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CLINICAL INVESTIGATION

Multicenter prospective observational study of fungal keratitis in Japan: analyses of in vitro susceptibility tests for combinations of drugs

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Abstract

Purpose To determine the effects of a combination of two antifungal drugs against causative fungi of fungal keratitis in Japan.

Study design Multicenter prospective observational study.

Methods Eighteen isolates of yeast-like fungi and 22 isolates of filamentous fungi collected by the Multicenter Prospective Observational Study of Fungal Keratitis in Japan were studied. Specially manufactured minimum inhibitory concentration (MIC) measurement plates were used to test the effectiveness of 10 combinations of two antifungal drugs against the isolates. The combinations were pimaricin (PMR) + voriconazole (VRCZ), PMR + fluconazole (FLCZ), PMR + miconazole (MCZ), PMR + micafungin (MCFG), VRCZ + FLCZ, VRCZ + MCZ, VRCZ + MCFG, VRCZ + amphotericin–B (AMPH-B), MCZ + FLCZ, and MCZ + MCFG. The checkerboard microdilution method was used, and the fractional inhibitory concentration (FIC) index was calculated based on the guidelines of The Clinical & Laboratory Standards Institute (CLSI).

Results In yeast-like fungi, additive effects were observed between PMR and MCFG in 77.8% of the isolates, and they were also observed between the azoles. Synergistic effects were observed on 11.1% of the isolates for MCZ and FLCZ. On the other hand, antagonistic effects were present between PMR and azoles with 88.9% between PMR and VRCZ, 72.2% between PMR and FLCZ, and 94.4% between PMR and MCZ. In filamentous fungi, additive effects were observed between PMR and MCFG in 40.9% of the isolates, and between VRCZ and MCZ in 40.9% of the isolates. Antagonistic effects were observed for PMR and the azoles.

Conclusions The combination of drugs prescribed for fungal keratitis incurs a possibility of synergistic, additive, indifferent, or antagonistic effects, depending on drug combinations and fungal strains.

Keywords Fungal keratitis · Drug susceptibility test · Fractional inhibitory concentration (FIC) index · Yeast-like fungi · Filamentous fungi

Introduction

New antifungal drugs are being continuously developed, and the treatment of fungal keratitis has changed accordingly. Fungal keratitis is now being treated by various combinations of antifungal drugs in Japan [1]. At the first stage of the Multicenter Prospective Observational Study of Fungal Keratitis in Japan, it was found that 83.5% of the cases were treated with two or more antifungal drugs [2]. In countries such as China [3] and India [4] with more cases of fungal keratitis than bacterial keratitis, a combination of antifungal drugs is being used, although monotherapy with natamycin (pimaricin)

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is prevalent partly because of the price and unavailability of the newly developed drugs. In these countries, the failure of medical treatment is frequent, and such cases have had to be treated surgically.

For systemic fungal infections, single-agent therapy is recommended in the guidelines of Japan [1] and the United States [5], although in some cases, combination therapy such as amphotericin B plus flucytosine has been used against cryptococcal meningitis. Because a single-agent antifungal treatment may not be effective in all cases, a combination of two or more antifungals needs be considered as an option. Combination antifungal therapy has several advantages including increased potency (synergy), broader spectrum, prevention of the emergence of resistance, and minimizing toxicity due to a reduced dosage. However, the use of combination antifungal therapy is not so common because of a lack of evidence, and the apprehension of antagonism and adverse effects.

In Japan, the combined use of antibacterial eye drops against bacterial keratitis is common, and the Guidelines for the Clinical Management of Infectious Keratitis recommend this treatment [6]. Based on the success of this combination therapy, the concept has been applied to the treatment of fungal keratitis, especially because the medical treatment of fungal keratitis is more difficult than bacterial keratitis. In combination use of antibacterial eye drops, there is supportive in vitro data for the effectiveness of combination therapy. Suzuki et al. studied combination therapy and drug susceptibility tests using the fractional inhibitory concentration (FIC) index [7]. In their study, the effect of combinations of levofloxacin (LVFX) with cefmenoxime (CMX), tobramycin (TOB), erythromycin, and chloramphenicol were investigated. Most combinations of two antibacterial drugs had additive effects, and no antagonistic effect were observed. The FIC index of LVFX and CMX against Gram-positive cocci, and that of LVFX and TOB against Gram-negative rods were the lowest, indicating these combinations are appropriate for clinical use. However, the evidence for fungal keratitis is scarce and conflicting [8–10].

The purpose of this study was to determine the in vitro susceptibility of yeast-like and filamentous fungi to 10 combinations of two antifungal drugs in the second and partially in the first stage of the Multicenter Prospective Observational Study of Fungal Keratitis in Japan. The results of the clinical data and statistical analyses are reported in a separate report [11].

Materials and methods

Patients

Patients who were examined in 15 participating institutions between April 1, 2015 and March 31, 2016 and were diagnosed with or clinically suspected of having fungal keratitis were studied. All who had signed an informed consent form to participate in this study were eligible. Patients from 11 ophthalmological institutions were enrolled. The demographics of the patients such as the clinical findings, are presented in detail in a separate report [11].

Samples and fungal strains

The samples used were collected from the patients, but samples from patients who were withdrawn by the reporting institution were excluded. Fungal strains that grew extremely poorly in culture and were difficult to test for drug susceptibility were excluded. Some strains of *Candida* spp. and *Fusarium* spp. kept refrigerated/frozen after being collected in the first stage of the Multicenter Prospective Observational Study of Fungal Keratitis in Japan conducted from November 1, 2011 to October 30, 2013 were included in the drug susceptibility tests after a re-isolation of the organism.

A total of 40 strains was used for combination effects of in vitro susceptibility test, which consisted of 19 strains out of isolated 72 strains from 133 patients at the first stage, and 21 strains out of isolated 22 strains from 22 patients at the second stage. Classification of fungi is as follows; 18 strains of yeast-like fungi; *C. parapsilosis*: 10 strains, *C. albicans*: 5 strains, *C. guilliermondii*: 2 strains, *C. tropicalis*: 1 strain, and 22 strains of filamentous fungi; *Fusarium* spp.: 15 strains, *Paecilomyces lilacinus*: 2 strains, *Scedosporium apiospermum*: 2 strains, *Aspergillus fumigatus*: 1 strain, *Beauveria bassiana*: 1 strain, *Phialemonium curvatum*: 1 strain.

Study design

This was a multicenter, prospective, observational study. Institutions and members participating in this study are listed at the end of this report.

