In Vitro Antifungal Activities of Amphotericin B, Fluconazole, Itraconazole, Terbinafine, Caspofungin, Voriconazole, and Posaconazole against 30 Clinical Isolates of Cryptococcus neoformans var. neoformans

Young Ki Lee* and Annette W. Fothergill

Seoul Government Research Institute of Public Health & Environment
1Fungus Testing Laboratory, Department of Pathology, The University of Texas Health Science Center at San Antonio
(Received June 4, 2003)

Aantifungal agents were tested against 30 clinical isolates of Cryptococcus neoformans var. neoformans using the NCCLS method (M27-A2). Posaconazole, itraconazole and amphotericin B had lower MIC than the remaining four antifungal agents. The MIC result for posaconazole was over 220-fold lower active than fluconazole. Fluconazole MICs for most isolates fell within the dose-dependant range. The overall MIC ranges and MICs, were amphotericin B (0.03-0.25; 0.25), fluconazole (0.5-64; 16), itraconazole (0.015-1; 0.125), terbinafine (0.06->2; 1), caspofungin (8-32; 32), voriconazole (0.015-0.5; 0.25), and posaconazole (0.015-0.25; 0.06 µg/ml), respectively. In conclusion, the MICs of these drugs did not exhibit any sign of an upward shift with the exception of fluconazole and tendency cross-resistance between the seven drugs was not observed. We conclude that in vitro resistance to antifungal agents has not significantly changed despite the recent wide-spread use of triazoles for long-term treatment of Cryptococcal meningitis.

KEYWORDS: Antifungal agents, Cryptococcal meningitis, Cryptococcus neoformans, MIC

In the 1980s with the increased incidence of AIDS, infections with C. neoformans was on the rise. However, during the 1990s due to the highly active antiretroviral therapy (HAART), incidence of Cryptococcosis was decreased. This remains one of the most serious mycoses that primarily affects HIV-infected patients and those receiving immunosuppressive treatment for cancer, organ transplantation, and other serious medical conditions (Brandt et al., 2001; Yildiran et al., 2000). The treatment of disease due to C. neoformans has improved dramatically over the last several decades because of establishment of antifungal agents; amphotericin B (1950s), flucytosine (1970s), fluconazole and itraconazole (1990s) (Saag et al., 2000; White et al., 1998). The antifungal drugs currently available for the treatment of invasive mycoses can be divided into 4 different classes on the basis of their mechanisms of action.

*Corresponding author <E-mail: pp99pp@dreamwiz.com>
This method is widely used in the evaluation of established antifungal agents because of its reproducible and convenient characteristics. To date, there have been few published reports of resistance to amphotericin B, itraconazole, terbinafine, caspofungin, voriconazole, or posaconazole and cross-resistant between these antifungal agents (Davey et al., 1998).

The purpose of this investigation was designated to assess the in vitro antifungal activities and trends of cross-resistance of 30 clinical isolates of Cryptococcus neoformans var. neoformans against seven drugs (amphotericin B, fluconazole, itraconazole, terbinafine, caspofungin, voriconazole, and posaconazole).

Materials and Methods

Isolates. A total of 30 clinical yeast isolates were used in this study. Isolates were identified to the species level by conventional morphological and biochemical methods and stored at 70°C on SABDDEX agar slants. Before testing, all isolates were subcultured on the sabouraud dextrose agar (Remel, Lenexa, KS) to ensure optimal growth characteristics.

Antifungal agents. The antifungal agents tested were amphotericin B (Bristol-Meyers Squibb, USA), fluconazole, voriconazole (Pfizer, USA), itraconazole (Janssen Pharmaceuticals, USA), posaconazole (Schering-Plough Research Institute, USA), caspofungin (Merk, USA), and terbinafine (Novartis, USA) and were provided as standard powders by the manufacturers. Stock solutions were prepared in 50% of polyethylene glycol (itraconazole, terbinafine, voriconazole, and posaconazole) or distilled water (amphotericin B, fluconazole, and caspofungin). Serial two-fold dilutions were prepared as recommended in the NCCLS approved standard M27-A2. The final concentrations ranged from 16 to 0.004 µg/ml. The MICs of fluconazole, itraconazole, terbinafine, caspofungin, voriconazole, and posaconazole were defined as 100% inhibition (optically clear).

