Activity of sertraline against Cryptococcus neoformans: in vitro and in vivo assays

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Abstract

Cryptococcus neoformans infection is an important cause of meningitis in HIV/AIDS endemic regions. Antifungals for its management include amphotericin B, flucytosine, and fluconazole. Recently, treatment of this mycosis with sertraline has been studied with variable clinical outcomes. The aim of the study was to assess the in vitro antifungal effect of sertraline against clinical isolates of Cryptococcus spp. as well as its in vivo activity in a murine model of cryptococcal meningoencephalitis. The in vitro susceptibility to fluconazole, amphotericin B, voriconazole and sertraline of 153 Cryptococcus spp. strains were evaluated according to CLSI procedures. Fungal tissue burden, serum antigenaemia and histopathology, together with the therapeutic efficacy of amphotericin B (3 mg/kg), fluconazole (15 mg/kg), and sertraline (3, 10, and 15 mg/kg) were evaluated in mice intracranially inoculated with one isolate of Cryptococcus neoformans. All strains were susceptible to the antifungals studied and exhibited growth inhibition with sertraline at clinically relevant concentrations. Sertraline at a dose of 15 mg/kg reduced the fungal burden in the brain and spleen with an efficacy comparable to that of fluconazole. In conclusion, sertraline exhibited an excellent in vitro-in vivo anti-cryptococcal activity, representing a possible new alternative for the clinical management of meningal cryptococcosis.

Key words: cryptococcal meningoencephalitis, Cryptococcus neoformans, sertraline, antifungal in vitro susceptibility, antifungal efficacy study.
Introduction

Cryptococcus meningitis globally causes approximately 25%–30% of deaths each year in AIDS patients, and it is always fatal without treatment. In Mexico, cryptococcosis of the central nervous system (CNS) is the second leading cause of opportunistic infections in patients with AIDS.

Progress in antifungal drug treatment for cryptococcal meningitis has been terribly slow. The cornerstone therapy, amphotericin B, was introduced in the late 1950s. The number of current antifungals is limited, and some of them have a poor CNS penetration. Standard treatment for cryptococcal meningitis includes amphotericin B (deoxycholate or liposomal), fluocytosine, and fluconazole. The search for new effective and widely distributed affordable drugs is imperative.

Sertraline is a potent antidepressant approved for the treatment of obsessive compulsive, panic and post traumatic disorders that belongs to the group of selective serotonin reuptake inhibitors. Its antifungal potential against Cryptococcus spp. has recently been demonstrated with fungicide concentrations below 10 μg/ml; less than that of other fungal genera such as Aspergillus and Candida. Additionally, it has been speculated that its combination with fluconazole might have a synergistic effect in vitro and in vivo on growth inhibition of Cryptococcus spp. In this study we sought to determine the in vitro antifungal effect of sertraline against Cryptococcus spp. as well as its in vivo activity in a murine model of cryptococcal meningoencephalitis.

Materials and methods

Clinical isolates

A total of 153 clinical isolates of Cryptococcus spp. collected from patients from six different hospitals in Northeast Mexico were used. These isolates were previously identified, genotyped by PCR fingerprinting, and stored as suspensions in sterile distilled water at room temperature and cultured for 48 h on Sabouraud-dextrose agar (SDA) slants (Difco, Detroit, MI, USA) at 37°C before use.

The strains were isolated from sterile fluids, 146 samples from cerebrospinal fluid (95.4%) and seven from blood samples (4.6%). All strains belonged to the species C. neoformans, with the following genotype distribution: VNI = 124 (81.0%), VNII = 15 (9.8%), VNIII = 8 (5.2%), and VNIIV = 6 (4.0%).

Antifungals and in vitro susceptibility testing

Fluconazole and voriconazole (Pfizer, Inc., New York, NY, USA), amphotericin B (Bristol-Myers Squibb, Princeton, NJ, USA) were obtained in reagent-grade powder. Sertraline (purity HPLC > 98%) was obtained through TCI America Chemical Solutions (TCI Chemicals Inc., New York, NY, USA). Antifungal susceptibility testing was conducted using the plate microdilution method according to document M27-A3 of the CLSI. The final drug concentrations ranged from 0.125 to 64 μg/ml for fluconazole and sertraline, and from 0.03 to 16 μg/ml for voriconazole and amphotericin B. Candida parapsilosis ATCC 22019 and C. krusei ATCC 6258 were included as quality control organisms. Although interpretative MIC breakpoints particularly for C. neoformans have not yet been established, Pfaffer et al. reported in a recent global epidemiological survey that 98–100% of C. neoformans isolates were susceptible to amphotericin B (MIC ≤ 1 μg/ml) and 75% of isolates from North America were susceptible to fluconazole (MIC ≤ 8 μg/ml).

