Effects of sertraline hydrochloride and fluconazole combinations on Cryptococcus neoformans and Cryptococcus gattii

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Our study evaluated the efficacy of a combination of fluconazole and the psychoactive compound sertraline against strains of Cryptococcus neoformans and Cryptococcus gattii. Using the checkerboard microdilution method based on CLSI M27-A2 guidelines, we determined the susceptibilities of each drug alone and in combination against 40 strains of serotypes A, D and AD in C. neoformans and 13 strains of serotype B C. gattii. The MIC ranged 10.8–43 and 2–64mg/l for sertraline and fluconazole, respectively. No difference in MIC for sertraline was observed among the serotypes. However, within C. neoformans, a significant difference in MIC for fluconazole was observed among the serotypes, as follows: AD > A > D (AD being the most resistant). Strains of C. gattii had MIC for fluconazole not significantly different from those of serotypes A and AD but significantly higher than serotype D. Synergy (FICI ≤ 0.5) was found for 31 strains, while the remaining 22 strains showed no difference. No evidence for antagonistic interaction was observed. Our study suggests the potential utility of fluconazole–sertraline combination therapy for cryptococcal infections.

Keywords: antifungal activity; sertraline; fluconazole; antifungal susceptibility testing; synergy; yeasts; Cryptococcus

Abbreviations: DMSO, dimethyl sulfoxide; CLSI, clinical laboratory standards institute; MIC, minimal inhibitory concentration; FIC, fractional inhibitory concentration; MAPK, mitogen-activated protein kinase; CFU, colony formation units

Introduction

Cryptococcus neoformans is a pathogenic basidiomycete yeast capable of causing meningoencephalitis in immunosuppressed individuals. It is a ubiquitous fungus that is commonly isolated from rotting wood and avian guano in the environment (Litvintseva et al. 2005; Nielsen et al. 2007). Cryptococcus gattii on the other hand is most frequently found in tropical and subtropical regions and is capable of infecting immunocompetent individuals. The largest outbreak of C. gattii occurred in British Colombia, Canada in 1999 (Kidd et al. 2005). Since then, C. gattii has been isolated from several parts of Pacific North America indicating a changing demographic for the organism (MacDougall et al. 2007; Dixit et al. 2009). Similarly, a recent study regarding the infection patterns in China discovered that C. neoformans might also be capable of infecting healthy, immunocompetent individuals (Chen et al. 2008). Infections by both species can result in significant morbidity and mortality, with mortality rates reaching as high as 60% 6 months after a meningeal infection for some populations. The high mortality serves as a grim reminder that our arsenal and tactics against this disease need to be expanded (Kambu et al. 2008).

Fluconazole remains the drug of choice for treating cryptococcosis and many other fungal infections. It has a high bioavailability, is well tolerated by most patients and can be administered orally. However, the maximum attainable plasma concentration with a 400-mg oral capsule is 13.5 mg/l or 4.92–17.6 μg/g in the brain (Fischman et al. 1993; Thaler et al. 1995). As such, infections with resistant strains may result in poor treatment outcomes unless other more potent drugs but with significant toxicities (i.e. amphotericin B) are used. In these scenarios, combination therapies may be necessary and preferable. The idea behind combination therapy is that a greater therapeutic efficacy can be achieved by producing a potent fungicidal outcome with reduced toxicity (due to reduced drug dosage) experienced by the patient (Barchesi et al. 2000). Moreover, the use of combinations of drugs may even present a synergistic result whereby the antifungal effect is more pronounced than any drug alone at the same concentration. Additionally, the use of combination treatments can help reduce the emergence of resistance. One of the most commonly used combination therapy against human pathogenic fungi is that of the amphotericin B/lipid A analog combinations (Schwarz et al. 2006, 2007).

