Short Report

Antifungal Susceptibility of Clinical Isolates and Artificially Produced Multi-azole-resistant Strains of Cryptococcus neoformans (formerly: Cryptococcus grubii) to Ravuconazole

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ABSTRACT

Ravuconazole (RVCZ) is a newly available human azole drug in Japan since 2018 and is a broad-spectrum antifungal agent that exhibits excellent activity against Candida albicans and Cryptococcus neoformans (formerly: Cryptococcus grubii). The drug is also highly active against isolates that are resistant to fluconazole (FLCZ). In the present study, the in vitro susceptibility to ravuconazole (RVCZ) of Japanese clinical isolates and multi-azole-resistant strains of C. neoformans was investigated using the Clinical & Laboratory Standards Institute (CLSI) M27-A3 test. The minimum inhibitory concentrations for the 14 clinical isolates and the multi-azole-resistant strains were 0.003125-0.125 mg/L and 0.25-0.5 mg/L for RVCZ, respectively. RVCZ is as effective as ITCZ and VRCZ for treating clinical isolates from cats and humans. Moreover, RVCZ is highly effective against multi-azole-resistant strains that encode a protein with a G344S substitution in ERG11. Consequently, RVCZ has considerable potential for use as a therapeutic agent for multi-azole resistant cryptococcosis.

Key words: antifungal susceptibility, azole, Cryptococcus neoformans, multi-azole resistance, ravuconazole

Introduction

Cryptococcosis is a common cause of systemic mycoses in cats and of zoonotic fungal infections1, 2). Feline cryptococcosis in Japan is typically attributed to Cryptococcus neoformans (former name; Cryptococcus grubii)3-6). Feline cryptococcosis is generally treated with azoles, such as fluconazole (FLCZ) and itraconazole (ITCZ), which are effective against human and animal cryptococcoses and have few side effects1, 2).

FLCZ resistance, however, has been reported in isolates from human cryptococcoses7-9). Moreover, we have also described the first case of feline cryptococcosis in which an FLCZ-resistant C. neoformans strain was isolated from a cat6). Subsequent work demonstrated that multi-azole-resistant strains can readily be isolated from FLCZ-resistant strains by culturing in medium containing voriconazole (VRCZ)10). The ERG11 genes from these multi-azole-resistant strains encode a protein with a G344S substitution10).

Ravuconazole (RVCZ) is a newly available human azole drug for human tinea unguium in Japan since 2018 and is a broad-spectrum antifungal agent that exhibits excellent activity against Candida albicans and Cryptococcus neoformans11, 12). Since RVCZ is also highly active against azoles that are resistant to FLCZ13), anti-fungal susceptibility of clinical isolates to RVCZ could be a major determinant of treatment outcome in Japan. However, the in vitro susceptibility of Japanese clinical isolates and azole resistance strains of C. neoformans to ravuconazole RVCZ has not been investigated.

In the present study, the in vitro susceptibility of Japanese clinical isolates and multi-azole resistance strains of C. neoformans to RVCZ was investigated using the Clinical & Laboratory Standards Institute (CLSI) M27-A3 test14). The clinical isolates and multi-azole resistant strains of C. neoformans examined in this study are listed in Table 1. The isolates were maintained on Sabouraud’s glucose agar (SGA) prior to being subjected to antifungal susceptibility tests. The broth microdilution (BM) assays to test the
susceptibility of C. neoformans to the antifungal drugs FLCZ (Pfizer Japan Inc., Tokyo, Japan), ITCZ (Sigma-Aldrich Japan Inc., Tokyo, Japan), VRZ (Pfizer), and RVCZ (Seren Pharmaceuticals Inc., Tokyo, Japan) were performed according to the CLSI M27-A3 guidelines. Minimal inhibitory concentrations (MIC) were determined after incubation at 35ºC for 72 h. For RVCZ, the MIC was defined as the lowest concentration that induced prominent inhibition of growth (approximately ≥50% inhibition).

The MICs for the isolates subjected to the CLSI M27-A3 test are summarized in Table 1. The mean MICs for the 14 clinical isolates were 1-64 mg/L for FLCZ, 0.125-0.5 mg/L for ITCZ, 0.125-0.5 mg/L for VRZ, and 0.003125-0.25 mg/L for RVCZ (Table 1).