Ethics statement

Murine experiments were performed with the approval of the Ethics and Research Committee of the School of Medicine of the Universidad Autónoma de Nuevo León (registration number: MI13-009). The experimental protocol was designed in conformity with the International Review Board regulations, following the recommendations of the Guidelines for the Care and Use of Laboratory Animals, and in agreement with Good Laboratory Practices. Care, maintenance and handling of the animals were in accordance with the Mexican regulations for animal experimentation (NOM-062-ZOO-1999).

Animals

Immunocompetent male CD1 mice, four weeks old (24–26 g weight) were purchased from the animal facility of the Universidad Autónoma Metropolitana (Mexico D.F., Mexico). A total of 114 animals were used. These were housed in ventilated cages of five mice each under specific pathogen-free conditions at the animal facility of the Department of Microbiology. All mice had access to sterile water and Purina rodent food ad libitum and were monitored daily for 15 days. The day/night cycle was 12 h/12 h. Before use, the animals were allowed to acclimatize for 5 days.

Experimental infection

The reference strain C. neoformans USC1597 (serotype A, genotype VNI) used in this study was kindly provided by the laboratory of Dr. Thomas F. Patterson in University of Texas Health Science Center at San Antonio, and had
been previously used in other studies.\textsuperscript{13-16} The serotyping and genotyping of the strain was done by multiplex PCR\textsuperscript{17} and a consensus multilocus typing scheme,\textsuperscript{18} respectively. To establish cryptococcal meningoencephalitis, mice were anesthetized by inhalation of 2\% isoflurane (Boise, ID, USA). Their heads were closely clipped, the area was swabbed with 70\% alcohol, and an adjusted inoculum of \textit{C. neoformans} at 1,500 cfu/mouse was delivered in a 0.06-ml volume through a 27-gauge needle fastened to a tuberculin syringe with a cuff to prevent penetration of more than 1 mm.\textsuperscript{16} A midline puncture through the cranial vault approximately 6 mm posterior to the orbit was made, the inoculum was injected, and the mice were allowed to recover.

### Compounds and chemotherapeutic schemes

Fluconazole and amphotericin B were purchased as Flucozan and Amfotericina B from PiSA, Mexico, while sertraline (TCI, Tokyo, Japan) was obtained as reagent-grade powder. Fluconazole was diluted with sterile water to the desired dose to use in animals. Amphotericin B was reconstituted in sterile water and diluted with sterile 5\% dextrose to the desired dose for animal administration. Sertraline hydrochloride was dissolved in a 0.9 volume of sterile water and 0.1 volume of sterile 10\times phosphate-buffered saline stock solution. Drug solutions were freshly prepared before animal administration. Mice were divided into six groups of nineteen animals per group: untreated controls; fluconazole 15 mg/kg by oral gavage; amphotericin B 3 mg/kg by intraperitoneal injection; or sertraline at 3, 10, and 15 mg/kg administered by intraperitoneal injection. The therapeutic schemes we adopted were according to previous studies.\textsuperscript{7,16} Given that it takes at least 5 to 7 days for sertraline to reach a steady state in the mouse brain,\textsuperscript{19} mice treated with any dose of sertraline started with a pretreatment scheme 7 days prior to Cryptococcus infection and treatment continued after the challenge. Fluconazole and amphotericin B were started 24 h after infection; fluconazole was administered once daily, while amphotericin B was given on days 1, 3, and 5 post-challenge. All drug treatments concluded on day 5 after infection.

### Serum antigen and tissue fungal burden

Cryptococcal serum antigen determinations were made in serum drawn from three mice per group on days 3, 5, and 7 post-challenge using a commercially available kit according to the manufacturer’s instructions (Cryptococcal Antigen Latex Agglutination System, Meridian Bioscience, Inc., Cincinnati, OH, USA). On the other hand, for tissue fungal burden determinations ten mice per group were terminated at day 7 post-challenge. After sacrifice, the brain and spleen of each mouse were aseptically removed, weighed and placed in 1.0 ml of sterile saline. The organs were homogenized in a tissue grinder (Polytron-Aggregate, Kinematica AG, Lucerne, Switzerland) and serially diluted 1:10 in sterile saline. Aliquots of 0.1 ml of the undiluted and diluted homogenates were then plated in duplicate onto SDA plates. Following 48–72 h of incubation at 37°C, colonies were enumerated and cfu per gram of tissue for each animal was calculated.