Ethical approval

The procedures used were approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Tottori University, Tottori, Japan as a representative facility and also by the IRB in each institute except the ones that did not have their own IRB. Informed consent was obtained prior to the procedure from all participants after an explanation of the procedures to be used.

Sample collection/transportation

Corneal scrapings were collected by the attending ophthalmologist using cotton swabs or spatulas, and separate

devices were used to directly inoculate them into a potato dextrose agar (LN-F) medium (Nikken Bio Co., Ltd.). The inoculated media were stored at room temperature, and shipped in containers with airtight stoppers to the infectious microbe testing section of the Laboratory for Clinical Investigation, Osaka University Hospital (Osaka University Laboratory) with the test request forms. Corneal scrapings collected similarly were used for cultures and smears for the direct microscopic examinations at the laboratories of the respective treating institutions. When a sample positive in a fungal culture test at the treating institution tested negative at the Osaka University Laboratory, this isolated fungal strain was additionally sent to the Osaka University Laboratory for evaluation. The patient information was anonymized in a linkable fashion.

Culture

The Osaka University Laboratory cultured the samples at 25° C for 3 weeks, and the samples showing growth of any fungi were propagated by pure culturing for identification and drug susceptibility testing.

Identification

In samples showing growth of yeast-like fungi, the organism was identified biochemically with API 20C AUX (API AUX; bioMérieux). Filamentous fungi were identified morphologically under a microscope after slide culture. Yeast-like fungi not identified definitively by API AUX and all filamentous fungi were sent to the Medical Mycology Research Center, Chiba University to determine the target gene sequences and identify the species by analyzing nucleotide sequence homologies.

Drug susceptibility testing

Drug susceptibility levels were measured by the broth microdilution method in accordance with the Clinical & Laboratory Standards Institute (CLSI) standards (M27-A3 and M38-A2). The susceptibility levels to 6 drugs individually and in combinations of 2 drugs were measured. The antifungal agents tested were fluconazole (FLCZ), miconazole (MCZ), voriconazole (VRCZ), micafungin (MCFG), amphotericin B (AMPH-B), and pimaricin (PMR; natamycin). The 2-drug combinations and drug concentrations tested are shown in Table 1.

Liquid fungal inoculums were prepared according to the CLSI methods. More specifically, yeast-like fungi were adjusted to a turbidity of McFarland standard number 0.5 with sterile saline and diluted 50-fold and then 20-fold with RPMI-1640 medium (Sigma -Aldrich) to give a final inoculum concentration of $1–5 \times 10^3$ CFU/mL.

For filamentous fungi, the conidia were collected from colonies formed on potato dextrose agar slant media with sterile saline supplemented with a small amount of Tween80 (Tween80 ~ 1 drop/sterile saline ~ 25 mL) and filtered with sterile gauze once to remove large clumps and mycelia. Then, the collected conidia were diluted 50-fold with RPMI-1640 medium to adjust the final inoculum concentration to 0.8×10^4 to 1×10^5 spores/mL and measured with a counting plate.

The drug susceptibility was tested at 35° C according to the CLSI standard method. Strains that did not grow at 35° C were cultured at 25° C. The CLSI standard method does not set judgement criteria for the AMPH-B, MCZ, and PMR susceptibility of yeast-like fungi and for the susceptibility of filamentous fungi to all drugs tested. Thus, we modified the CLSI criteria to prepare our own criteria (Table 2) [2, 12] used in this study. All minimum inhibitory concentration (MIC) plates used for the drug susceptibility tests (prepared by Eiken Chemical Co., Ltd.) were specially manufactured for the measurement of combination effects of antifungal drugs and confirmed to satisfy the quality control standards using CLSI-recommended quality control strains, viz., *Candida parapsilosis* ATCC 22,019 and *Candida krusei* ATCC 6258.

Judgment in measurements of combination effects

The CLSI recommends the use of the following endpoints: a complete growth inhibition for the susceptibility of yeast-like fungi to AMPH-B and filamentous fungi to VRCZ and AMPH-B; minimal effective concentration (MEC) for the susceptibility of filamentous fungi to MCFG; and IC_{50} for the susceptibility of yeast-like and filamentous fungi to other drugs. The MEC was specially set for the candins by CLSI, and defined as the lowest

Table 1 Drug combinations and concentrations (μg/mL)

Drug combinations	Drug1(concentration)	Drug2(concentration)
MCZ + MCFG	MCZ (0.06-4)	MCFG (0.004-4)
PMR + MCFG	PMR (0.12-8)	MCFG (0.004-4)
MCFG + VRCZ	MCFG (0.03-2)	VRCZ (0.008-8)
PMR + VRCZ	PMR (0.12-8)	VRCZ (0.008-8)
AMPH-B + VRCZ	AMPH-B (0.06-4)	VRCZ (0.008-8)
MCZ + VRCZ	MCZ (0.06-4)	VRCZ (0.008-8)
PMR + MCZ	PMR (0.12-8)	MCZ (0.008-8)
FLCZ + MCZ	FLCZ (0.06-4)	MCZ (0.008-8)
PMR + FLCZ	PMR (0.12-8)	FLCZ (0.008-8)
FLCZ + VRCZ	FLCZ (0.06-4)	VRCZ (0.002-2)

PMR: Pimaricin (Natamycin), MCFG: Micafungin, FLCZ: Fluconazole, MCZ: Miconazole, VRCZ: Voriconazole, AMPH-B: Amphotericin-B

Table 2 Criteria of Drug Susceptibility (Compatible to yeast-like fungi and filamentous fungi*[1]) (μg/mL)

	Micafungin (MCFG)	Amphotericin-B(AMPH-B)*[3]	Fluconazole (FLCZ)	Voriconazole (VRCZ)	Miconazole MCZ*[3]	Pimaricin (PMR)*[6]
S(susceptible)	≦2	≦1	≦8	≦1	≦1	≦16
I(intermediate)				> 2*[5]		
R(resistant)	> 2*[2]	> 1	> 8*[4]	≧4	> 1	> 16

*1 Criteria of yeast-like fungi in CLSI guide line are applied to filamentous fungi, because no CLSI criteria of filamentous fungi exist

* 2 'Not susceptible' in CLSI guide line, which is considered to be similar to resistant in this study

* 3 No criteria in CLSI guide line. These are tentatively set in this study based on the blood concentration after intravenous administration

* 4 Because of difficulty of high drug concentration setting, > 8 is considered to be R in this study

* 5 'dose-dependent susceptible' in CLSI guide line, which is considered to be similar to intermediate in this study

