Original Article

Effects of Culture Conditions (Microplate and Solvent) on \textit{in vitro} Antifungal Activity of Caspofungin Against \textit{Candida} Species

Yuhei Higashitsuji\textsuperscript{1}, Rie Ueno\textsuperscript{1}, Masami Ogawa\textsuperscript{2}, and Daniel Ruzicka\textsuperscript{1}

\textsuperscript{1}Medical Affairs, MSD K.K.
\textsuperscript{2}Japan Development, MSD K.K.

ABSTRACT

Effects of the type of microplates and solvent for preparation of caspofungin (CPFG) on antifungal susceptibility testing of CPFG against clinical isolates of \textit{Candida albicans}, \textit{Candida glabrata}, and \textit{Candida krusei} (20 strains each) by broth microdilution method according to the Clinical and Laboratory Standards Institute were evaluated. When CPFG was dissolved in water, MICs against the three \textit{Candida} species decreased 3.1-6.0-fold in surface-untreated microplates compared to those in treated microplates. When CPFG was dissolved in dimethyl sulfoxide, MICs against the three \textit{Candida} species decreased 1.3-2.5-fold in surface-untreated microplates compared to those in treated microplates. Differences in MICs according to the type of solvent did not exceed the difference for one dilution interval (0.5-2-fold MIC ratio) regardless of whether the microplate surface was treated or not. These findings suggest that differences in CPFG MICs may depend mainly on the type of surface treatment of assay microplates.

Key words: Antifungal susceptibility, \textit{Candida}, Caspofungin, Dimethyl sulfoxide, Microplate

I. Introduction

Echinocandin antifungal agents are a highly effective treatment option for candidiasis (candidemia, disseminated candidiasis, etc.) when disseminated fungal infection is suspected in association with febrile neutropenia and azole resistant infection\textsuperscript{1}. Reports of the increasing resistance of \textit{Candida glabrata} to echinocandin globally indicate that correct antifungal susceptibility testing of \textit{Candida} species against echinocandins is crucial for the success of treatment, and that antifungal stewardship is needed to reduce the resistance selection pressure in clinical settings\textsuperscript{2,3}.

In our analysis of an interim report of a post-marketing survey for caspofungin (CPFG) susceptibility testing against \textit{Candida} isolates in Japan, we found no resistant strain in a total of 510 strains of 6 \textit{Candida} species isolated between 2012 and 2014, based on clinical breakpoints (CBPs) according to the Clinical and Laboratory Standards Institute (CLSI) M27-S3\textsuperscript{4,5}. Based on the analysis of CBPs according to CLSI M60 1st ed.\textsuperscript{6} (M27-S4\textsuperscript{7}), however, many strains of \textit{C. glabrata} and \textit{Candida krusei} were found to be resistant to CPFG\textsuperscript{5}. Similar results were obtained with \textit{Candida} species isolated between 2015 and 2016\textsuperscript{5}.

Espinel-Ingroff et al.\textsuperscript{8} and Arendrup et al.\textsuperscript{9} pointed out that there was a significant variation in CPFG MICs between institutions and that this may result in errors in susceptibility testing based on CBPs revised in CLSI M27-S4\textsuperscript{7}. Therefore, the CLSI standard method in M60 1st ed.\textsuperscript{6} recommends that susceptibility testing to other echinocandins or genetic screening in resistant mutations should be conducted if a strain is found to be resistant or have intermediate resistance to CPFG because resistance to echinocandins is mainly induced by mutation of \textit{FKS} genes, and cross-resistance has been reported in the echinocandin antifungal agent class.

Recently, Fothergill et al. showed that surface-treated microplates were associated with higher CPFG MICs than non-surface-treated microplates in susceptibility against \textit{Candida} species\textsuperscript{10}. Different range and variability of CPFG MICs were also reported between the solvents for stock solution\textsuperscript{11}.