### Histology

For histopathological analysis, brains and spleens were collected from three animals per group on days 3, 5, and 7 post-inoculation and fixed with 10\% phosphate-buffered formalin. Samples were dehydrated, paraffin embedded, and sliced into 5-μm sections. The sections were then stained with mucicarmine, which stains the inner capsule of the yeast red and examined in a blinded fashion by light microscopy.

### Statistics

The analysis of variance with Tukey’s post test was used to assess differences in serum antigen and tissue fungal burden among experimental groups. Calculations and graphics were performed using GraphPad Prism version 5.03 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). P values ≤ .05 were considered statistically significant.

### Results

#### \textit{In vitro} antifungal susceptibility testing

The \textit{in vitro} antifungal susceptibilities of the \textit{C. neoformans} isolates are summarized in Table 1. All isolates were susceptible to voriconazole (range: 0.03–0.125, \textit{MIC}_{50}: 0.03, \textit{MIC}_{90}: 0.125), amphotericin B (range: 0.125–1, \textit{MIC}_{50}: 0.125, \textit{MIC}_{90}: 0.5) and fluconazole (range: 0.5–4, \textit{MIC}_{50}: 0.5, \textit{MIC}_{90}: 1), agreeing with recent reports.\textsuperscript{20,21} Voriconazole was the most effective antifungal. Sertraline has no cut-off value currently available; however, we found that the range of susceptibility was between 1 and 8 μg/ml with a \textit{MIC}_{50} of 2 μg/ml and a \textit{MIC}_{90} of 4 μg/ml, findings similar to those previously reported by Nayak and Xu\textsuperscript{6} and Zhai et al.\textsuperscript{7}

The \textit{in vitro} susceptibility testing of \textit{C. neoformans} USC1597 was: fluconazole, 1 μg/ml; voriconazole, 0.06 μg/ml; amphotericin B, 0.25 μg/ml; and sertraline, 4 μg/ml.
Table 1. Percent of fungal growth inhibition according to the MIC of 153 clinical isolates of Cryptococcus spp.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (μg/ml)</th>
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<tr>
<td></td>
<td>0.030</td>
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<tr>
<td>Amphotericin B</td>
<td>66.7</td>
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<tr>
<td>Voriconazole</td>
<td>79.7</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>81</td>
</tr>
<tr>
<td>Sertraline</td>
<td>40.5</td>
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</tbody>
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Figure 1. Fungal tissue burden of brain (a) and spleen (b) in mice infected by intracranial inoculation with 1,500 cfu/mouse of C. neoformans USC1597 by day 7 post-challenge. Each treatment group included 10 animals. Asterisks represent significant differences compared to controls, **P < 0.01 and ***P < 0.001. AMB, amphotericin B; FLC, fluconazole; SRT, sertraline.

In vivo antifungal efficacy evaluation

The results of fungal tissue burden are depicted in Figure 1. The brain fungal burden of the untreated controls ranged from 3.85 × 10⁴ to 1.75 × 10⁵ cfu/g (median: 9.75 × 10⁴ cfu/g) and, as shown in Figure 1A, there was a statistically significant reduction of the brain fungal load in infected animals treated with amphotericin B (median: 1.33 × 10² cfu/g), fluconazole (median: 1.3 × 10¹ cfu/g), and sertraline at doses of 10 mg/Kg (median: 3.48 × 10⁴ cfu/g) and 15 mg/Kg (median: 2.96 × 10¹ cfu/g) (P < .001). Regarding the spleen fungal burden, the therapeutic scheme with fluconazole and sertraline at a dose of 15 mg/kg significantly reduced fungal burden to 1.41 × 10¹ cfu/g (P < 0.01) and 9.28 × 10³ cfu/g (P < .001) with respect to the untreated animals, in which spleen fungal burden ranged from 1.2 to 5.8 × 10⁴ cfu/g (median: 2.51 × 10⁴ cfu/g) (Figure 1B).

Serum antigenaemia

Serum antigenaemia was negative for all experimental groups by day 3 post-inoculation and remained so by day 7 in the infected mice treated with amphotericin B, while by day 5 after intracranial challenge, antigen titers of 1:32, 1:64, 1:32, and 1:16 were present in animals under treatment with fluconazole or sertraline at 3 mg/kg, 10 mg/kg, and 15 mg/kg, respectively. Although an evident rise in antigen titers to 1:128 occurred in all treated groups by day 7, a statistically difference was not found with respect to the untreated control mice, which presented a titer of 1:256 (Figure 2).

