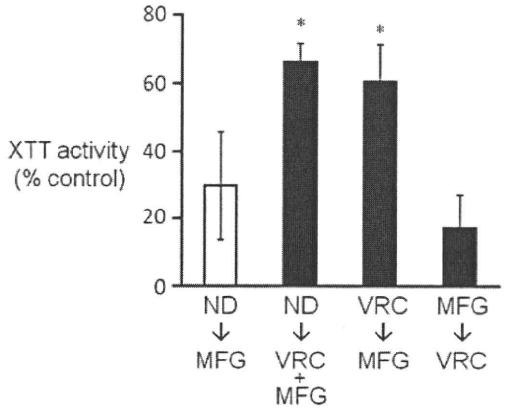

Fig. 3 Combination therapy with a time lag shows that VRC attenuates the effect of MFG even when the biofilm is no longer exposed to VRC. Each bar shows the mean percent of XTT activity compared to control (% control, $n = 8$ each). VRC alone (ND → VRC), the simultaneous combination of VRC and MFG (ND → VRC+MFG), and the serial combination of VRC followed by MFG (VRC → MFG) were less effective than MFG alone (ND → MFG) and the serial combination of MFG followed by VRC (MFG → VRC). There was no significant difference between ND → MFG and MFG → VRC. Data are representative of 3 independent experiments. ND, no drug; VRC, voriconazole; MFG, micafungin; ND → MFG, ND followed by MFG; ND → VRC + MFG, ND followed by VRC + MFG; VRC → MFG, VRC followed by MFG; MFG → VRC, MFG followed by VRC. $*P < 0.05$ compared to 0.5 μg/ml of MFG alone (ND → MFG).

The effect of a serial combination of VRC and MFG was also inferior to that of MFG alone

We added in our studies VRC and MFG sequentially with a time lag in between to examine whether the effect of MFG against biofilms is attenuated even when the biofilm is no longer exposed to VRC. The biofilms were first treated with VRC alone, MFG alone, or no drug (ND) for the first 24 h, and then sequentially with MFG alone, VRC alone, or a combination of VRC and MFG, respectively, for the next 24 h. The concentrations of MFG and VRC were 0.5 μg/ml and 0.125 μg/ml, respectively. The effects of a simultaneous combination of MFG and VRC (ND → VRC+MFG) and the serial combination of VRC followed by MFG (VRC → MFG) were less than that observed with MFG alone. This result shows that the effect of MFG is attenuated even when the biofilm is no longer exposed to VRC (Fig. 3). In contrast, the sequential treatment with MFG followed by VRC (MFG → VRC) was still as effective as MFG alone.

Inhibiting stress responses enhanced the effect of MFG against biofilms and reversed the antagonistic effect of VRC

The results of the time-lag experiment indicated that certain stress responses to VRC possibly modulated the sensitivity of biofilms to MFG. To investigate whether

inhibiting stress responses could reverse this antagonism, Hsp90 and proteins associated with it were inhibited pharmacologically. Maximum doses of Rad (16 μM), CsA (80 μM), Cer (8 μM), and NZ (32 μg/ml) in this study decreased the metabolic activity of the cells in the biofilms, but the decrease was never below 50% (data not shown). When low doses of Rad, CsA, Cer and NZ were added along with MFG and VRC, they effectively reversed the VRC antagonism and restored the effect of MFG against biofilms (Fig. 4a). However, the doses of Rad alone, CsA alone, Cer alone and NZ alone had little effect against the biofilms (Fig. 4b). This result implies that the resistance to MFG in the presence of VRC is possibly induced via stress responses. We also investigated whether these inhibitors enhanced the effect of antifungals against *Candida* biofilms. Rad, CsA, Cer, and NZ did not enhance the effect of VRC at the concentrations used in this study (Fig. 4b), but they enhanced the effect of MFG (Fig. 4c). The delayed addition (8 hours) of Rad also significantly decreased the antagonism, though the simultaneous addition showed better effects (Fig. 4d).

VRC induced UTR2 gene expression

To investigate whether VRC modulates an intracellular signal related to stress responses, the expression of the *UTR2* gene, which contains a calcineurin-dependent response element in its promoter [16], was examined by RT-PCR. After 24 h treatment with VRC, expression of the *UTR2* gene increased by 4-fold (Fig. 5).

Discussion

We studied the effect of the combination of VRC and MFG against biofilms and found that VRC antagonized the effect of MFG against biofilms but not against planktonic cells.