Similarly, the MICs for the multi-azole resistance strains were >64 mg/L for FLCZ, >32 mg/L for ITCZ, 1-4 mg/L for VRZ, and 0.25-0.5 mg/L for RVCZ (Table 1).

The strains Candida parapsilosis ATCC 22019 and Candida krusei ATCC 6258 were used as quality control isolates for the CLSI M27-A3 test assay. The MICs of azoles for C. parapsilosis (ATCC22019) were 2 mg/L for FLCZ, 0.125 mg/L for ITCZ, 0.063125 mg/L for VRZ, and 0.0625 mg/L for RVCZ after incubation at 35ºC for 48 h. The MICs of azoles for C. krusei (ATCC 6258) were >64 mg/L for FLCZ, 0.5 mg/L for ITCZ, 0.5 mg/L for VRZ, and 0.25 mg/L for RVCZ after incubation at 35ºC for 48 h. These results were within the range recommended by the CLSI document M27-A3.

To our knowledge, this is the first study to investigate the in vitro susceptibility to RVCZ of clinical isolates from feline cryptococcosis and multi-azole-resistant strains of C. neoformans. RVCZ was as effective as ITCZ and VRZ against clinical isolates from cats and humans (Table 1). A C. neoformans var. grubii strain, NUBS14020, was the first fluconazole (FLZ) -resistant strain isolated from a feline cryptococcosis in Japan. Subsequent work demonstrated that multi-azole-resistant strains are readily isolated from FLZ-resistant strains (NUBS14020-2, -3 and -5) by culturing in medium containing voriconazole (VRZ). RVCZ was the most effective against multi-azole-resistant strains (NUBS14020-2, -3 and -5) bearing a G344S substitution in ERG11. The affinity of the protein encoded by ERG11 for RVCZ may differ from that for FLCZ, ITCZ, and VRZ. Therefore, RVCZ has considerable therapeutic potential for treating multi-azole resistant cryptococcosis. An increase in antifungal drug resistance in clinical isolates of Cryptococcus neoformans has been reported around the world. We therefore believe that RVCZ will become the primary antifungal agent for treating human and animal cryptococcosis.

Table 1. Strains and minimal inhibitory concentrations (MICs) (mg/L) of anti-fungal drugs

| Strain number | Origin                           | FLCZ | ITCZ | VRZ | RVCZ |
|---------------|----------------------------------|------|------|-----|------|
| NUBS18003     | Feline cutaneous cryptococcosis   | 8    | 0.25 | 0.125 | 0.03125 |
| NUBS18004     | Feline systemic cryptococcosis    | 8    | 0.5  | 0.25 | 0.03125 |
| NUBS18005     | Feline systemic cryptococcosis    | 8    | 0.5  | 0.25 | 0.03125 |
| NUBS18006     | Feline cutaneous cryptococcosis   | 16   | 0.5  | 0.25 | 0.0625 |
| NUBS18007     | Feline cutaneous cryptococcosis   | 16   | 0.5  | 0.5  | 0.0625 |
| NUBS18008     | Feline systemic cryptococcosis    | 16   | 0.125| 0.125| 0.125  |
| NUBS18009     | Feline systemic cryptococcosis    | 32   | 0.25 | 0.5  | 0.0625 |
| NUBS18010     | Feline systemic cryptococcosis    | >64  | 0.5  | 0.25 | 0.125  |
| NUBS18011     | Feline systemic cryptococcosis    | 16   | 0.125| 0.125| 0.03125 |
| NUBS18012     | Feline cutaneous cryptococcosis   | 8    | 0.5  | 0.25 | 0.125  |
| NUBS18013     | Human cutaneous cryptococcosis    | 8    | 0.125| 0.25 | 0.03125 |
| NUBS18014     | Human cutaneous cryptococcosis    | 4    | 0.25 | 0.125| 0.03125 |
| NUBS18015     | Human cutaneous cryptococcosis    | 1    | 0.25 | 0.125| 0.03125 |
| NUBS14020     | Feline cutaneous cryptococcosis   | >64  | 0.5  | 0.25 | 0.125  |
| NUBS14020-2   | Multi-azole-resistant strain      | >64  | >32  | 4    | 0.5    |
| NUBS14020-3   | Multi-azole-resistant strain      | >64  | >32  | 1    | 0.5    |
| NUBS14020-5   | Multi-azole-resistant strain      | >64  | >32  | 1    | 0.25   |

aNUBS: Nihon University College of Bioresource Sciences
bFLZ-resistant strain
cReference6
dThese strains were obtained by culturing in medium containing voriconazole10.
Declaration of interest

Rui Kano is applying for patent royalties (2019-73394) in Japan on the results of this study.