* 6 No criteria in CLSI guide line. These are tentatively set based on the corneal penetration data of 5% eye drops and 1% ophthalmic ointment in epithelial defect model of mouse[12]

Table 3 Criteria for reading end point

Drug combinations	Reading end point	
	Yeast-like Fungi	Filamentous Fungi
MCZ + MCFG	IC_{50}*[2]	Sufficient reduction of growth*[1]
PMR + MCFG		
MCFG + VRCZ		
PMR + VRCZ		100% inhibition
AMPH-B + VRCZ		
MCZ + VRCZ		Sufficient reduction of growth *[1]
PMR + MCZ		
FLCZ + MCZ		
PMR + FLCZ		
FLCZ + VRCZ		

*1 Observation with a microscope (×40)

*2 50% inhibition concentration compared with drug-free growth control turbidity by turbimeter (655 nm)

concentrations of candins leading to the growth of small, rounded, and compact hyphal forms as compared to the hyphal growth seen in the growth control well. These judgements about MIC and MEC values of drugs tested individually were made according to M27-A3 and M38-A2 of the CLSI. However, judgments for the combined use of 2 drugs were made following two endpoints (Table 3): IC_{50} (50% inhibition concentration based on the absorbance at 655 nm measured with a Novapath microplate reader (Bio-Rad) or Multiskan GO (Thermo Fisher Scientific) for the susceptibility of yeast-like fungi for all combinations; complete growth inhibition points for the susceptibility of filamentous fungi to PMR + VRCZ and VRCZ + AMPH-B; and points confirmed to show sufficient reduction of growth under a microscope at × 40 for the susceptibility of filamentous fungi to other combinations. Endpoints used for determination of the combined effects were the IC_{50} when IC_{50} was the endpoint for one of the two drugs and points producing 100% growth inhibition only when complete growth inhibition was the endpoint for both drugs.

For all strains tested, the combined effects were determined at 48 h after the initiation of the culture. The CLSI recommends that the susceptibility of yeast-like fungi to MCFG be determined 24-h after beginning the culture, the drug susceptibility of filamentous fungi such as *Aspergillus* spp. and *Paecilomyces variotii* after 21–26 h of culture and the drug susceptibility of *Scedosporium* spp. after 46–72 h of culture. However, the drug susceptibility levels of all fungal strains were determined after 48-h culture because the aim of this study was to determine the effects of a combination of two drugs.

Evaluation of combination effects

The fractional inhibitory concentration (FIC) indices were calculated as follows (Fig. 1):

$$\mathrm{FIC} = \left[\text{MIC of Drug 1 in combination}/\text{MIC of Drug 1 alone}\right] + \left[\text{MIC of Drug 2 in combination}/\text{MIC of Drug 2 alone}\right].$$

This equation was used to determine if the observed effects were synergistic, additive, indifferent, or antagonistic as defined. Two drugs were synergistic when the FIC was ≤ 0.5, additive when 0.5 < FIC ≤ 1, indifferent when 1 < FIC ≤ 2, and antagonistic when FIC was > 2 [13]. The combination effect was judged to be undeterminable when the effect could not be characterized because the MIC and MEC were below the lowest concentration or above the

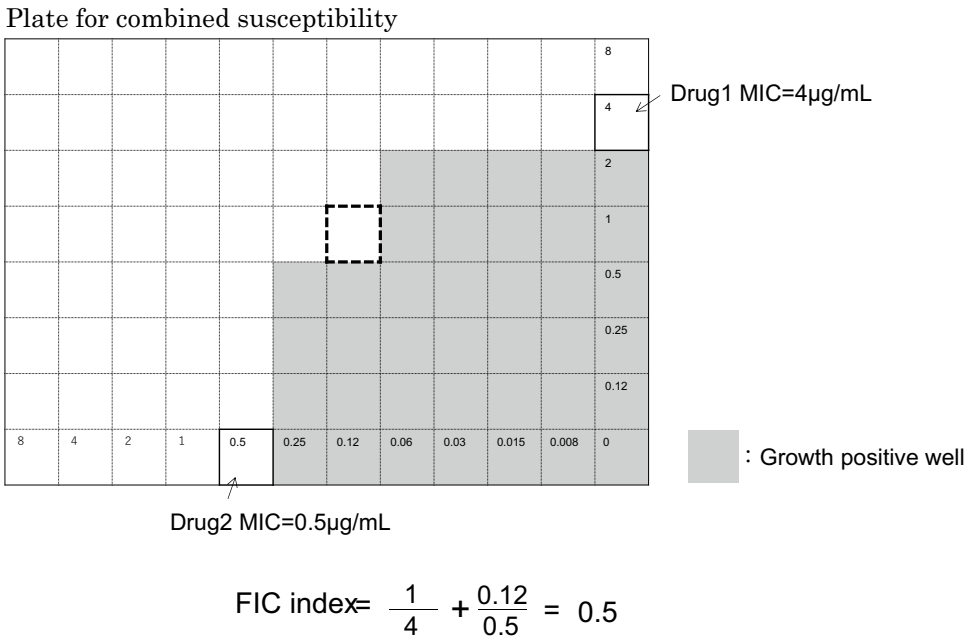

Fig. 1 Method of calculating the fractional inhibitory concentration (FIC) index. A checkerboard microtitration method is used to determine the minimum inhibitory concentration (MIC) using specially manufactured plates for the measurement of the combination effects of the antifungal drugs and the FIC. The indices were calculated as follows: FIC = [MIC of Drug 1 in combination/MIC of Drug 1 alone] + [MIC of Drug 2 in combination/MIC of Drug 2 alone]

highest concentration of the combination effect measurement plate.

Results

In vitro drug susceptibility test of single antifungal drug for isolated fungi

All 18 strains of the yeast-like fungi were susceptible to all 6 drugs (Table 4). More specifically, there was 100% susceptibility of S except 1 strain with susceptibility of R to MCZ. However, in filamentous fungi, the rates of susceptibility of S to MCFG, AMPH-B, FLCZ, VRCZ, MCZ, and PMR were 18.2%, 45.5%, 4.5%, 31.8%, 18.2%, and 100%, respectively. The rates of susceptibility of R to MCFG, AMPH-B, FLCZ, VRCZ, MCZ, and PMR were 81.8%, 54.5%, 95.5%, 50.0%, 81.8%, and 0%, respectively (Table 5). Five drugs other than PMR proved ineffective for filamentous fungi in vitro. Although VRCZ has been recommended for keratitis due to filamentous fungi including *Fusarium* spp. [1, 6], one-half of the isolates were resistant to VRCZ.