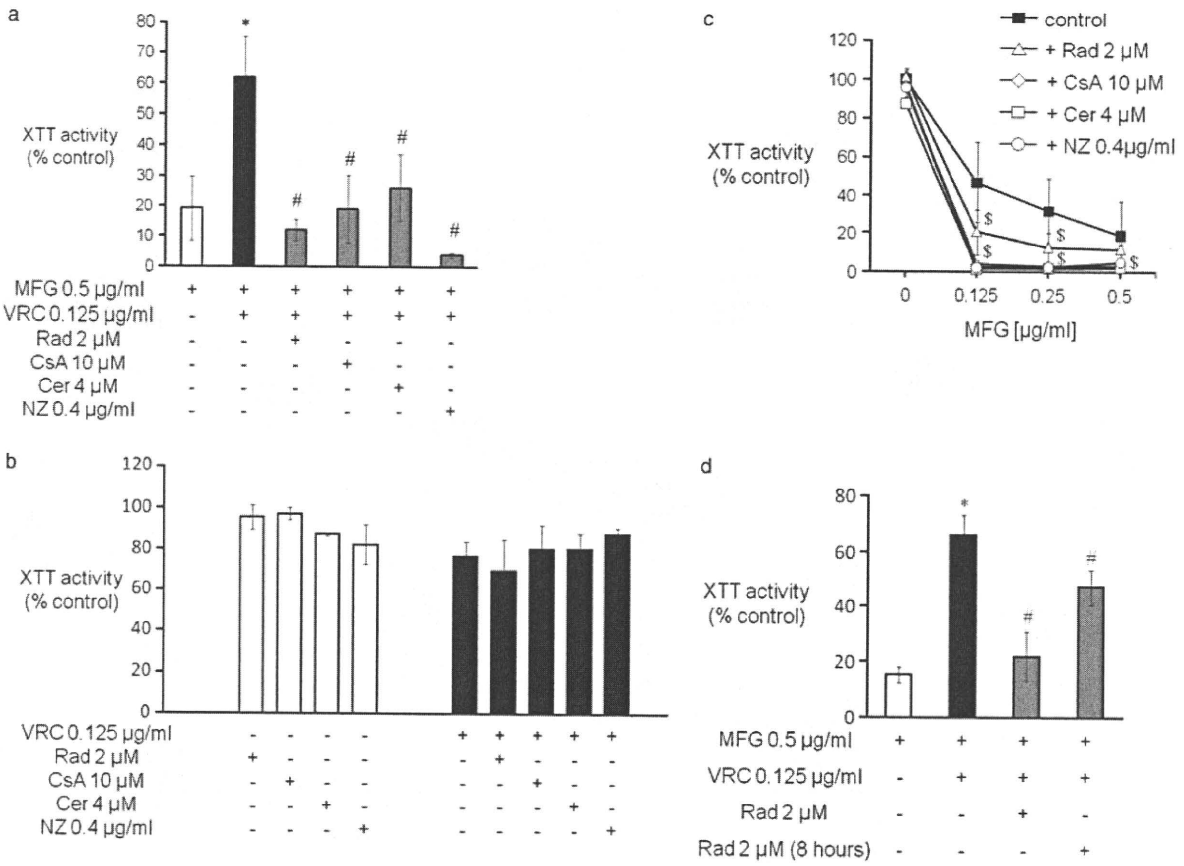

Fig. 4. Pharmacological inhibition of stress responses related to cell wall integrity enhances activity against biofilms. (a) Low doses of Rad, CsA, Cer, and NZ also reverse VRC-induced antagonism of MFG. (b) Rad, CsA, Cer, and NZ do not drastically reduce the XTT activity of biofilms even in combination with VRC. (c) Low doses of Rad, CsA, Cer, and NZ enhance the effect of MFG against *Candida* biofilms. (d) The delayed addition (8) of Rad also significantly decreases the antagonism, though the simultaneous addition shows better effects.VRC, voriconazole; MFG, micafungin; Rad, radicicol; CsA, cyclosporine A; Cer, cercosporamide; NZ, nikkomycyn Z. $*P < 0.05$ compared to 0.5 μg/ml of MFG alone. $\#P < 0.05$ compared to 0.5 μg/ml of MFG + 0.125 μg/ml of VRC. $\$P < 0.05$ compared to each corresponding dose of MFG alone.

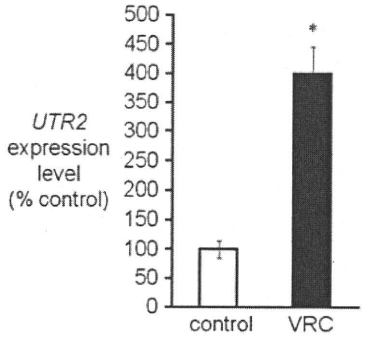

Fig. 5. VRC induces *UTR2* gene expression. The expression of *UTR2*, which contains a calcineurin-dependent response element in its promoter, elevates to 4-fold after 24 h treatment with VRC. VRC, voriconazole. $*P < 0.05$ compared to control.

This antagonism could be referred to as MFG-resistance induced by VRC because VRC attenuated the effect of MFG against *Candida* biofilms. This result is consistent with those described in recent reports [8–10]. We also demonstrated that pharmacological inhibition of Hsp90 reduced this VRC-induced MFG-resistance of biofilms (Fig. 4a). Cowen *et al.* recently reported that an Hsp90 inhibitor enhanced the fungicidal efficacy of an azole [17,18]. However, in our study, Hsp90 inhibition did not enhance the effect of VRC but rather enhanced the efficacy of MFG (Fig. 4b, c).

This discrepancy in the results can be explained on the basis of the Hsp90-related stress responses in fungi, which appear to enhance cell wall integrity [19]. Fungal cell walls consist of glucan, chitin, and several proteins, including mannoprotein. Echinocandins inhibit glucan synthase and disrupt the cell wall, thus causing cell death. To compensate for the loss of function of glucan synthase due to severe inhibition by echinocandins, fungal cells tend to increase the synthesis of chitin instead of glucan as a stress response [20,21]. Taking into consideration this information and our result (Fig. 4c), we speculate that Hsp90-related stress responses might play an important role in increasing cell wall integrity when cell wall synthesis is disrupted by MFG, especially in biofilms. The same mechanism seems to work when the cell membrane is disrupted by some azoles, e.g., chitin synthesis is increased as a secondary effect of the inhibition of sterol synthesis [22]. In planktonic cells, such a mechanism is useful for resisting damage to both the cell membrane and the cell wall. However, our result (Fig. 4b) suggests that in biofilms, increasing cell wall integrity is not useful for preventing damage to the cell membrane. Therefore, while the VRC-induced increase in cell wall integrity is of little use in itself, it induces MFG-resistance in *Candida* biofilms, which in turn increases cell survival. In addition, VRC-induced MFG-resistance disappeared after incubation in drug-free medium (data not shown). Therefore, this resistance is not acquired through a genetic change.

Transcription analysis using RT-PCR of *UTR2* implies that calcineurin dependent response was induced by VRC. Thus, VRC might temporally modulate intracellular signals, which increases cell wall integrity and protect *Candida* cells in biofilms from MFG. The difference in the importance of cell wall integrity in planktonic cells and biofilms might explain the discrepancy between our result and those described by Cowen *et al.* [17].

In order to qualify our results for clinical relevance, *in vivo* work, and, ultimately, clinical studies will be needed, but our results may provide several important pieces of information. First, a combination of MFG and VRC may be ineffective against some *Candida* infections related to biofilms. Second, inhibiting stress responses could enhance the effect of MFG against *Candida* biofilms. Third, combining inhibitors of stress responses could reverse the antagonism of MFG by VRC.

In conclusion, our results showed that the mechanism underlying the antagonism of MFG by VRC could be related to stress responses and cell wall integrity. Elucidation of the molecular aspects of this mechanism may help discover newer strategies to effectively combat fungal infections.

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Declaration of interest: We declare that we have no conflicts of interest.

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Chronic aspergillus infections of the respiratory tract: diagnosis, management and antifungal resistance

Koichi Izumikawa, Takahiro Takazono and Shigeru Kohno

Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

Correspondence to Koichi Izumikawa, MD, PhD, Nagasaki University Graduate School of Biomedical Sciences, Department of Molecular Microbiology and Immunology, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
Tel: +81 95 819 7276; fax: +81 95 849 7285; e-mail: koizumik@nagasaki-u.ac.jp

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Purpose of review

Chronic pulmonary aspergillosis (CPA) is a relatively rare, slowly progressive pulmonary syndrome due to *Aspergillus* spp. which requires specific knowledge in terms of disease entity, diagnosis, management and azole resistance. This review focuses on the recent understanding of CPA entity and the emergence of azole resistance in CPA.

Recent findings

Due to complexities related to patients' background and limited pathological evidence, the disease entity of CPA was incomprehensive and numerous names were previously used. The disease entities and nomenclature of subtypes of CPA have recently been proposed, though previous literature had grouped several different forms of CPA together. Recent advances in the methodology of susceptibility testing have indicated increasing azole resistance in *Aspergillus* spp. CPA is potentially involved in producing azole resistance and associated with poor response to azoles.

Summary

Since there are few publications regarding CPA, there are still many unanswered questions. However, updating of disease entity will promote the clinical and basic research in this field. Moreover, the emergence of antifungal drug resistance of *Aspergillus* is becoming a major concern. Thus, more evidence and research regarding drug resistance are required to improve the outcome of CPA.

Keywords

aspergilloma, azole resistance, CCPA, CNPA

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Introduction

Chronic pulmonary aspergillosis (CPA) is defined as slowly progressive pulmonary syndrome. Compared to invasive pulmonary aspergillosis (IPA), the pathophysiology of CPA ranges widely from aspergilloma to chronic necrotizing pulmonary aspergillosis (CNPA), also known as, subacute IPA, semi-invasive aspergilloma, symptomatic pulmonary aspergilloma or *Aspergillus* pseudotuberculosis in previous literature [1–4]. Allergic bronchopulmonary aspergillosis is a hypersensitivity disease of the lungs associated with inflammatory destruction of respiratory tract to *Aspergillus* spp. [5] and distinct from CPA through its chronicity. Due to the complex of CPA patients' clinical background, minimal pathological evidence and a low number of cases, these forms of CPA sometimes mixed up and the disease entity is currently confused. For example, Du made comment that CNPA is suitable diagnosis instead of IPA in certain published case report article [6,7]. Thus, the disease entity of *Aspergillus* infection, especially chronic forms of aspergillosis, is sometimes vague, particularly in the absence of typical clinical features and course. As described before, subcategorizing CPA is difficult, such that CPA can represent 'complex pulmonary aspergillosis' rather than 'chronic pulmonary aspergillosis'.