Antifungal susceptibility testing. The reference microdilution method M27-A2 was used for in vitro antifungal testing. Final drug concentrations ranged from 16 to 0.03 µg/ml for amphotericin B, from 64 to 0.125 µg/ml for fluconazole and caspofungin, from 8 to 0.015 µg/ml for itraconazole, voriconazole, and posaconazole, and from 2 to 0.004 µg/ml for terbinafine. A final inoculum of 0.5–2.5 x 10⁸ cells was prepared using a spectrophotometer. Each microdilution well containing 100 µl of the 2x drug concentrations was inoculated with 100 µl of the diluted (2x) inoculum suspension (final volume in each well was 200 µl). Growth and sterility control wells were included for each isolate tested. Candida parapsilosis ATCC 22019 and Candida krusei ATCC 6258 were used as quality control organisms and were included each time that a set of isolates was tested (Pfaller et al., 1995). After inoculation, plates were incubated for 72 h at 35°C and readings were taken daily. Absorbance was determined spectrophotometrically at 630 nm after agitation of the plates. The MICs of fluconazole, itraconazole, voriconazole, posaconazole and terbinafine were defined as the lowest drug concentration exhibiting approximately 50% reduction of growth compared with the control well (Rex et al., 2001). The MICs of amphotericin B and caspofungin were defined as the lowest drug concentration giving 100% inhibition (optically clear).

Results

Testing was done by the NCCLS broth microdilution method. The MIC ranges for amphotericin B, fluconazole, and itraconazole were within the expected ranges for the quality control strain, Candida parapsilosis ATCC 22019, Candida krusei ATCC 6258 (data not shown). Ranges for the other antifungal agents have not yet been established. Table 1. summarizes the in vitro antifungal activities of 30 clinical isolates of C. neoformans var. neoformans to seven drugs.

The results are reported as MIC ranges and the MIC required to inhibit 50% of the isolates, respectively. A broad range of MICs were observed for both fluconazole and itraconazole. Fluconazole MICs ranged between 0.5 and 64 µg/ml, while itraconazole ranged between 0.015 and 1 µg/ml. The MIC₅₀ results for caspofungin and posaconazole were also within the expected ranges for the quality control strains.

Table 1. In vitro antifungal activities of amphotericin B, fluconazole, itraconazole, terbinafine, caspofungin, voriconazole, and posaconazole against clinical isolates of Cryptococcus neoformans var. neoformans

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>MIC₅₀ (µg/ml)</th>
<th>Range</th>
<th>MIC₅₀</th>
<th>Range</th>
<th>MIC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>0.03–0.25</td>
<td>0.25</td>
<td>0.03–0.25</td>
<td>0.25 (0.167)</td>
<td></td>
</tr>
<tr>
<td>Fluconazole</td>
<td>0.25–64</td>
<td>16 (11.8)</td>
<td>0.5–64</td>
<td>16 (10.5)</td>
<td></td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.015–2</td>
<td>0.125 (0.066)</td>
<td>0.015–1</td>
<td>0.125 (0.061)</td>
<td></td>
</tr>
<tr>
<td>Terbinafine</td>
<td>0.06–2</td>
<td>0.5 (0.483)</td>
<td>0.06–2</td>
<td>1 (0.558)</td>
<td></td>
</tr>
<tr>
<td>Caspofungin</td>
<td>8–32</td>
<td>32 (18.4)</td>
<td>8–32</td>
<td>32 (18.4)</td>
<td></td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.015–0.5</td>
<td>0.25 (0.162)</td>
<td>0.015–0.5</td>
<td>0.25 (0.182)</td>
<td></td>
</tr>
<tr>
<td>Posaconazole</td>
<td>0.015–0.25</td>
<td>0.06 (0.046)</td>
<td>0.015–0.25</td>
<td>0.06 (0.046)</td>
<td></td>
</tr>
</tbody>
</table>

²MICs were determined after 72 h of incubation for isolates of C. neoformans var. neoformans under the NCCLS conditions (NCCLS, 1997).