Combination effects of two antifungal drugs for yeast-like fungi using in vitro drug susceptibility tests

The percentage of synergistic, additive, indifferent, antagonistic, and undeterminable effects against yeast-like fungi are presented in Table 6. Additive effects were observed between PMR and MCFG in 77.8% of the isolates, between MCZ and MCFG in 44.4% of the isolates, and those were also observed between azoles, such as between VRCZ and

Table 4 Drug Susceptibility of Yeast-like Fungi

		MCFG	AMPH-B	FLCZ	VRCZ	MCZ	PMR
MIC_{90}(μg/mL)		1	1	8	0.12	1	8
S	No. of strain (%)	18 (100.0)	18 (100.0)	18 (100.0)	18 (100.0)	17 (94.4)	18 (100.0)
I	No. of strain (%)						
R						1 (5.6)	

S: Susceptible, I: Intermediate, R: Resistant

Eighteen strains are used as follows; *C. parapsilosis*: 10 strains, *C. albicans*: 5 strains, *C. guilliermondii*: 2 strains, *C. tropicalis*: 1 strain

Table 5 Drug Susceptibility of Filamentous Fungi

		MCFG	AMPH-B	FLCZ	VRCZ	MCZ	PMR
MIC_{90}(μg/mL)		8	4	> 8	8	> 8	> 8
S	No. of strain	4	10	1	7	4	22
	(%)	(18.2)	(45.5)	(4.5)	(31.8)	(18.2)	(100.0)
I	No. of strain				4		
	(%)				(18.2)		
R	No. of strain	18	12	21	11	18	0
	(%)	(81.8)	(54.5)	(95.5)	(50.0)	(81.8)	(0.0)

S: Susceptible, I: Intermediate, R: Resistant

Twenty-two strains are used as follows; *Fusarium* spp.: 15 strains, *Paecilomyces lilacinus*: 2 strains, *Scedosporium apiospermum*: 2 strains, *Aspergillus fumigatus*: 1 strain, *Beauveria bassiana*: 1 strain, *Phialemonium curvatum*: 1 strain

Table 6 The Rate of Combination Effects against Yeast-like Fungi (%)

Drug 1	PMR				VRCZ				MCZ	
Drug 2	VRCZ	FLCZ	MCZ	MCFG	FLCZ	MCZ	MCFG	AMPH-B	FLCZ	MCFG
Synergistic	0	0	0	0	0	0	0	0	11.1	0
Additive	5.6	16.7	0	77.8	66.7	61.1	16.7	33.3	77.7	44.4
Indifferent	5.6	11.1	5.6	22.2	27.8	5.6	38.9	22.2	11.1	33.3
Antagonistic	88.9	72.2	94.4	0	5.6	0	11.1	27.8	0	5.6
Undeterminable	0	0	0	0	0	33.3	33.3	16.7	0	16.7

18 strains are used as follows; *C. parapsilosis*: 10 strains, *C. albicans*: 5 strains, *C. guilliermondii*: 2 strains, *C. tropicalis*: 1 strain

FLCZ in 66.7% of the isolates, between VRCZ and MCZ in 61.1% of the isolates, and between MCZ and FLCZ in 77.7% of the isolates. In addition, synergistic effects were observed between MCZ and FLCZ in 11.1% of the isolates.

The curves of the cumulative growth inhibition rates of each drug in drug combinations with mainly synergistic or additive effects are shown in Fig. 2. In all combinations, the curve for each drug is shifted to the left, i.e., the lower side of the MIC indicating a good combination effect.

On the other hand, antagonistic effects were prevalent between PMR and the azoles; in 88.9% of the isolates between PMR and VRCZ, 72.2% of the isolates between PMR and FLCZ, and 94.4% of the isolates between PMR and MCZ (Table 6). The curves of the cumulative growth inhibition rates of each drug in the drug combinations with mainly antagonistic effects are shown in Fig. 3. In these 3 combinations, the curves for PMR are shifted to the right, the higher side of MIC, indicating that the antifungal effects of PMR were reduced by the azoles. However, the curves for the azoles are at the same position both with and without PMR. This indicates that the antifungal effects of azoles are not affected by PMR.

Combination effects of two antifungal drugs for filamentous fungi using in vitro drug susceptibility tests

The percentage of synergistic, additive, indifferent, antagonistic, and undeterminable effects of the combination of two antifungal drugs against filamentous fungi are presented in Table 7. Although the rate of undeterminable effects was high because of the higher MICs than in measurable concentration for a single or combination effect measurement plates, additive and antagonistic effects were observed in a pattern similar to those for the yeast-like fungi. Additive effects were observed between PMR and MCFG in 40.9% of the isolates and between VRCZ and MCZ in 40.9% of the isolates. The curve of the cumulative growth inhibition rates of each drug in these two combinations had a shift to the left, lower side of MIC, indicating a synergistic combination especially for the combination of VRCZ and MCZ (Fig. 4). On the other hand, antagonistic effects were observed between PMR and the azoles; 54.5% of the isolates between PMR and VRCZ, and 50.0% of the isolates between PMR and MCZ. In combinations of PMR and FLCZ, only 18.2% of the isolates had antagonistic effects because the rate of undeterminable effect was high at 77.3%.