The lack of standardization with CPA leads to difficulties in diagnosis, establishing treatment regimens and conducting other clinical studies. Additionally, antifungal drug-resistant *Aspergillus* spp. is becoming a major clinical concern that will certainly influence the morbidity and mortality of CPA patients who usually require long-term antifungal treatment.

The review focuses on the current understanding of CPA, including its diagnosis, management and drug resistance.

Disease entity

The chronic forms of pulmonary aspergillosis were originally established in early 1980s by Binder *et al.* [1] as CNPA and as semi-invasive aspergillosis by Gefter *et al.* [2]. It is characterized as slowly progressive inflammatory pulmonary syndrome due to *Aspergillus* spp. Patients usually possess underlying pulmonary diseases (e.g.

tuberculosis sequelae, bronchiectasis, chronic obstructive pulmonary disease, cystic lesions and pulmonary fibrosis) with destruction of lung tissues, usually with low-grade immunosuppression (e.g. low-dose steroid administration, diabetes, collagen diseases, renal disorders or alcohol).

In the last decade, new nomenclature and definition of chronic forms of aspergillosis have been proposed [4,8,9] and recent guidelines from Infectious Diseases Society of America (IDSA) have indicated three major subtypes of chronic forms of pulmonary aspergillosis, namely CNPA (categorized in invasive form of aspergillosis), chronic cavitary pulmonary aspergillosis (CCPA) and aspergilloma [10]. Aspergilloma was traditionally classified as simple or complex in the surgical literature and complex aspergilloma is recently treated as CCPA in current IDSA guidelines [10,11]. CCPA is defined as the occurrence of multiple cavities with or without fungus ball, in association with detectable serum *Aspergillus* antibodies, pulmonary and systemic symptoms, and elevated inflammatory markers. Aspergilloma is defined as a conglomeration of *Aspergillus* hyphae, fibrin, and cellular debris within a pre-existing pulmonary cavity or an ectatic bronchus, with positive serum *Aspergillus* antibodies. As described before, the distinction between aspergilloma and other forms of CPA may be semantic but reflect the singularity of the aspergilloma against the multiple cavities which is typical for CPA [9]. An apparent pathological difference between aspergilloma (simple) and other forms of CPA is that there is no absolute tissue invasion of hyphal elements of *Aspergillus* in simple aspergilloma, whereas hyphal invasion to lung parenchyma but not to blood vessels may occasionally be seen in CNPA and CCPA [3,9]. As Hope *et al.* [9] indicated that apparent distinct entities do not exist for this syndrome and these forms usually overlap and previous articles regarding CNPA included these chronic forms of aspergillosis. Recent IDSA guidelines and textbook have indicated the differences between CNPA and CCPA are the prolonged time frame (CNPA: 1–3 months vs. CCPA: more than 3 months) [12], although this difference is arbitrary, and the genetic predisposition of defects in innate immunity such as mannose-binding lectin and surfactant protein A in CCPA patients [13–15]. Toll-like receptors (TLRs) play critical roles in innate immunity and Carvalho *et al.* [15,16] recently reported that polymorphism (Asp299Gly) of the TLR-4 gene is significantly associated to CCPA [odds ratio (OR) 3.46; $P = 0.003$]. Establishing the precise disease entity of CPA, whereas challenging, is important in conducting clinical trials, developing tools for diagnosis and treatment. Complexities regarding the background of patients with CPA, coinfection with other microorganisms and status of immunosuppression may interfere with establishment of the simple entity of CPA. Due to these reasons, previous and even current articles regarding CPA may include all forms of CNPA, CCPA and even aspergilloma. The authors describe CCPA and CNPA as CPA in this review, as this approach has been used by others [11,12].

Epidemiology

There are at least a hundred species in *Aspergillus* spp., and most common pathogenic species to humans are *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*. *A. fumigatus* is the most common cause of the vast majority of CPA cases. Though recent trend in causative agents of CPA is not known, *A. fumigatus* occurred in 82.6–91.3% of CPA cases in previous studies [4,17–19]. *A. flavus* is the second most common cause of all forms of aspergillosis [20]. Although the clinical presentation of CPA caused by *A. flavus* does not differ from CPA by other *Aspergillus* spp., it rarely causes CPA including aspergilloma for unknown reasons [21,22]. *A. lentulus* and *A. udagawae* are classified together with *Aspergillus* section *Fumigati* and relatively low-susceptible to antifungals including amphotericin B [23–25]. More importantly, these species used to be misidentified as *A. fumigatus* by morphological observation; however, recent molecular techniques enable identification of these species [26]. Although no data are currently available for the frequency of infections with these species in CPA patients, previous epidemiological data might include and label these uncommon species as *A. fumigatus*.

Clinical features and diagnosis

Chronic pulmonary aspergillosis and aspergilloma usually occur in middle-aged to elderly patients with chronic pulmonary underlying diseases. Pre-existing or residual cavities after mycobacterial infection are common dwelling site of *Aspergillus* and these cavitary lesions are located mostly in upper lobes [27–29]. Other pulmonary diseases that resulted in forming cavitation such as emphysematous bullae, chronic obstructive pulmonary diseases, bronchiectasis, pulmonary cysts sarcoidosis, histoplasmosis and rheumatoid nodules can be a cause of CPA and aspergilloma [9,29]. CPA slowly progress in months and even years, causing lung destruction such as progressive cavitation, fibrosis and pleural thickening [2,30,31]. CPA patients often present with chronic pulmonary or systemic symptoms (usually for longer than 3 months), such as weight loss, productive cough, chronic sputum, hemosputum or hemoptysis [4,32]. Single or multiple fungus balls might be seen in these cavitary lesions. Whereas patients with aspergilloma usually demonstrate no disease-specific symptoms, however, hemoptysis occurs in 50–90% of patients, occasionally becoming life-threatening massive hemoptysis in 30% [9,28]. Though no large-scale epidemiological data, Nam *et al.*

[32] reported a 5-year survival rate in CPA patients of around 50%.

Serum *Aspergillus* precipitins test detecting antibodies (IgG) to *Aspergillus* are usually positive [8,33] in CPA patients, including those with aspergilloma. Recent study indicated that the test was positive in more than 95% of cases [32], whereas another study indicated 89.3% positivity compared to only 50% with *Aspergillus* galactomannan antigen ELISA tests [34•]. Although *Aspergillus* galactomannan antigen ELISA tests have been approved for the diagnosis of IPA, with a sensitivity of 79% and a specificity of 86% in meta-analysis [35], little data are available for its utility in CPA patients and, hence, it is currently not considered useful in CPA diagnosis. The utility of testing bronchoalveolar lavage specimens by *Aspergillus* galactomannan antigen ELISA tests varies in the reports and is still controversial; there being no available data for CPA cases. Other serological tests, such as β-D-glucan tests, have not been evaluated in CPA patients. There is an urgent need to develop newer serological tool for diagnosis and for reflecting clinical progression of CPA cases.

Sputum cultures, bronchoalveolar lavage cultures, and surgical biopsy specimens usually reveal the causative organism and may support diagnosis. However, as Aspergilli are ubiquitous in the environment, careful evaluation is required to confirm colonization or infection in the culture-positive results. Pathological examination by transbronchial biopsies, surgical biopsy and computed-tomography-guided biopsies are confirmatory methods for CPA diagnosis; however, they may not be applied to all cases. Thoracoscopic or open-lung biopsies are rarely performed mainly due to the risk of complication from underlying pulmonary diseases [27,33].