Taking all these findings into account, we evaluated the effects of surface treatment of microplates and the solvent for preparation of CPFG solution using \textit{Candida albicans}, \textit{C.}}
glabrata, and C. krusei collected in the post-marketing survey for CPFG to investigate these two factors responsible for differences in CPFG MICs determined by the CLSI broth microdilution method.

II. Materials and methods

1. Fungal strains

Twenty strains each of C. albicans, C. glabrata, and C. krusei isolated from specimens collected from patients suspected to have fungal infections at medical institutions in Japan (mainly Kanto District) between January 2012 and December 2016 were used. Strains were isolated generally from blood and respiratory samples, including sputum. In accordance with CLSI M60 1st ed. (M27-S3) CBPs, all strains were found to be susceptible to micafungin.

All the strains were collected in compliance with the ethical guidelines for epidemiological research of the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare. Privacy of patients was strictly protected in data correction.

2. Drug

CPFG (MSD K.K. Tokyo, Japan; Lot No.: 003M041) was used.

3. Susceptibility testing

The broth microdilution assay was performed according to CLSI M27-A3. Either purified water or dimethyl sulfoxide (DMSO: Kanto Chemicals Co., Inc.) was used as the solvent for the preparation of stock CPFG solution. When CPFG was dissolved in purified water, CPFG solution was diluted with the medium before the assay. When DMSO was used, CPFG solution was diluted with DMSO so that the final concentration of DMSO in all the assay media was 1%. CPFG MICs assayed in surface-treated (treated; Code No. 3870-096) and untreated (untreated; Code No. 3875-096) U-shaped bottom microplates manufactured by IWAKI (AGC TECHNO GLASS Co., Ltd.) were compared.

Candida parapsilosis ATCC 22019 and C. krusei ATCC 6258 were assayed as quality control strains. CPFG MICs obtained in the assays with these strains were found to be within the permitted range.

4. Data analysis

MICs, MICgeometric mean MIC were calculated from the susceptibility of each strain. Ratios of MICs obtained with the same strain under each assay condition and their arithmetic means were calculated.

Susceptibility of each strain to CPFG was evaluated based on the CLSI M60 1st ed. (M27-S4) CBPs. CBPs of C. albicans and C. krusei to CPFG in CLSI M60 1st ed. were classified as susceptible (S) at MIC of 0.25 µg/mL or lower, intermediate (I) at 0.5 µg/mL, and resistant (R) at 1 µg/mL or higher, while CPFG of C. glabrata were classified as S at 0.12 µg/mL or lower, I at 0.25 µg/mL, and R at 0.5 µg/mL or higher. CBPs of Candida species to CPFG in CLSI M27-S3 were classified as susceptible at 2 µg/mL or lower, and nonsusceptible at more than 2 µg/mL.

III. Results

1. Effect on susceptibility of various Candida species to CPFG

Results of effect on CPFG MICs of the microplate surface treatment (treated or untreated) and solvent used to dissolve CPFG (water or DMSO) are shown in Table 1.

1) Treated or untreated microplates

When CPFG was dissolved in water, CPFG MICs for C. albicans, C. glabrata, and C. krusei in untreated microplates were 3.1-, 6.0-, and 3.7-fold lower than the respective MICs in treated microplates. The effect of surface treatment on CPFG MICs assay was greatest for C. glabrata. When CPFG was dissolved in DMSO, CPFG MICs for C. albicans, C. glabrata, and C. krusei in untreated microplates were 1.3-, 1.7-, and 2.5-fold lower than the respective MICs in treated microplates. The effect of surface treatment on CPFG MICs assay was greatest for C. krusei. The difference in CPFG MICs between treated and untreated microplates was less when dissolving in DMSO than in water.

2) Solvent

CPFG MICs against C. albicans, C. glabrata, and C. krusei in treated microplates were 1.6-, 2.0-, and 1.6-fold lower when CPFG was dissolved in water than when it was dissolved in DMSO. CPFG MICs against these Candida species in untreated microplates were 0.63-, 0.6-, and 1.0-fold lower when CPFG was dissolved in water than when it was dissolved in DMSO. Effects of both DMSO and water against each species did not exceed the difference for one dilution interval (0.5-2 fold MIC ratio).