In this study, a combination of sertraline hydrochloride and fluconazole was tested for efficacy and interaction against strains of C. neoformans and C. gattii. This drug combination was selected based on several recent reports.
showing that sertraline displayed antifungal activities against Saccharomyces, Candida and Aspergillus species (Lass-Flörl et al. 2001a,b, 2003; Ericson et al. 2008). However, no studies have been conducted, to the best of our knowledge, on the efficacy of sertraline against any Cryptococcus species. Sertraline is a commonly prescribed psychotropic drug that functions as a selective serotonin re-uptake inhibitor (SSRI). It acts specifically on the 5-HT neuronal transporter protein (SERT) found in humans (Barchiesi et al. 2005). It is a competitive inhibitor of serotonin that functions on the serotonin-binding domain of the 5-HT protein (Koe et al. 1990).

To examine the effects of sertraline and fluconazole/sertraline combination on C. neoformans and C. gattii, we screened 40 strains of C. neoformans representing three distinct serotypes A, D, and AD. In addition, 13 strains of C. gattii, a close relative of C. neoformans were included. The C. gattii strains all belonged to serotype B. We were interested in whether the different species or serotypes would show different patterns of drug responses to fluconazole, sertraline and/or to a combination of the two drugs. Any observed differences would also provide evidence that each serotype might employ different regulatory mechanisms to deal with cell stress and might be related to their different virulence properties in mammalian hosts (Barchiesi et al. 2005). Since fluconazole targets 14-α-demethylase and sertraline does not (Lass-Flörl et al. 2003; Rodero et al. 2003), we expect no evidence of cross-resistance between the two drugs. Moreover, due to the distinction in targets, we expect to see a synergistic interaction between the drugs particularly against fluconazole-resistant isolates. As was shown for the amphotericin B/flucytosine combinations, the two drugs interacted more synergistically against more fluconazole-resistant strains (Schwarz et al. 2006, 2007).

A finding of synergy would help in the development of a more aggressive treatment option against cerebral mycotic infections. Additionally, the potential patterns of antifungal drug responses among strains and serotypes might lead to the development of therapies targeted towards specific serotypes, which may help improve the success rate of treating systemic cryptococcal infections. Aside from the health benefits, targeted therapies could also reduce costs. A recent report has indicated that the treatment of mycotic infections amounts to $2.6 billion USD annually in US healthcare spending alone (Cowen and Steinbach 2008).

Materials and methods

Preparation of drug stock solutions

Sertraline was obtained from Sigma-Aldrich Canada and Fluconazole (Diflucan®) was purchased from Pfizer (New York, USA). Sertraline was dissolved in DMSO to a final concentration of 2870 mg/l. Fluconazole was bought as a prepared i.v. solution at 2000 mg/l in saline. The stock solutions were stored at room temperate (25 °C) as indicated by the manufacturers.

Isolates

A total of 53 isolates were tested in this study, including 40 C. neoformans strains and 13 C. gattii strains (Table 1). These strains were originally obtained from the Center for Disease Control and Prevention in the US and the University of British Columbia (Kidd et al. 2005; Brandt et al. 1996; Yan et al. 2002). We also included two CLSI recommended quality control (QC) reference strains Candida parapsilosis ATCC22019 and Candida krusei ATCC6258. The type strain of C. neoformans var. grubii, H99, was also included as a third QC strain throughout our testing. Each strain was removed from the ~80 °C freezer and subcultured onto yeast extract peptone dextrose (YEPD; 2% dextrose, 1% yeast extract, 1% peptone) plates. The subcultures were further streaked onto fresh YEPD and allowed to grow at 30 °C for 3–5 days. Three to five colonies were removed from each plate, inoculated into 4 ml of YEPD broth (Difco) and incubated overnight (~12 h) in a rotating incubator at 120 rpm at 37 °C. The OD_{530} of the overnight cultures were obtained and standardized to 0.5 McFarland units in YEPD broth prior to inoculation for drug susceptibility testing.