Although various histopathological alternations in CPA have been reported, apparent histopathological definition of CPA has not been established to date [3,36,37]. Generally, CPA is characterized by chronic inflammation of the cavity wall, the presence of hyphae consistence with *Aspergillus* spp. and necrotic lung tissue with or without fungus ball [36]. Hyphae invading to adjacent lung parenchyma, but without angioinvasion (common findings with IPA) by *Aspergillus*, can be seen in CPA except simple aspergilloma [4,9]. The presence of organizing lesions without fungal components around the cavity is also characteristic of CPA but not IPA [37].

Diagnosis of CPA is made synthetically based on these findings of clinical, radiological, mycological and serological factors. Denning *et al.* [4] proposed enrollment criteria for prospective clinical studies of CPA and much of recent literature basically follow this criteria.

Management

Due to the recent development of new antifungal drugs, such as voriconazole (VRCZ) and echinocandins, the management of CPA has changed. Before 2000, only itraconazole (ITCZ) capsules and intravenous amphotericin B formulations were available for treatment of CPA [27].

The latest IDSA guidelines for the treatment of aspergillosis recommend oral VRCZ or ITCZ for CNPA and CCPA as primary treatment [10]. Surgical resection is recommended for simple aspergilloma cases [10,28,38]. Attempts to resect CPA lesions, however, are difficult due to pre-existing underlying diseases, such as the sequelae of tuberculosis infections and other factors (e.g. poor general status due to complications), which contribute to the morbidity and mortality. Recent studies indicated lower mortality and better survival rates in selected CPA (complex aspergilloma) cases compared to previous literature [39,40]. However, evidence is insufficient and, hence, surgery should be reserved for those who develop severe hemoptysis. Bronchial artery embolization (BAE) to occlude the causative vessels in CPA patients with hemosputum or hemoptysis is another option for treatment of CPA in case surgical treatment is contraindicated. However, BAE is only temporarily effective due to the presence of collateral vascular channels at the site of bleeding.

Although no large-scale randomized studies for the management of CPA have been conducted, medical therapy is the standard therapy for CPA. Additionally, the following clinical factors have not been standardized or even described in IDSA guidelines: timing of the initiation of treatment, duration of treatment and timing of discontinuation of treatment for CPA. Only few case series reports are currently available. From the early 1990s to date, the reported efficacy of oral ITCZ is somewhat variable, with a range of 30–82.1%, a duration of administration of approximately 4–12 months and adverse effects being seen in 16–33% [4,32,41–43]. In the last decade, the reported efficacy of oral VRCZ in terms of response rate ranged from 53 to 65% with several months' administration, and the frequency of adverse effects which resulting in discontinuation of therapy ranged from 9 to 27% [44–46]. The efficacy of ITCZ and VRCZ in these studies was mostly assessed by clinical, radiological and mycological improvement at the end of treatment or regular interval, regardless of whether there was a partial or complete response. Posaconazole is a new triazole drug with a wide spectrum of activity including zygomycoses. Although its efficacy has been proved in salvage therapy for IPA patients and prophylaxis of invasive fungal diseases in patients with neutropenia and transplantation, no clinical data are as yet available for CPA cases [47,48]. Its

activity against *Aspergillus* spp. and oral formulation are both suitable for CPA treatment.

Since relatively prolonged administration of triazoles is the mainstay of CPA treatment, appropriate use of drugs based on pharmacokinetics/pharmacodynamics properties and therapeutic drug monitoring (TDM) is logically important. The pharmacokinetics/pharmacodynamics parameter of triazoles is the ratio of the area under the concentration–time curve (equal to total exposure per dosing interval) to the minimum inhibitory concentration (AUC/MIC) [49,50]. The purpose of TDM is to maximize the efficacy and minimize the toxicity of therapeutic agents. Pascual *et al.* [51] reported the relationship between trough concentrations and successful treatment as well as toxicity of VRCZ. A trough level of 1 mg/l was associated with a 70% probability of successful outcome in invasive mycoses, whereas that of 6 mg/l resulted in approximately 20% probability of central nervous system toxicity [51]. In recent studies regarding TDM studies of ITCZ and VRCZ, trough level of between 1–2 mg/l and 0.5–1.5 mg/l, respectively, are recommended for successful treatment of fungal diseases [52]. A trough level of 5 mg/l of ITCZ is associated with a 26% probability of an adverse effect [53]. These proposed target concentrations are, however, based upon limited data and not from prospective randomized studies. Thus, recent data suggest that TDM of triazoles may be useful in management.

There are limited published data on the use of other classes of antifungal agents in the treatment of CPA. The efficacy of intravenous micafungin (MCFG) was reported in two small series of CPA cases by Kohno *et al.* [54] and Izumikawa *et al.* [55], in which the success rate of treatment was 12/22 (duration of treatment 11–57 days) and 7/9 (duration of treatment 29–96 days) cases, respectively. Only one randomized control study of CPA therapy, comparing intravenous VRCZ and intravenous MCFG was presented, which indicated favorable response rate with both MCFG (60%) and VRCZ (53%) [56], though the utility of intravenous antifungal drugs for CPA treatment is not known. No studies on the use of combination use of antifungals to treat CPA, apart from a small number of case reports, have been reported.

Overall crude response rate of CPA to antifungal drugs such as ITCZ, VRCZ and MCFG is approximately 50–70%; however, we should realize that the definition of CPA, evaluation of response to drugs, endpoints of each study and the duration of treatment were varied in these published reports.

Evaluating response to antifungal drugs in CPA is challenging, since it usually takes several weeks to months for noticeable improvement of respiratory signs, symptoms and radiological findings. Additionally, no recommendations for commencement and discontinuation of treatment, dose and route of drugs are established. More data from case series or randomized clinical trails are urgently required.

There are small numbers of case studies using interferon-γ as adjunctive therapy for CPA with or without couse of antifungals. Although all four cases responded well [4,57], its utility has not been established.

Antifungal resistance

Azole-resistant *A. fumigatus* is increasing and becoming one of major clinical concerns in the treatment of *Aspergillus* infection [58,59••,60,61••]. The data regarding azole resistance are mainly originated from United Kingdom and The Netherlands and no worldwide epidemiological data are available. Azole resistance reported by Howard *et al.* [61••] from the United Kingdom group is largely in the groups of patients with CPA. Azole resistance in *A. fumigatus* has not been focused in the last decade, there being only few studies after the first report of ITCZ-resistant strains in 1997 by Denning *et al.* [62]. Recent advances in standardizing susceptibility testing by Clinical and Laboratory Standards Institute (M38-A2) and European Committee on Antimicrobial Susceptibility Testing [63,64], and establishment of interpretative cut-offs [65] as well as clinical breakpoints [59••], have greatly enhanced clinical and basic research. The resistant mechanisms against antifungal drugs including azoles are described elsewhere [66]. The primary resistance mechanism to azoles is the mutation in the target protein, 14 alpha-demethylase coded by *cyp51A*, and several hotspots of mutation are already confirmed [61••]. It is notable that many isolates resistant to ITCZ are usually cross-resistant to posaconazole (74%) and VRCZ (65%) [61••]. Although the molecular approach for revealing azole resistance is well studied, it is still unknown how resistance is evolved in nature. Verweij *et al.* [67] reported the possibility that environmental fungicide (azoles) use may induce mutations in *cyp51A* and these environmental strains become a cause of aspergillus infection of human [68]. On the contrary, some molecular epidemiological analyses indicated that drug resistance is acquired in infecting strains within the human lung rather than inhalation of environmental azole-resistant strains [61••,69]. Importantly, many azole-resistant strains were isolated from CPA patients with aspergilloma and many of whom had been exposed to azoles for an extended duration (1–30 months) [61••,70]. Although oral administration of azoles is the mainstay of treatment of CPA, long-term administration potentially induces azole resistance. Due to higher cross-resistance rate to azoles, intravenous amphotericin B or echinocandins are only alternative options for the treatment of CPA with azole-resistant

strains. Studies on optimizing triazole regimens with pharmacokinetics/pharmacodynamics and TDM data to prevent mutations of *cyp51A* and acquire maximum efficacy are desired. Developing new oral or intravenous antifungal drugs which do not show cross-resistance to current azoles are also required.