2. Susceptibility evaluation based on CBPs

Based on CLSI M27-S3 CBPs, all strains tested in this study were susceptible to CPFG. Results of susceptibility to CPFG based on CLSI M60 1st ed. (M27-S4) are shown in Table 1. Ten strains (50%) of C. albicans were classified as S when CPFG was dissolved in water and treated microplates were used. Under the other assay conditions wherein CPFG was dissolved in water and untreated microplates were used, and CPFG was dissolved in DMSO and both treated and untreated microplates were used, however, all C. albicans strains were classified as susceptible to CPFG. In case of C.
glabrata and C. krusei, all strains were either I or R regardless of the type of solvent when treated microplates were used. No strain was classified as S. However, all strains were classified as either S or I when surface-untreated microplates were used, with no strain classified as R.

### IV. Discussion

There are several issues in determination of CPFG MICs by broth microdilution standard method due to the fact of having significant differences among assay laboratories. It may therefore be difficult to evaluate susceptibility based on the current CBPs. In addition, these issues may affect revisions of the future CBPs based on accurate and stable susceptibility surveillance data. No clear single factor—such as CPFG lot, stock solution solvent, CPFG powder storage method, medium, or evaluation method—was found to influence CPFG MICs, while there were some reports of the effects of the surface treatment of microplates and the solvent for preparation of drug substance. The present study focused on the surface treatment of the microplates (treated or untreated) and the solvent used to dissolve the drug (water or DMSO) as factors that may affect the susceptibility of three Candida species to CPFG.

Microplates used in susceptibility testing have generally been developed to culture animal cells in vitro. They are as transparent as glass and composed of polystyrene resins with easy processing characteristics. Because they have little affinity to animal cells without surface treatment due to their surface hydrophobicity, microplates should be hydrophilized by plasma treatment, corona discharge treatment, etc. to adhere to culture cells. In our study, culture yeast cells were macroscopically observed on the bottom of surface-treated microplates with U-shaped wells with all the three Candida species, while they were observed as a uniform cells suspension in each well in untreated microplates. Agitating the microplates did not cause any apparent difference in turbidity (cell growth) between the two types of microplates (data not shown). These findings suggest that all three Candida species may grow as uniformly suspended (planktonic) cells without sedimentation in untreated microplates, while they may grow as cells attached to the bottom of wells in treated microplates.

In the present study, MICs of CPFG dissolved in water were lower with a 3-6-fold difference in untreated microplates compared to those in treated microplates. It was reported that culturing Candida strains for 24 hours in treated microplates at high cell concentrations resulted in the formation of biofilms at the bottom of the microplate well and a decrease in activities of antifungal agents, which might partially explain the results in this study. Further studies are needed to determine whether biofilm formation of yeast cells contributes to differences in CPFG MICs between treated and untreated microplates. As in the present study, the study conducted by Fothergill et al. showed that CPFG MICs for echinocandin-susceptible strains without mutation of FKS genes were lower in untreated microplates compared to those in treated microplates. The CLSI standard method revised in 2017 (CLSI M27 4th ed.) recommends the use of untreated microplates in susceptibility testing.