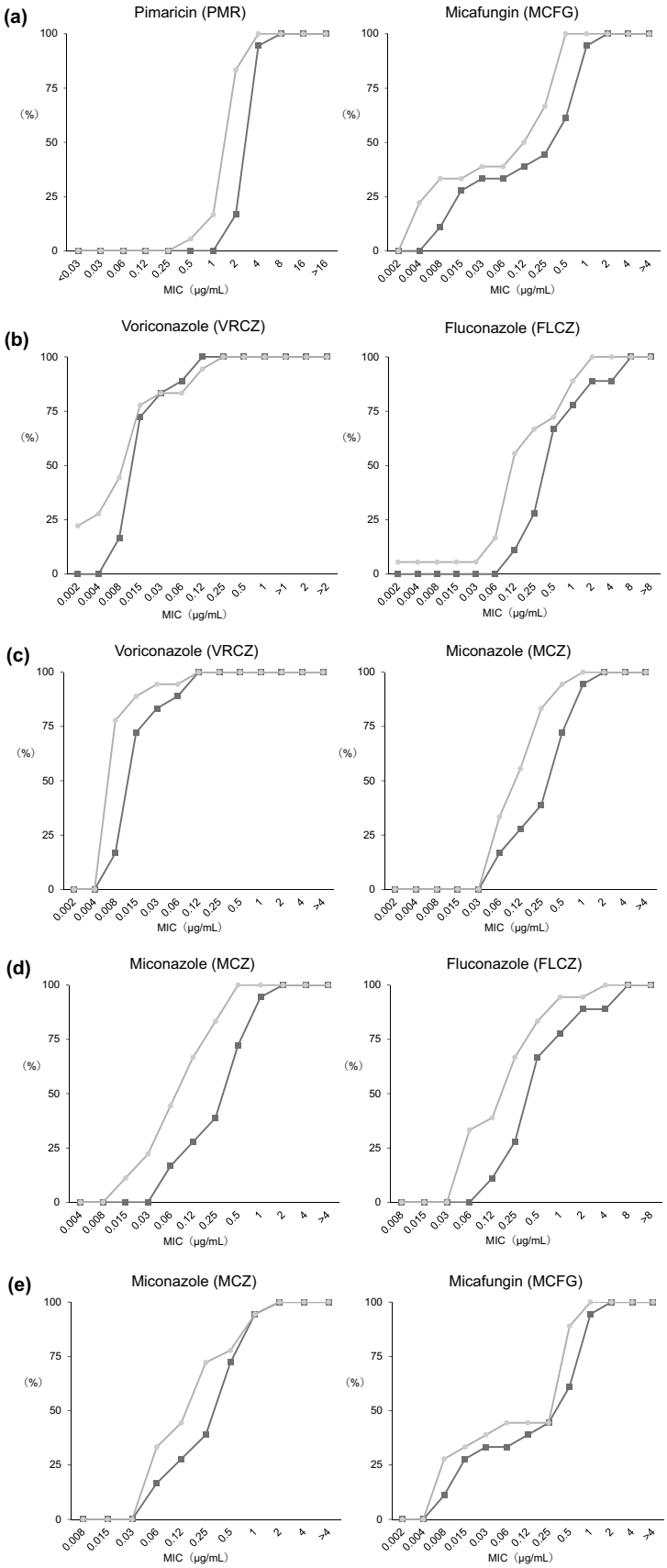

Fig. 2 Curves of the cumulative growth inhibition rates of each drug in drug combinations with mainly synergistic or additive effect against yeast-like fungi. a) pimaricin (PMR) and micafungin (MCFG). b) voriconazole (VRCZ) and fluconazole (FLCZ). c) voriconazole (VRCZ) and miconazole (MCZ). d) miconazole (MCZ) and fluconazole (FLCZ). e) miconazole (MCZ) and micafungin (MCFG). The horizontal axis is the minimum inhibitory concentration (MIC). The vertical axis is the percentage of strains with MIC less than that indicated on the horizontal axis. Black line: single drug. Gray line: combination of two drugs. Curve of each drug is shifted to the left along with combination, indicating synergistic or additive effect

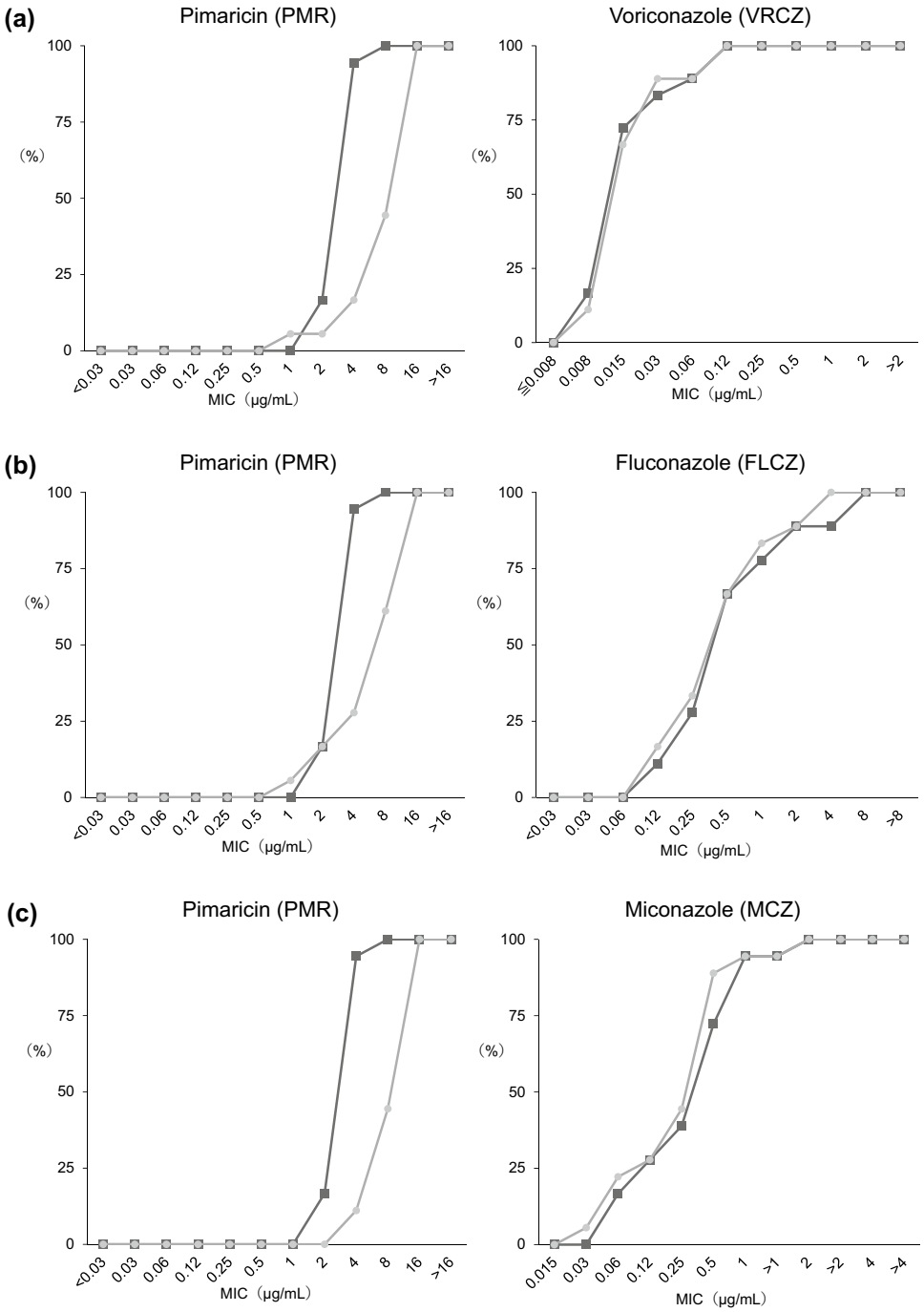

Fig. 3 Curves of the cumulative growth inhibition rates of each drug in drug combinations with mainly antagonistic effect against yeast-like fungi. a) pimaricin (PMR) and voriconazole (VRCZ). b) pimaricin (PMR) and fluconazole (FLCZ). c) pimaricin (PMR) and miconazole (MCZ). The horizontal axis is the minimum inhibitory concentration (MIC). The vertical axis is the percentage of strains with MIC less than that indicated on the horizontal axis. Black line: single drug. Gray line: combination of two drugs. Curves of pimaricin are shifted to the right along with combination with azoles, indicating that azoles interfere with antifungal effects of pimaricin. On the other hand, the curves of azoles in combination with pimaricin are not shifted, indicating that pimaricin does not interfere with antifungal effects of azoles