A. fumigatus is sensitive to amphotericin B and echinocandins *in vitro* and no emergence of resistance to these drugs has been reported [71]. Other species of *Aspergillus*, such as *A. terreus* and *A. nidulans*, are resistant to amphotericin B [71], although the incidence of CPA due to these species is not high.

Conclusion

Studies regarding CPA are quite limited. Newer disease entities of CPA will somewhat increase and promote clinical and basic studies. The emergence of azole-resistant *Aspergillus* is becoming a major issue in the treatment of aspergillosis, such that new antifungal drugs should be developed and new regimens of antifungal drugs will be required.

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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

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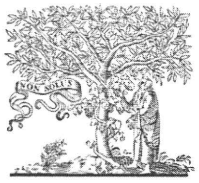

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Intravenous micafungin versus voriconazole for chronic pulmonary aspergillosis: A multicenter trial in Japan

Shigeru Kohno [a,*], Koichi Izumikawa [a], Kenji Ogawa [b], Atsuyuki Kurashima [c], Niro Okimoto [d], Ryoichi Amitani [e], Hiroshi Kakeya [a], Yoshihito Niki [f], Yoshitsugu Miyazaki [g], Japan Chronic Pulmonary Aspergillosis Study Group (JCPASG)

[a] *Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan*
[b] *Department of Pulmonary Medicine, National Hospital Organization Higashinagoya National Hospital, Nagoya, Japan*
[c] *Department of Respiratory Diseases, National Hospital Organization Tokyo National Hospital, Tokyo, Japan*
[d] *Center of Respiratory Diseases, Kawasaki Medical School Kawasaki Hospital, Okayama, Japan*
[e] *Department of Respiratory Medicine, Osaka Red Cross Hospital, Osaka, Japan*
[f] *Department of Clinical Infectious Diseases, Showa University, Tokyo, Japan*
[g] *Department of Chemotherapy and Mycoses, National Institute of Infectious Diseases, Tokyo, Japan*

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Summary Chronic pulmonary aspergillosis (CPA) is slowly progressive inflammatory pulmonary syndrome due to *Aspergillus* spp. The evidence regarding CPA treatment is limited. We conducted a randomized, multicenter, open-label trial comparing intravenous micafungin (MCFG) of 150–300 mg once daily with intravenous voriconazole (VRCZ) of 6 mg/kg twice on Day 1 followed by 4 mg/kg twice daily for the treatment of 107 in patients with CPA to compare the efficacy and safety of both drugs as initial treatment in Japan. Treatment effectiveness was defined by clinical, mycological, radiological and serological responses 2 weeks after the initial administration and at the end of therapy. The total of 50 and 47 patients were assigned to the MCFG and VRCZ groups, respectively. The difference in efficacy rates between MCFG and VRCZ was not significant, either after 2 weeks [68.0% vs. 58.7%; the absolute difference, 9.3% with a 95% confidence interval (CI), −9.97 to 28.58, $P = 0.344$] or at the end of therapy (60.0% vs. 53.2%; the absolute difference, 6.8% with a 95% CI, −12.92 to 26.54, $P = 0.499$). In the safety evaluation, fewer adverse events occurred in the MCFG than

* Corresponding author. Tel.: +81 95 (819) 7273; fax: +81 95 (849) 7285.
E-mail address: s-kohno@nagasaki-u.ac.jp (S. Kohno).

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VRCZ group (26.4% vs. 61.1%, $P = 0.0004$). MCFG was as effective as VRCZ and significantly safer than as an initial treatment of CPA. (UMIN Clinical Trials Registry number, UMIN000001786.)

Introduction

Chronic forms of pulmonary aspergillosis (CPA) are characterized as a slowly progressive inflammatory pulmonary syndrome due to *Aspergillus* spp.[1,2] Several names of these chronic forms of disease have been proposed: semi-invasive aspergillosis,[3] chronic necrotizing pulmonary aspergillosis (CNPA),[4] simple or complex aspergilloma, and chronic cavitary and fibrosing pulmonary aspergillosis (CCPA and CFPA).[5] As Hope et al.[6] indicated apparently distinct entities do not exist for this syndrome and these forms usually overlap. The common characteristics of these forms, however, consist of 1) underlying pulmonary disorders, 2) the status of low-grade immunosuppression, and 3) less than severe findings of angioinvasion in histopathology.[1,5,7]

Numerous clinical cases and few retrospective studies have been reported for the treatment of CPA;[8–12] however, there are no large-scale clinical trials that have been conducted. Although the latest Infectious Diseases Society of America (IDSA) guidelines for the treatment of aspergillosis recommend oral azoles for CPA as primary treatment,[13] the utility of antifungal injections for CPA treatment is not known. Intravenous antifungal agents may possess important role as an induction therapy followed by oral antifungal drugs as maintenance therapy for CPA cases. Several kinds of intravenous antifungal agents active to *Aspergillus* spp. are available. Micafungin (MCFG) is new semisynthetic lipopeptide antifungal drug in the echinocandin family that has been developed in Japan.[14] Several *in vitro* and *in vivo* studies have shown high levels of antifungal activity against *Aspergillus* spp.[15–17] Voriconazole (VRCZ), from a newer generation of triazoles, has also potent anti-aspergillus activity *in vivo* and *in vitro*.[18,19]

Our study is the first prospective large-scale trial comparing intravenous MCFG and intravenous VRCZ as induction therapy in CPA patients who require immediate treatment.

Methods

Patients

From April 1, 2006, to November 30, 2008, we enrolled patients with CPA at 24 Japanese hospitals in this study, which was approved by the ethics committee at each hospital. Every patient provided written informed consent. Astellas Pharma Inc. (Tokyo, Japan) supported the study with a grant; Astellas, Co. Ltd. was not involved in the design of study, the enrollment of patients, the collection, analysis, interpretation of the data or preparation of the manuscript. All authors vouch for the completeness and accuracy of the data presented.