³Mean MIC₅₀.
conazole at both 48 and 72 h remained the same, while results for the remaining antifungal agents were similar to within +1 dilution. According to the MIC<sub>v</sub> values obtained at 72 h of incubation, posaconazole gave the lowest values (0.06 µg/ml), followed by itraconazole (0.125 µg/ml), amphotericin B (0.25 µg/ml), and voriconazole (0.25 µg/ml), respectively. The MIC<sub>v</sub> results for posaconazole was over 220-fold lower active than fluconazole, while amphotericin B, displayed nearly identical activity to voriconazole.

Discussion

To date, there are a few studies on the susceptibility of C. neoformans, however direct comparisons of MIC data between studies are complicated by variability in test methods (Saag et al., 2000). The NCCLS M27-A2 method includes details for testing C. neoformans but does not specify any interpretive breakpoints for amphotericin B, fluconazole, itraconazole, terbinafine, caspofungin, voriconazole, and posaconazole against C. neoformans, except for fluconazole (Kirkpatrick et al., 1998; Rex et al., 2001). Therefore, this study tested the in vitro antifungal activity of seven drugs using M27-A2, which is commonly used worldwide, and examined their resistance properties through comparing the results of other researches. Overall, according to the MIC<sub>v</sub> values using the microdilution method, posaconazole gave the lowest values (0.06 µg/ml), followed by itraconazole (0.125 µg/ml), amphotericin B (0.25 µg/ml), and voriconazole (0.25 µg/ml), respectively. These results on the in vitro susceptibilities of these antifungal agents against C. neoformans were similar to those published earlier (Pfaffer et al., 2001). According to the results of a study by Brandt et al. (2001) on amphotericin B, fluconazole and itraconazole using the same test method, itraconazole appeared to be most active as its MIC range and MIC<sub>v</sub> were 0.06–0.5 and 0.125 µg/ml, respectively, followed by amphotericin B (0.125–0.5, 0.25 µg/ml) and fluconazole (2–8, 4 µg/ml). There was no remarkable resistance property found. In addition, in a test by Pfaffer et al., 2001, the MIC range and MIC<sub>v</sub> of posaconazole, itraconazole and fluconazole were 0.015–1; 0.12, 0.03–1; 0.25, and 0.25–128; 8 µg/ml respectively, similar to the results of the previously mentioned. There are also many test reports showing similar results although the tests used different method (micromodulation method). According to the results of a study by Franzot and Hamdan, 1996, using antifungal agents against clinical and environmental isolates, the MIC range and MIC<sub>v</sub> of itraconazole, amphotericin B and fluconazole were 0.03–0.25; 0.125, 0.25–1; 0.5, and 0.5–16; 4 µg, and in a test by Yildiran et al., 2002, posaconazole appeared to be most active (MIC range: MIC<sub>v</sub> ≥0.015≤≥8; ≤0.015), followed itraconazole (0.015–2; 0.015), voriconazole (0.125–0.125; ≤0.125), amphotericin B (0.125–0.5; 0.5) and fluconazole (≤0.125≤≥64; 1 µg/ml). This is similar to the results of the present study, however, the MIC<sub>v</sub> of voriconazole and amphotericin B in tests by Yildiran et al., 2002, and Ana, 1998, were opposite to those presented here. In a test by Ryder et al., 1998, (micromodulation method), the MIC range and MIC<sub>v</sub> of terbinafine against C. neoformans were 0.06–0.125; 0.125 µg/ml respectively, which were fourfold different from the results of the present test. Caspofungin is not recommended for Cryptococcal meningitis (Roling et al., 2002) was included in this study simply because of the possibility of cross resistance. When comparing the result of the susceptibility of the seven antifungal agents used in this study with those of previous studies relating to C. neoformans, a dose-dependant response, as the MIC<sub>v</sub> of fluconazole increased slightly (16 µg/ml), but the increase was not regarded as a remarkable enhancement of resistance.

In conclusion, our results showed that the MIC ranges and MIC<sub>v</sub>s of these seven antifungal agents did not increase significantly compared to the other studies despite the recent widespread use of triazoles for long-term treatment of cryptococcal meningitis and evident possible cross-resistant between these drugs was not noted.

References