Drug susceptibility testing

A modified version of the CLSI M27-A2 broth microdilution method was employed to test the drug combination. Briefly, serial two-fold dilutions of both fluconazole and sertraline were performed with concentrations ranging 0.25–128 mg/l and 1.3–86 mg/l, respectively. Sertraline was diluted in dimethyl sulfoxide (DMSO), while fluconazole was diluted in double distilled water. Both drugs were distributed in a 96-flatwell clear-bottomed microtitration plate (Costar 96-well tissue culture plate; Corning Inc., New York, NY, USA) with the final DMSO concentration not exceeding 3% (v/v) per well. A preliminary test involving a checkerboard of varying concentrations of DMSO in combination with fluconazole demonstrated that DMSO inhibited growth by 50% at the concentration of 5% (v/v) and caused complete growth inhibition at concentrations ≥ 7.5% (v/v) against C. neoformans strain H99. However, there were no synergy between DMSO and fluconazole (FICI = 1.03). Each well was inoculated with approximately 1.0 × 10^{3}–2.0 × 10^{5} CFU/ml. The plates were incubated at 37 °C for 72 h. The MIC for both fluconazole and sertraline was determined as the initial most prominent decrease in turbidity as indicated by CLSI. The fractional inhibitory concentration (FIC) was determined as the well that showed the most prominent decrease in turbidity relative to the other combination wells. If more than one well
showed similar decreases in turbidity, the well that yielded the lowest FIC Index (FICI) value was used since this would represent the optimal fluconazole:sertraline ratio for in vitro growth inhibition. The FICI value was calculated for each strain using the formula: $FICI = (FIC_{\text{Fluconazole}}/\text{MIC}_{\text{Fluconazole}}) + (FIC_{\text{Sertraline}}/\text{MIC}_{\text{Sertraline}})$, where the FIC represents the MIC of the drug in combination. A FICI $\leq 0.5$ was considered synergistic, >0.5-4.0 indifference and > 4.0 antagonistic (Odds 2003).

**Statistical analysis**

The Minitab Database Software (MDS) 14.0 was used to perform Mann–Whitney tests to determine serotype differences in their MICs to each drug alone and in combination (i.e. FIC). The Mann–Whitney test was also used to determine serotype differences in drug interactions by comparing the FICI values for strains of each serotype. A $p < 0.05$ was considered statistically significant.

The MDS 14.0 software was also used to calculate Pearson correlation coefficient values to determine the relationship between the fluconazole or sertraline susceptibility and the FICI within this population of 53 strains. A $p < 0.05$ was considered significant for all tests.

**Results and discussion**

**Efficacy of the sertraline/fluconazole combination**

While there are exceptions in correlating in vitro data with in vivo outcomes for most antibiotics, including antifungals, the general trend for combination therapy shows that synergy or indifference demonstrated in vitro is usually indicative of positive outcomes in vivo (Johnson et al. 2004; Mukherjee et al. 2005). Our study found no evidence of antagonistic interaction (FICI $\geq$ 4.0) between the sertraline and fluconazole combination against strains of the *C. neoformans* species complex. A large number of isolates (31/53) responded with synergy (FICI $\leq$ 0.5) to the sertraline/fluconazole combination. Of these 31 strains, 10 belonged to serotype A (of 15 serotype A strains total tested), nine to serotype AD (of 13 strains total), nine to serotype B (of 13 strains total) and three to serotype D (of 12 strains total).

The remaining 22 strains demonstrated no significant interaction (i.e. 0.5 < FICI < 4.0). Thus, we expect this combination to work effectively in vivo to produce positive treatment outcomes. Moreover, our linear regression analysis found a weak, but significant, negative correlation between the susceptibility to fluconazole and the FICI within this population of isolates (Figure 1a). A similar trend was also observed with regards to a strain’s susceptibility to sertraline (Figure 1b). The Pearson’s correlations for fluconazole and sertraline MICs to the FICI were −0.296 ($p < 0.05$) and −0.355 ($p < 0.01$), respectively. Our analyses indicate that the more resistant a strain is to either fluconazole