In the cumulative growth inhibition rates of each drug in the 3 combinations, curves of PMR were shifted to the right slightly indicating that the antifungal effects were slightly reduced by the azoles. However, the curves of the azoles were at the same position with and without PMR, which indicates that the antifungal effects of azoles were not affected by PMR (Fig. 5). These phenomena were not as clear as yeast-like fungi because the antifungal effects of single drugs against filamentous fungi are poorer than against yeast-like fungi, and the rates of undeterminable effects were high.

Discussion

Our results show that additive and synergistic effects were observed between the azoles, and antagonistic effects were observed between PMR and the azoles. These results were more distinct for the yeast-like fungi than the filamentous fungi. Although in filamentous fungi, the MIC of azoles were too high to accurately evaluate the combination effects, similar trends of combination effects to yeast-like fungi

Table 7 The Rate of Combination Effects against Filamentous Fungi (%)

Drug 1	PMR				VRCZ				MCZ	
Drug 2	VRCZ	FLCZ	MCZ	MCFG	FLCZ	MCZ	MCFG	AMPH-B	FLCZ	MCFG
Synergistic	0	0	0	0	0	4.5	0	0	0	4.5
Additive or synergistic	4.5	0	4.5	4.5	0	4.5	0	0	0	9.1
Additive	0	4.5	9.1	40.9	0	40.9	13.6	9.1	4.5	4.5
Indifferent	40.9	0	0	9.1	0	4.5	9.1	45.5	4.5	13.6
Antagonistic	54.5	18.2	50.0	4.5	18.2	0	9.1	45.5	4.5	0
Undeterminable	0	77.3	36.4	40.9	81.8	45.5	68.2	0	86.4	68.2

Twenty-two strains are used as follows; *Fusarium* spp.: 15 strains, *Paecilomyces lilacinus*: 2 strains, *Scedosporium apiospermum*: 2 strains, *Aspergillus fumigatus*: 1 strain, *Beauveria bassiana*: 1 strain, *Phialemonium curvatum*: 1 strain

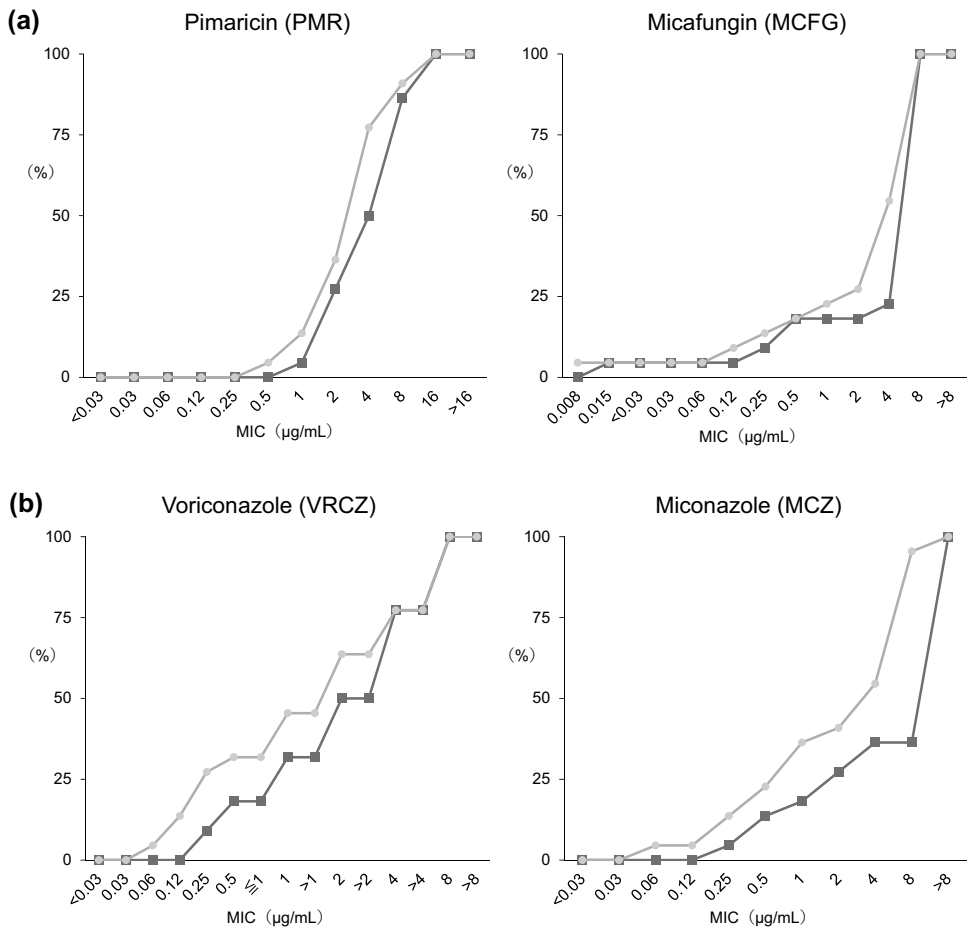

Fig. 4 Curves of the cumulative growth inhibition rates of each drug in drug combinations with mainly synergistic or additive effect against filamentous fungi. a) pimaricin (PMR) and micafungin (MCFG). b) voriconazole (VRCZ) and miconazole (MCZ). The horizontal axis is the minimum inhibitory concentration (MIC). The vertical axis is the percentage of strains with MIC less than that indicated on the horizontal axis. Black line: single drug. Gray line: combination of two drugs. Curve of each drug is shifted to the left along with combination, indicating synergistic or additive effect

were observed. However, we must carefully consider that the results are species-dependent, and the current data of filamentous fungi were influenced by the high percentage of *Fusarium* spp. (68.2%).