Patients were eligible for enrollment if they were at least 20 years old and had been given a diagnosis of CPA. The diagnostic criteria of Hope et al. for CPA including chronic CNPA, CCPA, CFPA and complex aspergilloma were used in this study.[6] Proposed enrollment criteria for prospective clinical studies of CPA by Denning with minor modifications were used for this trial.[5] Patients with CPA had to fulfill the following conditions: (1) the existence of at least one of the symptoms in the complex consisting of fever, weight loss, sputum, cough, hemoptysis, fatigue, and shortness of breath; (2) new infiltrates, cavity formation, or expansion of pre-existing cavities with or without peri-cavitary infiltrates and adjacent pleural thickening; (3) at least one positive result of serologic tests including the Platelia *Aspergillus* test (Fujirebio, Tokyo, Japan) for detecting *Aspergillus* galactomannan antigen by enzyme-linked immunosorbent assay (cut-off value, 1.0), the *Aspergillus* immunodiffusion system (Microgen Bioproducts, Ltd., Camberley, United Kingdom) for anti-*Aspergillus* antibody detection, and the (1,3)-β-D-glucan test (cut-off value, 11.0 pg/ml; Wako Pure Chemical Industries, Osaka, Japan; and cut-off value, 20 pg/ml for the Fungitec G Test, Seikagaku Corporation, Tokyo, Japan) and/or any positive evidence of the existence of *Aspergillus* spp. by molecular diagnosis, culture, or pathological findings; (4) positive findings of at least one of the inflammation markers such as white blood cell (WBC) counts, value of C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR); (5) lack of improvement of symptoms or signs after at least 3 days administration of broad-spectrum antibiotics. The following patients were excluded from the study: (1) patients who received MCFG or VRCZ within one month before the time of enrollment, (2) patients with simple aspergilloma, invasive pulmonary aspergillosis, or allergic bronchopulmonary aspergillosis, (3) patients with infectious diseases other than aspergillosis, (4) pregnant patients, (5) patients who did not provide informed consent, and (6) patients with liver, kidney, or heart failure, which fulfilled the Grade II level defined in the Common Toxicity Criteria grading system of the National Cancer Institute.[20]

Procedures

The patients were allocated to either the MCFG or VRCZ treatment group. The randomization of the minimization method was performed at a centralized web site by attending physicians after obtaining the informed consent from each patient. Random numbers were generated by the computer. Stratification factors of severity and past history of treatment of CPA including aspergilloma were used. Severity was defined as mild, moderate and severe. The factors that were used for determining severity were (1) serum albumin value (less than 2.5 g/dl), (2) SpO_2 less than 90% or PaO_2 less than 60 Torr, (3) impaired oral administration of drugs, (4) expansion of newer infiltration shadow on chest X-ray film that was more than half of the lung field, (5) the existence of severe complications

such as malignancies, cerebral vascular disorders, hepatic diseases, kidney diseases or heart failure, and (6) administration of corticosteroids. Mild severity was defined as the patient possessing none of the above factors. Moderate severity was defined as the patient possessing only one of the factors listed above. Severe severity was defined as the patient possessing at least two of the factors listed above. The randomization schedule was generated by a computer to ensure balanced treatment allocation. Briefly, when the difference of number of allocated patients between the MCFG and VRCZ groups was less than two, the patients were completely randomized. Hence, when the difference was more than three, the patients were allocated to the smaller number therapy group at 90% probability or the bigger number therapy group at 10% probability.

VRCZ was given intravenously at 6 mg/kg every 12 h for 24 h, and then 4 mg/kg every 12 h. MCFG was given at 150–300 mg per day. Dose of MCFG was accepted to be decreased due to patient's body weight by the decision of the attending physician. The reason that the dose of MCFG was not fixed in this study was that no data was available about the dose effect of MCFG in the treatment of pulmonary aspergillosis. Patients received treatment for at least 2 weeks with a maximum duration of 4 weeks.

Patients were followed up until 4 weeks after the first administration. Clinical assessments were made daily during treatment. Clinical laboratory tests were run once every week. Radiological and mycological investigations were performed at 2 weeks and at the end of treatment with a maximum 4 weeks. Concurrent treatment with antibiotics was prohibited in this study.

The primary efficacy end point was response to treatment, which was classified as 'success' or 'failure' at the end of administration (at least 2 weeks of administration with a maximum duration of 4 weeks). Each case was carefully reviewed by the investigators. All clinical response criteria were defined as previously reported with slight modifications.[8] Briefly, four groups of factors, consisting of (1) clinical, (2) laboratory, (3) radiological, and (4) mycological, were assessed. The efficacy was evaluated for each of the four groups of factors regardless of whether there was a partial or complete response. (1) Clinical factors were clinical symptoms and signs such as hemosputum, cough, dyspnea and fever which were recorded individually by attending physicians and assessed by a data-review committee. Clinical improvement was defined by reduction or disappearing of these symptoms or signs. (2) Laboratory data such as CRP level, WBC counts, and ESR were independently assessed by a data-review committee. Laboratory data improvement was defined by reduction levels of these inflammatory markers. The transition of titer of anti-*Aspergillus* antibody was not evaluated. (3) Radiological factors were also evaluated individually by a data-review committee. Briefly, improvement was defined only if there was apparent improvement in the newly appeared lesions on radiological images; no transitional changes in lesions, were not considered improvements (4) Mycological factors were determined by culture or histopathological tests using clinical samples such as sputum, bronchoalveolar lavage fluid, and percutaneous aspiration biopsy samples. Mycological improvement was defined by disappearing of *Aspergillus* from clinical samples. The members of the data-review committee were not blinded to the treatment assignment on the evaluation of the outcomes or side effects.

A 'success' with respect to clinical response at the end of the treatment was defined as follows: improvement in at least two of the four groups of factors without deterioration in other two groups of factors. A 'failure' was defined as a clinical response that did not match with the 'success' definition.

The secondary efficacy end point was response to treatment, which was classified as 'success' or 'failure' at the end of 2 weeks administration. All four groups of factors were used as in the primary endpoint; however, the criterion of "success" was changed as follows: improvement in at least one of the four groups of factors without deterioration in other groups of factors.

Adverse events were recorded from randomization until the last day of treatment. These events were classified according to the Common Toxicity Criteria grading system of the National Cancer Institute.[20]

Statistical analysis

The primary objective was to show the superiority of each drug in the per-protocol population. Because number of CPA patients, data for the efficacy of antifungals to CPA and budget were limited, the sample size for this clinical trial was set on the basis of the frequency of adverse events of both drugs. On the basis of the rate of adverse events, which averaged 35% for MCFG and 62% for VRCZ, a power of 0.80 and the requirement to show the superiority of one drug to the comparator group with a significance of 5.0% for the final analysis were set. A total sample size of 100 patients (50 assigned MCFG and 50 assigned VRCZ) satisfying the criteria for the per-protocol population was calculated to be necessary.

The intention-to-treat population was defined as patients who underwent randomization. The modified intention-to-treat population was defined as patients who received at least one dose of the study drug they were initially assigned to receive and who had a baseline diagnosis of CPA. The per-protocol population was defined as patients who were confirmed to have a baseline diagnosis of CPA, the availability of an investigator's assessment of overall treatment at the end of therapy, at least 14 doses of one of the study drugs, and no prohibited medication. The population included in the safety analysis consisted of all patients who received their initial study drug. The investigator's assessments of overall treatment success at the end of therapy and at the end of the first 2 weeks were analyzed with a two-sided 95% confidence intervals (CI). Noninferiority of MCFG could be concluded if the two-sided 95% CI for the difference in the proportions (MCFG minus VRCZ) had a lower boundary above −15%. Categorical variables were tested by the Fisher's exact test and were expressed as frequencies and proportions. Continuous variables were tested by the Student's *t*-test and were expressed as means and ranges. A *P*-value of <0.05 was considered statistically significant.

Results

Enrollment and baseline characteristics of the patients

A total of 107 patients were recruited by 24 centers in Japan during study period. A total of 53 and 54 patients were assigned to the MCFG and VRCZ group, respectively; these patients comprised the intention-to-treat population. Trial profile was presented in Fig. 1. All 107 patients were recruited for safety analysis. Four and six patients were excluded from the intention-to-treat population and the modified intention-to-treat population, respectively. One case in the VRCZ-treated group lacked the assessment data at the first 2 weeks of treatment; therefore, this case was not evaluated at the secondary endpoint but was evaluated at the primary endpoint. The demographic characteristics and underlying conditions of the patients in the intention-to-treat population are summarized in Table 1. The MCFG and VRCZ groups were well matched and no significance difference was observed in the intention-to-treat and modified intention-to-treat populations (data not shown).