| Strain | Source          | Serotype |
|--------|-----------------|----------|
| 92-31  | CDC             | A        |
| 92-28  | CDC             | A        |
| 92-21  | CDC             | A        |
| 92-29  | CDC             | A        |
| 92-2   | CDC             | A        |
| 92-45  | CDC             | A        |
| 92-101 | CDC             | A        |
| 92-13  | CDC             | A        |
| 92-10  | CDC             | A        |
| 92-30  | CDC             | A        |
| 92-1   | CDC             | A        |
| 92-3   | CDC             | A        |
| 92-43  | CDC             | A        |
| 92-56  | CDC             | A        |
| 92-293 | CDC             | A        |
| H99    | Yan et al. 2002 | A        |
| 92-32  | CDC             | D        |
| 92-134 | CDC             | D        |
| 92-77  | CDC             | D        |
| JEC20  | Yan et al. 2002 | D        |
| 92-76  | CDC             | D        |
| 92-27  | CDC             | D        |
| 92-198 | CDC             | D        |
| 92-178 | CDC             | D        |
| 92-138 | CDC             | D        |
| 92-170 | CDC             | D        |
| 92-119 | CDC             | D        |
| 92-337 | CDC             | D        |
| 92-47  | CDC             | AD       |
| 92-74  | CDC             | AD       |
| 92-66  | CDC             | AD       |
| 92-46  | CDC             | AD       |
| 92-190 | CDC             | AD       |
| 92-62  | CDC             | AD       |
| 92-5   | CDC             | AD       |
| 92-26  | CDC             | AD       |
| 92-280 | CDC             | AD       |
| 92-283 | CDC             | AD       |
| 92-304 | CDC             | AD       |
| 92-328 | CDC             | AD       |
| 92-354 | CDC             | AD       |
| F3050  | Kidd et al. 2005| B        |
| RB2    | Kidd et al. 2005| B        |
| WM178  | Kidd et al. 2005| B        |
| A3MR674| Kidd et al. 2005| B        |
| A4M364 | Kidd et al. 2005| B        |
| RB28   | Kidd et al. 2005| B        |
| WM179  | Kidd et al. 2005| B        |
| RM005  | Kidd et al. 2005| B        |
| R270   | Kidd et al. 2005| B        |
| MC-S-115| Kidd et al. 2005| B        |
| RB67   | Kidd et al. 2005| B        |
| 92-71  | Kidd et al. 2005| B        |
| 92-350 | Kidd et al. 2005| B        |
| ATCC22019 Hamilton General Hospital | Candida parapsilosis |
| ATCC6258 Hamilton General Hospital | Candida krusei |
or sertraline, the more likely it will respond synergistically to the combination therapy (Figure 1a and b). Consequently, this combination could be ideal to treat non-responsive infections.

Sertraline hydrochloride is a psychoactive drug that can be used to treat obsessive compulsive disorder, panic disorder and depression. The dosage administered ranges from 50 to 200 mg/day in oral form. The 50 mg/day dose yields a mean 24 h plasma concentration of 0.006 mg/l, while the 200 mg/day dose yields a mean plasma concentration of approximately 0.107 mg/l (Pfizer Inc. 2002; Nagy et al. 2004). However, the concentration achievable in the brain is greater than 40 times that in the plasma (DeVane 1992). In our collection of strains, the highest concentration of sertraline required in the in vitro combination treatment was 10.75 mg/l. Concordantly, of the 28 strains (MIC_Fluconazole ≥ 16 mg/l) that were predicted to fail with fluconazole monotherapy, eight strains could potentially be successfully treated by the sertraline/fluconazole combination (FIC_Fluconazole<16 mg/l and FIC_Sertraline < 4.28 mg/l). This is based on the assumption that the in vitro and in vivo sertraline susceptibilities are directly correlated. Despite a salvage rate of approximately 28% (8/28), short-term use of sertraline has more manageable adverse effects than the use of amphotericin B. In a study by Nagy et al. (2004), the most significant adverse effect due to sertraline was one case of eisonophilia among 17 healthy volunteers. The most frequently observed adverse effects were nausea and diarrhea (6/17). This might be preferable to acute renal toxicity commonly experienced with amphotericin B treatment. Our analyses suggest that further studies regarding the in vivo susceptibilities of C. neoformans and C. gattii to sertraline/fluconazole combination therapy are warranted. Moreover, if the functional groups of sertraline could be modified to improve its pharmacokinetic parameters and increase its fungicidal potency, the salvage rate might increase significantly.