References

1) Sykes JE and Malik R: Cryptococcosis. In Infectious Diseases of Dog and Cat (Green CE, ed) 4th ed, pp. 621-634, Elsevier Saunders, St. Louis, 2012.
2) Reiss E, Shaday MJ, Lyon GM: Cryptococcosis. In Fundamental Medical Mycology, pp. 303-331, Wiley-Blackwell, New Jersey, 2012.
3) Kano R, Nakamura Y, Watari T, Tsujimoto H, Hasegawa A: A case of feline cryptococcosis treated with itraconazole. Mycoses 40: 381-383, 1997.
4) Kano R, Fujino Y, Takamoto N, Tsujimoto H, Hasegawa A: PCR detection of the Cryptococcus neoformans CAP59 gene from a biopsy specimen from a case of feline cryptococcosis. J Vet Diagn Invest 13: 439-442, 2001.
5) Okabayashi K, Kano R, Watanabe T, Hasegawa A: Serotypes and mating types of clinical isolates from feline cryptococcosis in Japan. J Vet Med Sci 68: 91-94, 2006.
6) Kano R, Okubo M, Yanai T, Hasegawa A, Kamata H: First Isolation of azole-resistant Cryptococcus neoformans from feline cryptococcosis. Mycopathologia 180: 427-433, 2015.
7) Rodero L, Mellado E, Rodriguez AC, Salve A, Guelfand L, Cahn P, Cuenca-Estrrella M, Davel G, Rodriguez-Tudela JL: G484S amino acid substitution in lanosterol 14-alpha demethylase (ERG11) is related to fluconazole resistance in a recurrent Cryptococcus neoformans clinical isolate. Antimicrob Agents Chemother 47: 3653-3656, 2003.
8) Smith KD, Achan B, Hullsieck JJ, McDonald TR, Okagaki LH, Alhadab AA, Akampurita A, Rhein JR, Meya DB, Boulware DR, Nielsen K; ASTRO-CM/COAT Team: Increased antifungal drug resistance in clinical isolates of Cryptococcus neoformans in Uganda. Antimicrob Agents Chemother 59: 7197-7204, 2015.
9) Chen YC, Chang TY, Liu JW, Chen FJ, Chien CC, Lee CH, Lu CH: Increasing trend of fluconazole-non-susceptible Cryptococcus neoformans in patients with invasive cryptococcosis: a 12-year longitudinal study. BMC Infect Dis 15: 277, 2015.
10) Kano R, Okubo M, Hasegawa A, Kamata H: Multi-azole-resistant strains of Cryptococcus neoformans var. grubii isolated from a FLZ-resistant strain by culturing in medium containing voriconazole. Med Mycol 55: 877-882, 2017.
11) Fung-Tomc JC, Huczko E, Minassian B, Bonner DP: In vitro activity of a new oral triazole, BMS-207147 (ER-30346). Antimicrob Agents Chemother 42: 313-318, 1998.
12) Yamazumi T, Pfaffer MA, Messer SA, Houston A, Hollis RJ, Jones RN: In vitro activities of ravuconazole (BMS207147) against 541 clinical isolates of Cryptococcus neoformans. Antimicrob Agents Chemother 44: 2883-2886, 2009.
13) Shalini K, Kumar N, Drabu S, Sharma PK: Advances in synthetic approach to and antifungal activity of triazoles. Beilstein J Org Chem 7: 668-677, 2011.
14) Clinical 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) Canto E, Espinel-Ingroff A, Pemán J: Trends in antifungal susceptibility testing using CLSI reference and commercial methods. Expert Rev Anti Infect Ther 7: 107-119, 2009.