Base-line characteristics of the infection and the serological findings

Characteristics of the patients with CPA such as symptoms, signs, and inflammation markers and the results of the serum tests prior to the administration of study drugs in the per-protocol populations are summarized in Table 2. There were no significant differences in the clinical characteristics including symptoms, laboratory findings and the serological test result between the MCFG and VRCZ groups.

The results of the culture test and biopsy of respiratory specimens are shown in Table 3. The positive rate of culture tests remained at only 50%.

Response

The average administration time was 23.6 days for the MCFG group and 20.6 days for the VRCZ group without statistical difference ($P = 0.105$). The average dose of MCFG was 167.4 mg/day (range, 75–300 mg/day) and that for VRCZ was 8 mg/kg/day (range, 214–613 mg/day). There were four cases, in which 300 mg/day of MCFG was used in this study by the decision of the attending physicians. There was a single case in which 75 mg/day of MCFG was used due to low body weight of the patient. No single patient underwent surgical resection of the pulmonary legions in this study during the evaluation therapy.

The outcome at end of treatment in the per-protocol populations was not significantly different between the MCFG and VRCZ group (Table 4). In the intention-to-treat population, success rates were 56.6% (30 of 53 patients) with MCFG and 46.3% (25 of 54 patients) with VRCZ (absolute difference, 10.3%; 95% CI, −8.63 to 29.25). The response rates of each group of factors, (clinical, laboratory, radiological, and mycological) are shown in Table 4. There was no significant difference in the response rates among all factors between the MCFG and VRCZ groups.

The outcome at the end of the first 2 weeks of treatment was not significantly different between the MCFG and the VRCZ group. In the intention-to-treat population, success rates were 64.2% (34 of 53 patients) with MCFG and 50.0% (27 of 54 patients) with VRCZ (absolute difference, 14.2%; 95% CI, −4.61 to 32.91). The response rates of the four

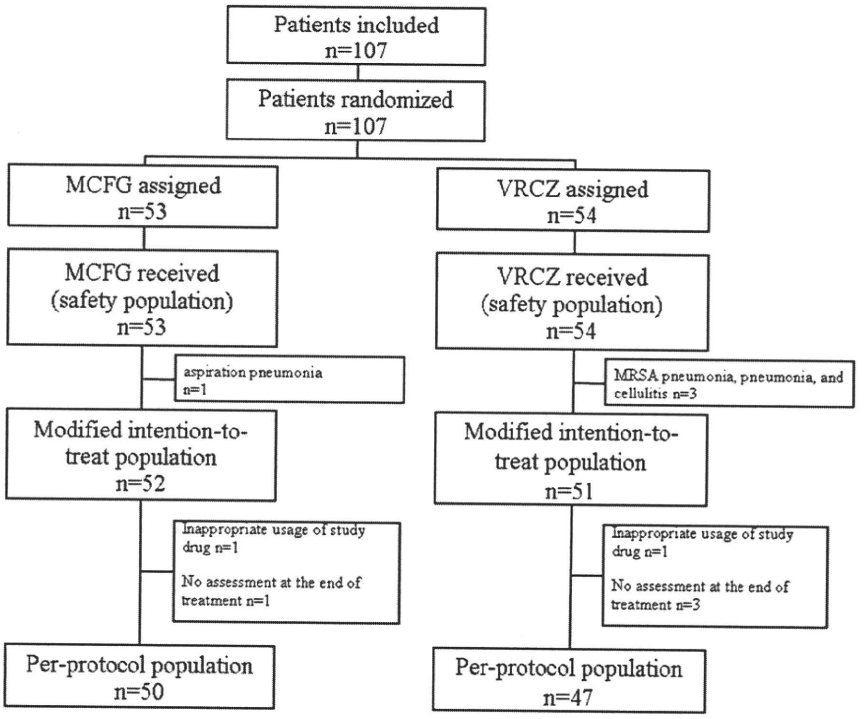

Figure 1 MCFG, micafungin; VRCZ, voriconazole; MRSA, methicillin-resistant *Staphylococcus aureus*.

Table 1 Characteristics of the patients in the intention-to-treat population.

characteristic	overall (n = 107)	micafungin (n = 53)	voriconazole (n = 54)	P
Age – yr				
Mean	70.9	72.1	69.9	0.163
Range	47–88	48–87	50–88	
Sex – no. (%)				
Male	83 (77.6)	37 (69.8)	46 (85.2)	0.057
Female	24 (22.4)	16 (30.2)	8 (14.8)	
Height – cm				
Mean	159.5	159.3	159.6	0.447
Range	134.5–179.0	138.0–179.0	134.5–169.5	
Weight – kg				
Mean	45.7	44.9	46.4	0.831
Range	28.0–74.2	28.0–68.0	29.1–74.2	
Underlying condition – no. (%)				
Tuberculosis sequelae	56 (52.3)	30 (56.6)	26 (48.1)	0.441
Chronic obstructive pulmonary disease	22 (20.6)	10 (18.9)	12 (22.2)	0.812
Diabetes	13 (12.1)	4 (7.5)	9 (16.7)	0.236
Others	16 (43.0)	22 (41.5)	24 (44.4)	0.520

groups of evaluated factors were not significantly different (Table 4).

Because the lower 95% confidence limit for the difference between MCFG and VRCZ at both endpoints settings was below zero, MCFG was considered to be neither inferior nor superior to VRCZ.

Safety

Table 5 shows the treatment-related adverse events and reasons for discontinuation from the study in patients who received at least one dose of study drug. Significantly fewer adverse effects were observed in the MCFG group than in the VRCZ group. Hepatic events were the most frequent adverse events in both the MCFG and VRCZ groups (8 cases in the MCFG group and 15 cases in the VRCZ group). There were four cases in which VRCZ was discontinued due to severe hepatic events (at least one of the values of glutamic oxaloacetic transamidase, glutamic pyruvic transamidase, alkaline phosphatase, or gamma-glutamyl transpeptidase exceeded 2.5 times the value of the upper normal limit). Various visual events including photophobia, xanthopsia, abnormal vision, defective color vision, vision blurred, and visual disturbance occurred only in the VRCZ group and all visual events were transient and resolved without intervention. All of the adverse effects other than visual events recovered during the treatment or after the end of treatment, except in one case with alkaline phosphatase elevation, which was mild.

Discussion

In the current study, we recruited patients who basically satisfied the criteria for the clinical study of CPA proposed by Denning et al.[5] The inclusion criteria in this study were designed to recruit patients who required immediate treatment. Another consideration was made using broad-spectrum antibiotics prior to registration to minimize registering patients with bacterial infections in addition to aspergillosis.

The latest IDSA guidelines for the treatment of aspergillosis recommend oral VRCZ or ITCZ for CPA as primary treatment,[13] however there is no a large-scale clinical trial data to support recommendation. Although the utility of antifungal injections for CPA treatment is not known, intravenous antifungal agents may be used as an induction therapy followed by oral antifungal drugs as maintenance therapy for CPA cases. Additionally, intravenous antifungal drugs will be needed if the patients are refractory to oral antifungal drugs or in severe status. Our study is the first large-scale clinical trial comparing intravenous MCFG and intravenous VRCZ as induction therapy in CPA patients who require immediate treatment. It may be ideal to use intravenous drugs as long as possible, however longer usage is not reasonable due to its high cost. Our study design was to use intravenous antifungals for several weeks (max 4 weeks) in order to see these antifungals may have power to stabilize or improve clinical status of CPA patients with active inflammatory signs.