### Table 1. Effect of solvent and microplate for caspofungin MIC determined by CLSI standard method

| Organism | Test method | No. of isolates and MICs (µg/mL) | MIC (µg/mL) | Category (%) |
|----------|-------------|---------------------------------|-------------|--------------|
|          |             | 0.06 0.12 0.25 0.5 1 2          |             | S I R |
| *Candida albicans* | D.W. treated | 3 7 10 | 0.32 | 3.1 1.6 100 50 50 0 |
|           | D.W. untreated | 4 16 | 0.11 | 1.6 1.0 100 0 0 0 |
|           | DMSO treated | 1 2 17 | 0.22 | 1.3 0.63 100 0 0 0 |
|           | DMSO untreated | 2 5 13 | 0.18 | 1.0 0.63 100 0 0 0 |
| *Candida glabrata* | D.W. treated | 7 13 | 0.78 | 6.0 2.0 0 0 0 100 |
|            | D.W. untreated | 17 3 | 0.14 | 2.0 1.0 85 15 0 0 |
|            | DMSO treated | 6 14 | 0.41 | 1.7 0.6 0 30 70 0 |
|            | DMSO untreated | 20 | 0.25 | 0.6 0 100 0 0 0 |
| *Candida krusei* | D.W. treated | 2 16 2 | 1.0 | 3.7 1.6 0 10 90 0 |
|             | D.W. untreated | 1 15 4 | 0.28 | 3.7 1.0 80 20 0 0 |
|             | DMSO treated | 12 8 | 0.66 | 2.5 1.0 0 60 40 0 |
|             | DMSO untreated | 17 3 | 0.28 | 2.5 1.0 85 15 0 0 |

D.W.: distilled water, DMSO: dimethyl sulfoxide
1): Twenty strains isolated in Japan from 2012 to 2016 were tested.
2): MIC ratio was calculated based on the MIC for the same isolate.
3): Frequency of susceptible (S), intermediate (I), and resistant (R) strains were calculated according to CLSI M60 1st ed. (M27-S4).
polystyrene microplates based on these findings. However, treated microdilution plates are used in the EUCAST antifungal MIC method for yeasts (EUCAST E DEF 7.3.1)\textsuperscript{20}.

CPFG is readily soluble in water because it has a cyclic hexapeptide structure with amphiphilic acyl side chains\textsuperscript{21}. However, the solvent of echinocandin stock solution in CLSI M27-S4 was changed from water to DMSO\textsuperscript{17}. When CPFG was dissolved in DMSO, CPFG MICs for various Candida species were decreased and became less variable\textsuperscript{17}. This is thought to be attributable to the fact that the electric permittivity of DMSO is lower than that of water, and it results in better solubility of echinocandin antifungal agents, better dispersion in the medium, and better distribution to target enzymes in the cell\textsuperscript{17}. Using the EUCAST standard method, Alastruey-Izquierdo et al. reported that DMSO as a solvent for CPFG stock solution produced lower MICs than when water alone was used\textsuperscript{17}. In the present study, we evaluated the effects of water and DMSO on CPFG MICs and found that differences in CPFG MICs did not deviate from possible errors due to manual reading (within one dilution interval or 0.5-2-fold MIC ratio) regardless of the assay microplates. Unlike previous reports, therefore, our findings showed that the effects of solvent on CPFG’s MICs are limited.

In conclusion, our findings suggest that differences of CPFG MICs for Candida may greatly depend on the treatment of assay microplates. An assay method that gives reliable results with no inter-laboratory variability needs to be developed to adequately evaluate CPFG susceptibility based on CBPs.

Conflict of interest

Yuhei Higashitsuji, Rie Ueno, Masami Ogawa, and Daniel Ruzicka are employees of MSD K.K.