Although studies investigating the effects of in vitro combinations of antifungals against clinical isolates from fungal keratitis are few [8–10], numerous i*n vitro* studies are reported using clinical isolates from other types of fungal infections [14–32].

Other studies report synergism between the azoles. Mikami et al. report synergistic effects against *Candida albicans*, but not against *Candida krusei*, or the combination of MCZ and FLCZ [14]. Spader et al. report synergistic effects against *Fusarium* spp. for a combination of VRCZ and itraconazole (ICZ) (50%), VRCZ and FLCZ (50%), VRCZ and MCZ (50%), and VRCZ and ketoconazole (KCZ) (50%) [15].

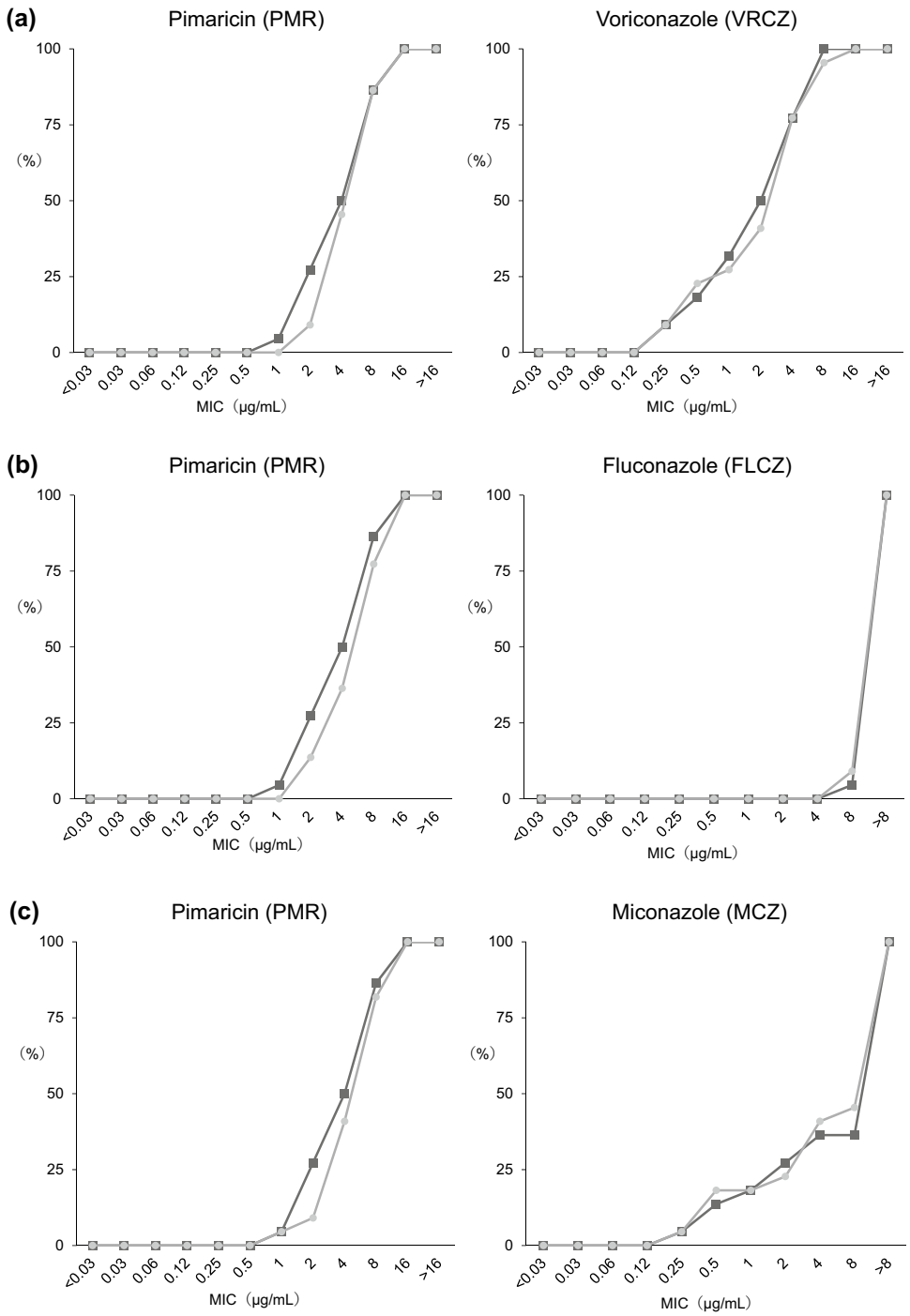

Fig. 5 Curves of the cumulative growth inhibition rates of each drug in drug combinations with mainly antagonistic effect against filamentous fungi. a) pimaricin (PMR) and voriconazole (VRCZ). b) pimaricin (PMR) and fluconazole (FLCZ). c) pimaricin (PMR) and miconazole (MCZ). The horizontal axis is the minimum inhibitory concentration (MIC). The vertical axis is the percentage of strains with MIC less than that indicated on the horizontal axis. Black line: single drug, Gray line: combination of two drugs. Curves of pimaricin are shifted slightly to the right along with combination with azoles, indicating that azoles interfere the antifungal effects of pimaricin. On the other hand, the curves of azoles in combination with pimaricin are not shifted, indicating that pimaricin does not interfere with antifungal effects of azoles

In the combination effects of polyenes and azoles against yeast-like fungi, most in vitro studies report antagonism between AMPH-B and azoles [16–20], with a few exceptions [21]. These results are consistent with those of the antagonism between PMR and azoles in the present study. Moreover, prior exposure to an azole resulted in the reduction of AMPH-B antifungal activity in vitro [22–24]. An in vivo study also reveals this antagonism between AMPH-B and azoles. In a rabbit model of endocarditis and pyelonephritis, pre-exposure to FLCZ before AMPH-B treatment was slower in clearing fungi from the tissues than AMPH-B alone [23].