Analysis of serotype differences

There has been some debate as to whether differences exist among the serotypes of the C. neoformans species complex in their antifungal susceptibilities. Several studies reported that serotype B strains were less susceptible to fluconazole than serotype A strains (Khan et al. 2007; Gomez-Lopez et al. 2008; Torres-Rodriguez et al. 2008), while others found no difference (Chang et al. 2006; Tay et al. 2006; Thompson et al. 2008). In our study, a significant difference was found between the susceptibility of serotypes A, AD, D and B to fluconazole alone. Specifically, serotype AD strains were more resistant to fluconazole than serotype A strains (p < 0.05) which in turn were more resistant than serotype D strains (p < 0.01). Additionally, serotype B strains were also significantly more resistant to fluconazole than serotype D strains (p < 0.01) but no difference was found between serotype B and the other two C. neoformans serotypes A and AD. Interestingly, this serotype-specific difference was statistically not significant when comparing FIC_Fluconazole between the three C. neoformans serotypes A, D and AD. However, while C. gattii serotype B had a similar value to serotype AD, its FIC_Fluconazole was significantly higher than both serotype A and serotype D (p < 0.05). Table 2 shows their geometric means and ranges.

In contrast to those for fluconazole, we found no differences in susceptibility to sertraline among the serotypes in the C. neoformans species complex. This pattern was also seen for the concentration of sertraline required in the fluconazole/sertraline combination as well, with the exception of a significant difference between serotypes AD and D. Specifically, the FIC_Sertraline for serotype AD strains were significantly higher than those for serotype D strains (p < 0.05). Furthermore, there was also no evidence of cross-resistance between sertraline and fluconazole – no significant correlation was found between MIC_Fluconazole and MIC_Sertraline or between FIC_Fluconazole and FIC_Sertraline (data not shown).

We would like to mention that the number of strains for each examined serotype is relatively small and that different samples might show different patterns. However,
Table 2. Geometric means (range) of the minimum inhibitory concentrations (MIC), fractional inhibitory concentrations (FIC), and fractional inhibitory concentration index (FICI) among the four serotypes of the *C. neoformans* species complex.

| Serotype | MIC- Fluconazole (mg/l) | MIC-Sertraline (mg/l) | FIC – Fluconazole (mg/l) | FIC – Sertraline (mg/l) | FICI       |
|----------|-------------------------|-----------------------|--------------------------|-------------------------|-----------|
| A (n=15) | 10.1 (4.0–64.0)         | 17.9 (10.75–43.0)     | 2.0 (0.25–32.0)          | 3.9 (1.34–10.75)        | 0.473     |
| D (n=12) | 4.1 (2.0–16.0)          | 20.3 (10.75–43.0)     | 1.7 (0.25–4.0)           | 2.5 (1.34–5.38)         | 0.550     |
| AD (n=13)| 17.8 (4.0–64.0)         | 19.3 (5.40–43.0)      | 3.2 (1.0–16.0)           | 4.3 (1.34–10.75)        | 0.444     |
| B (n=13) | 16.0 (4.0–64.0)         | 15.6 (10.75–43.0)     | 3.4 (1.0–8.0)            | 3.3 (1.34–10.75)        | 0.453     |

Conclusions

Our study demonstrated that fluconazole/sertaline combinations could be a promising duo in the treatment of Cryptococcal meningitis. Moreover, the more resistant a strain is to either sertaline or fluconazole, the more likely it is to respond synergistically to the combination. We also revealed that the four serotypes of the *C. neoformans* species complex showed overall different susceptibilities to fluconazole with AD>A>D and B>D (AD being the most resistant). However, this pattern of differences disappeared in the presence of sertraline for *C. neoformans*. Interestingly, *C. gattii* was more resistant to fluconazole than serotypes A and D in the presence of sertraline. Since sertaline inhibits extracellular phospholipase activity, we hypothesize that the variability in fluconazole susceptibility might be due to differential roles of phospholipase-activated pathways among the serotypes.
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