References

1) Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, Reboli AC, Schuster MG, Vazquez JA, Walsh TJ, Zaoutis TE, Sobel JD: Clinical practice guideline for the management of Candidiasis: 2016 update by Infectious Diseases Society of America. Clin Infect Dis 62: 409-417, 2016.
2) Wiederhold NP: Antifungal resistance: current trends and future strategies to combat. Infect Drug Resist 10: 249-259, 2017.
3) Arendrup MC, Perlin DS: Echinocandin resistance: an emerging clinical problem? Curr Opin Infect Dis 27: 484-492, 2014.
4) Clinical and Laboratory Standards Institute: Reference method for broth dilution antifungal susceptibility testing of yeasts; Informational supplement, 3rd ed, CLSI document M27-S3, CLSI, Wayne, PA, 2008.
5) Amano H, Kurokawa T, Miyasaka T, Kondo T, Kawai A, Hara M: Survey of susceptibility of Candida and Aspergillus species to caspofungin in Japan. Jpn J Chemother 65: 17-26, 2017. [in Japanese]
6) Clinical and Laboratory Standards Institute: Performance standards for antifungal susceptibility testing of yeasts; 1st ed, CLSI supplement M60, CLSI, Wayne, PA, 2017.
7) Clinical and Laboratory Standards Institute: Reference method for broth dilution antifungal susceptibility testing of yeasts; Informational supplement, 4th ed, CLSI document M27-S4, CLSI, Wayne, PA, 2012.
8) Ueno R, Ikeda H, Ogawa M, Higashitsuji Y, Hattori J, Yoshinari T, Maekawa S: In vitro susceptibility study of clinical isolates of Candida and Aspergillus species to caspofungin (Additional Report): a post-marketing surveillance report. Jpn J Chemother 67: 651-659, 2019. [in Japanese]
9) Espinel-Ingroff A, Arendrup MC, Pfaller MA, et al: Inter-laboratory variability of caspofungin MICs for Candida spp. using CLSI and EUCAST methods: Should the clinical laboratory be testing this agent? Antimicrob Agents Chemother 57: 5836-5842, 2013.
10) Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope WW: Breakpoints for antifungal agents: an update from EUCAST focusing on echinocandins against Candida spp. and triazoles against Aspergillus spp. Drug Resist Updat 16: 81-95, 2013.
11) Fothergill AW, McCarthy DI, Albataineh MT, Sanders C, McElmeel M, Wiederhold NP: Effects of treated versus untreated polystyrene on caspofungin in vitro activity against Candida species. J Clin Microbiol 54: 734-738, 2016.
12) Alastruey-Izquierdo A, Gómez-López A, Arendrup MC, Lass-Flörl C, Hope WW, Perlin DS, Rodriguez-Tudela JL, Cuenca-Estrella M: Comparison of dimethyl sulfoxide and water as solvents for echinocandin susceptibility testing by the EUCAST methodology. J Clin Microbiol 50: 2509-2512, 2012.
13) The Japanese Society of Clinical Microbiology: Practical enforcement for ‘Ethical Guidelines for Epidemiological Research’. J Jpn Soc Clin Microbiol 12: 255, 2002. [in Japanese]
14) Clinical and Laboratory Standards Institute: Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved standard, 3rd ed, CLSI document M27-A3, CLSI, Wayne, PA, 2008.
15) Kawamura K: Cell culture surface. Membrane 30: 171-173, 2005. [in Japanese]
16) Pierce CG, Uppuluri P, Tristan AR, Worman FL Jr, Mowat E, Ramage G, Lopez-Ribot JL: A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat Protoc 3: 1494-1500, 2008.
17) Walraven CJ, Bernardo SM, Wiederhold NP, Lee SA: Paradoxical antifungal activity and structural observations in biofilms formed by echinocandin-resistant Candida albicans clinical isolates. Med Mycol 52: 131-139, 2014.
18) Chavez-Dozal AA, Jahng M, Rane HS, Asare K, Kulkarny VV, Bernardo SM, Lee SA: In vitro analysis of flufenamic acid activity against Candida albicans biofilms. Int J Antimicrob Agents 43: 86-91, 2014.
19) Clinical and Laboratory Standards Institute: Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved standard, 4th ed, CLSI document M27, CLSI, Wayne, PA, 2017.
20) The European Committee on Antimicrobial Susceptibility Testing (EUCAST): Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts. In EUCAST Definitive document E.DEF 7.3.1, European Society of Clinical Microbiology and Infectious Diseases, Basel, 2017.

21) Balkovec JM, Hughes DL, Masurekar PS, Sable CA, Schwartz RE, Singh SB. Discovery and development of first in class antifungal caspofungin (CANCIDAS\textsuperscript{®})—A case study. Nat Prod Rep 31: 15-34, 2014.