On the other hand, in the combination of polyenes and azoles against filamentous fungi, there was no consensus in the results of different in vitro studies. The results of 3 studies investigating the combination effects of PMR and azoles against *Fusarium* isolates from keratomycosis are conflicting. Li et al. [8] report an antagonism in PMR

and ICZ in 52.6% of the isolates and PMR and FLCZ in 60.5% of the isolates supporting our data. On the other hand, Al-Hatmi et al. [9] report no antagonism observed for any combination of PMR and VRCZ, PMR and ICZ, or PMR and MCFG, and the combination of PMR and VRCZ was synergistic for 70% of the *Fusarium* strains. In their report, the MIC endpoints were different from ours and Li et al. which probably affected the results of the degree of combination effects [25]. Also, the *Fusarium* spp. used in their study was different from ours and that of Li et al. Generally, combination effects are very dependent on the fungal strains, therefore this difference of causative species of *Fusarium* can also explain the differences in the results. Recently, Sradhanjali et al. [10] reported that there was no antagonism in any combination of PMR and VRCZ against various filamentous fungi nor in yeast-like fungi; synergism was observed in 23.1% of the *Fusarium* spp. including *Fusarium solani* and 33.3% of the Candida spp. Although their definition of antagonism was set at FIC > 4, different from ours, it is difficult to explain the reason for the discrepancies between their results and ours by this one difference.

In the combination effects of AMPH-B and azoles against filamentous fungi, mainly *Aspergillus* spp., antagonism is reported in some studies [25, 26] and synergism in others [8, 27, 28]. Meletiadis [29] report that the concentration of AMPH-B affected its interaction with ICZ, and synergy was found for combinations with low concentrations of AMPH-B, and antagonism was found for combinations with high concentrations of AMPH-B, similar to our findings. Animal studies report antagonism between AMPH-B and azoles in models of aspergillosis. [33–35].

Theoretically, azoles inhibit ergosterol synthesis, and this action depletes the ergosterol, the binding site of polyenes in cytoplasmic membrane of fungi. Therefore, polyenes cannot act properly under azoles. Maesaki et al. [30] claim that the pretreatment of azoles (FLCZ, ICZ, KCZ) other than MCZ followed by AMPH-B resulted in antagonistic effect against *Aspergillus fumigatus*, and the pretreatment of AMPH-B followed by azoles resulted in synergistic effects or indifference, using evaluation of wet weight change of mycelial cells. In our study, the antifungal effects of PMR were reduced by azoles for filamentous fungi and yeast-like fungi, in accords with other studies and theories. Also, in the neuropenic murine model of *Aspergillus* lethal infection, pretreatment by KCZ completely abolished the protective effect of AMPH-B [36].

It is difficult to prove the benefit of combination treatment of polyenes and azoles in clinical studies. In a clinical retrospective study of invasive aspergillosis with hematologic malignancy, the effects of both liposomal AMPH-B treatment and liposomal AMPH-B with ICZ treatment were poor with no difference [37]. Although the VRCZs are effective against keratomycosis caused by filamentous fungi including *Fusarium* spp., no beneficial effects were observed in combination of 5% PMR eye drops and oral VRCZ compared with 5% PMR alone in a large scale multicenter, double-masked, placebo-controlled, randomized clinical study [38]. It is also reported that concurrent use of 5% pimaricin and 2% econazole did not appear to offer additional benefits over historical control with monotherapy of 5% PMR for the management of fungal keratitis [39].

In the combination effects of the candins and azoles, Nishi et al. [31] report synergistic effects of MCFG and FLCZ in 11% of the isolates, and MCFG and VRCZ in 15% of the isolates, respectively, and the latter had synergistic effects against *Candida glabrata* in 63% of the isolates. The review of Vazquez et al. [32] concludes that candins and azoles against *Aspergillus* species were mostly synergistic or additive. In our study, additive effects were also observed between MCFG and MCZ in 44.4% of the yeast-like fungi.

In the combination effects of candins and polyenes, Nishi et al. [31] report on the combination effects of MCFG and AMPH, indifferent for 85% of isolates with no antagonism. However, a reduction of the MIC was partly observed with the combination. The review by Vazquez et al. [32] summarizes the effects of combinations of candins and polyenes against *Aspergillus* species. They report mostly synergistic or additive effects. In our study, additive effects were observed between MCFG and PMR in 77.8% of the yeast-like fungi and 40.9% of filamentous fungi.

Statistical analyses were performed to determine the association between the use of 10 combinations of antifungal drugs and the clinical outcomes, such as the healed date (date antifungal eye drops were reduced to three times totally) and best-corrected visual acuity at 3 months after the first visit. However, neither significant nor useful results were obtained (data not shown). Even in monotherapy, the association between in vitro drug susceptibility and clinical outcomes are complicated and difficult to prove [40]. Despite the lack of complete agreement of the in vitro findings with those of earlier studies, and no proof of clinical outcomes, the possibility of antagonism and synergism should not be ignored. In cases of yeast-like fungi, combinations of azoles are good choices for treatment because most azoles are effective against yeast-like fungi (Table 4). Moreover, synergism can be expected from most in vitro studies including ours, and adverse effects of PMR can be avoided. The most popular combination in Japan, especially for keratomycosis due to filamentous fungi, is PMR and VRCZ, recommended in the Guidelines for Management of Deep-seated Mycoses [1]. We reconsidered the use of this combination based on the results of our study and others. In monotherapy, PMR is superior to VRCZ, as proved in a prospective, double-masked, randomized, controlled, clinical trial [41]. There is a possibility that preexposure of azoles weakened the

effects of PMR; this possibility is supported by the results of our study and others, although it depended on the fungal strains and methods of measurement and evaluation. Thus, when keratomycosis due to filamentous fungi is suspected, especially in superficial corneal layer cases, monotherapy by PMR is recommended to avoid the antagonism of VRCZ. In cases with spreading into the deeper corneal layers; however, the effect of PMR is unreliable because of poor penetration of PMR [42]. In such cases, the additional effect of VRCZ in deep layers might be expected because of its deep penetration [43, 44]. MCFG has synergistic effects with PMR in vitro, which is supported theoretically that candins inhibit the synthesis of 1,3-β-D-glucan in cell walls which is very different from mechanism of action of polyenes. Despite this synergistic combination effect in vitro, cooperative combination effects are not expected in vivo, because the systemic distribution of MCFG to the avascular parts of the eye tissues is poor [45, 46]. In addition, the penetration of MCFG eye drops into the cornea is probably not sufficient because of its high molecular weight.

Although our study has several limitations, including limited number of clinical isolates, discrepancies of the results between ours and other in vitro studies, absence of correlation with the clinical prognosis, and differences between in vitro and clinical situations, the current results should be considered in the management of fungal keratitis both in Japan and in other countries.

In conclusion, our in vitro combination study of antifungal agents has shown that azoles act synergistically against some clinical isolates of fungal keratitis. However, PMR and azoles often produce antagonistic effects against certain clinical isolates of fungal keratitis. In Japan, a combination of antifungal drugs is generally used for the treatment of fungal keratitis, however the combination of drugs must be carefully considered because of these in vitro variable combination effects.

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