The reason that the treatment success rates of both MCFG and VRCZ were decreased at the end of treatment compared to those of the first 2 weeks of administration is that the definition of treatment success was different. The criteria for "success" at the end of treatment were stricter than those after 2 weeks of administration by the protocol. No deterioration and improvement in at least two of the clinical, laboratory, radiological, and mycological factors were required for "success" clinical efficacy assessment at the end of therapy. Lack of improvement in two factors was still allowed if the other two factors were improved for the "success" evaluation after 2 weeks of administration. The number of deterioration cases in clinical, radiological, and

Table 2 Clinical characteristics, laboratory and serological findings of the patients in the per-protocol population.

characteristic	overall	micafungin	voriconazole	*P*
Body temperature (°C)				
Number of cases evaluated	95	48	47	
Mean	37.1	37.0	37.2	0.094
Range	36.2–39.0	36.2–38.2	35.3–39.0	
Respiration rate (/min)				
Number of cases evaluated	61	28	33	
Mean	19.7	18.8	20.5	0.181
Range	12.0–38.0	12.0–28.0	12.0–38.0	
Symptoms (%)				
Number of cases evaluated	97	50	47	
Cough	86 (88.7)	45 (90.0)	41 (87.2)	
Sputum	82 (84.5)	45 (90.0)	37 (78.3)	
Hemosputum	13 (13.4)	7 (14.0)	6 (12.8)	
Hemoptysis	0 (0)	0 (0)	0 (0)	
SpO_2 (%)				
Number of cases evaluated	92	47	45	
Mean	96.5	96.6	96.3	0.304
Range	92.0–100.0	92.0–100.0	93.0–99.0	
WBC (/mm^3)				
Number of cases evaluated	97	50	47	
Mean	7381.4	7051.8	7732.1	0.308
Range	2600–27600	2600–14500	3200–27600	
CRP (mg/dl)				
Number of cases evaluated	94	49	45	
Mean	4.8	4.3	5.4	0.277
Range	0.0–21.0	0.0–16.9	0.1–21.0	
ESR (mm/hr)				
Number of cases evaluated	59	30	29	
Mean	79.8	84.0	75.4	0.408
Range	7.0–156.9	16.0–138.0	7.0–156.9	
β-D-glucan/WAKO (pg/ml)				
Number of cases tested	47	24	23	
Positive	9 (19.1%)	7 (29.2%)	2 (8.7%)	0.137
Mean	12.5	17.7	7.1	
Range	0.6–158.3	0.6–158.3	1.0–33.8	
β-D-glucan/Fungitec G test (pg/ml)				
Number of cases tested	40	19	21	
Positive	11 (27.5%)	5 (26.3%)	6 (28.6%)	1.000
Mean	24.1	22.0	26.0	
Range	0.8–171.0	5.0–83.0	0.8–171.0	
Platelia *Aspergillus* test				
Number of cases tested	79	41	38	
Mean	0.75	0.8	0.7	0.570
Range	0.1–5.0	0.1–5.0	0.1–3.9	
Platelia *Aspergillus* test (%) cut off: 1.0				
Number of cases tested	88	46	42	
Positive	24 (27.3%)	15 (32.6%)	9 (21.4%)	0.338
Aspergillus immunodiffusion system (%)				
Number of cases tested	79	40	39	
Positive	70 (88.6%)	36 (90.0%)	34 (87.2%)	0.737

Table 3 Mycological findings of patients in the per-protocol population.

	overall (n = 84)	micafungin (n = 45)	voriconazole (n = 39)
CULTURE			
A. fumigatus	30 (35.7%)	18	12
A. niger	4 (4.8%)	0	4
A. terreus	1 (1.2%)	1	0
Aspergillus spp.	7 (8.3%)	3	4
overall *Aspergillus* spp.	42 (50.0%)	22	20
BIOPSY			
filamentous fungi	2 (2.4%)	1	1

n: number of cases in which culture tests were performed on any respiratory specimen.

mycological factors was few at 2 weeks and the end of treatment. However, 20%–30% of all cases showed deterioration in laboratory factors in both the MCFG and VRCZ groups at 2 weeks and the end of the treatment, although there were no statistical differences between the MCFG and VRCZ groups (data not shown). These data indicated that the effective rate at 2 weeks and the end of treatment of both drugs might be increased if the serological factors were not counted for evaluation. Because the clinical aspects of CPA are complicated, establishing a reasonable assessment system of the clinical effectiveness of antifungals with objective factors is also challenging. The limitation of this study is that there was no longer follow-up data after the discontinuation of the intravenous drugs. Many of the patients had received the following oral antifungals such as ITCZ capsules or VRCZ tablets after the trial. The relapse rate is important for understanding an ultimate outcome of the patients with CPA and required in the future study.

The overall effective rate of both drugs however, was only around 60% with average duration periods about 3 weeks. The response rates of evaluation factors after the first 2 weeks and at the end of treatment indicated that longer treatment will increase the response rate of all factors with almost 10% of improve rate gained except *Aspergillus* eradication. There were four cases in which a high dose of MCFG (300 mg/day) was used in this study. Three of four patients were successfully treated and there were no major adverse reactions. However, this data is not showing dose effect of MCFG, the population of patients taking a high dose of MCFG is too small to compare the dose effect of MCFG in patients with CPA.

Our results indicated apparently fewer adverse effects in the MCFG than VRCZ group, even though the average administration period was longer by 3 days. The incidence rate of adverse events was still significantly lower in MCFG group even with exclusion of visual events, which are unique to VRCZ. VRCZ is metabolized via the cytochrome P450 (CYP) 2C19 isozyme in liver[21] and the CYP2C19 mutant type is generally found in 60%–70% of Asian populations including Japanese,[22] which indicates that these poor metabolizers of VRCZ are likely to have more serious liver dysfunction. Our current data showed significantly more occurrence of liver dysfunction in the VRCZ than MCFG group. Although the MCFG group showed a lower incidence rate of adverse effects, one case with disseminated intravascular coagulation (DIC) was reported as a severe adverse effect. DIC occurred 4 days after the discontinuation of MCFG in this case and the patient recovered. Monitoring blood concentration of VRCZ, not performed in current study, might support further understanding of frequency and severity of adverse effects.

Table 4 Treatment success and response rate of clinical, laboratory findings, radiological, and mycological factors at the end of the treatment and two weeks after initial administration in the per-protocol population.

	micafungin	voriconazole	Difference in proportion (95% CI)	P
At the end of treatment (primary endpoint)				
Number treated successfully (%)	30 (60.0)	25 (53.2)	6.8% (−12.92 to 26.54)	0.543
Clinical	35 (70.0)	34 (72.3)	–	0.826
Laboratory findings	25 (50.0)	25 (53.2)	–	0.840
Radiological	25 (50.0)	21 (44.7)	–	0.685
Mycological	9 (18.0)	8 (17.0)	–	1.000
At the first two weeks (secondary endpoint)				
Number treated successfully (%)	34 (68.0)	27 (58.7)	9.3% (−9.97 to 28.58)	0.399
Clinical	29 (58.0)	27 (58.7)	–	1.000
Laboratory findings	24 (48.0)	20 (43.5)	–	0.686
Radiological	20 (40.0)	16 (34.8)	–	0.675
Mycological	8 (16.0)	4 (8.7)	–	0.361