Histopathological analysis

There was no evidence of consistent histologic alterations in the brain or spleen, irrespective of day post-inoculation. There was an absence of cryptococcal cells in the spleen regardless of the experimental group and the day after challenge. However, abundant encapsulated yeast cells foci
accompanied by evident inflammatory infiltrates were observed in the meninges of untreated control mice by day 7 post-infection and considerably less in animals treated with fluconazole and any dose of sertraline (Figure 3). In addition, there were no mucicarmin positive cells compatible with *Cryptococcus* in the brain tissue of mice treated with amphotericin B.

**Discussion**

*Cryptococcus* spp. remains one of the major causes of meningitis in various geographical regions and it is associated with a high mortality in patients with HIV/AIDS and in recipients of solid organ or hematopoietic stem cells transplants.\(^4,22\) Resistance to the standard treatment has been already reported.\(^23,24\) The use of voriconazole, a drug evaluated in some studies for strains resistant to fluconazole,\(^25\) although effective, is too expensive and its availability is reduced to hospitals in urban centers. Overall, the search for new antifungals that can be widely distributed, have an affordable cost and appropriate antimicrobial activity for use in the treatment of cryptococcal meningocerebralitis is imperative. In this sense, sertraline has shown antibacterial, antiparasitic, antiviral and antitumoral properties.\(^6,7,26\) Additionally, this drug reaches levels in CNS 20–40 times higher than in blood\(^19,27,28\) and has the advantage of being inexpensive, available worldwide and well tolerated with few side effects even after prolonged use.\(^29\)

Our findings are consistent with previous reports, which demonstrate growth inhibition of *Cryptococcus* spp. with sertraline, obtaining a MIC range of 1–8 μg/ml.\(^6,7\) Levels of clinical relevance since concentrations achieved after administration of standard doses of 50–200 mg/day in blood reach a range varying between 55 and 250 ng/ml.\(^27\) Furthermore, it has been shown in some studies that this concentration is 20 to 40 times higher in cerebrospinal fluid,\(^28\) validating the potential therapeutic use of sertraline for cryptococcal meningitis. One of the most valuable aspects of sertraline as a potential anti-cryptococcal drug is its superior ability to accumulate in the CNS relative to other antifungals, being necessary more days of treatment to achieve optimal drug levels. This property is particularly critical in the treatment of cryptococcosis, given that the fungus preferentially proliferates in brain tissue.\(^7\)

The animal models represent a valuable useful approach to mimic the progression and clinical signs of certain infections in humans, in which the infectious burden can be a sensitive parameter for studies of drug efficacy, comparative virulence or disease progression.\(^30\) Different techniques have been widely utilized for the study of fungal infections associated with the CNS. Intracerebral models of infection are particularly used to test therapeutic options for treatment of diverse human mycosis.\(^30\) This animal model represents a better option to neurotropic infection than intraperitoneal, intranasal, or intratracheal inoculation route for evaluation of

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**Figure 3.** Representative histopathological mucicarmin-stained brain sections of mice intracranially challenged with 1,500 cfu/mouse of *C. neoformans USC1597* by day 7 post-inoculation without treatment (a) and subjected to different treatments: fluconazole 15 mg/kg (b), amphotericin B 3 mg/kg (c), sertraline 3 mg/kg (d), sertraline 10 mg/kg (e) and sertraline 15 mg/kg (f). Original magnification ×400, metric bar 50 μm.
cryptococcal meningoencephalitis, due to a better emula-
tion of the human infection.15

As was expected, the inoculum concentration we used
allowed infection establishment without animal death, as
previously published.15 During the course of the disease,
untreated mice experienced light weight loss, while ani-
mals treated with amphotericin B maintained their initial
weight and those animals treated with fluconazole and
any dose of sertraline showed some weight gain. This is
in accordance with findings by Larsen et al.13 Addition-
ally, hyperactive/overstimulated behavior was observed
in mice treated with sertraline. Otherwise, slight pilo-er-
etion episodes were sporadically noted in animals, principally
by day 6 post-challenge, ocular alterations and motor im-
pairment were not detected in either group throughout
the study. We found that independently of the evident in-
crease in cryptococcal antigen titer by day 7 post-infection, treatment
with amphotericin B was able to reduce 2-logs the
fungal tissue burden in brain (P < .001) and practically
sterilized the spleen of mice under this therapeutic regi-
men. On the other hand, treatment with sertraline at a
dose of 15 mg/kg originated a 1-log reduction of fungal
tissue burden both in the brain and spleen (P < .001) with
respect to untreated control animals. In conclusion, treat-
ment with sertraline at a dose of 15 mg/kg reduced the
fungal burden in the brain and spleen with an efficacy com-
parable to that of fluconazole, as recently reported by Zhai
et al. in a murine model of systemic cryptococcosis.7 The
fungicidal activity of sertraline and its synergy with flucon-
azole against Cryptococcus spp. has already been observed
in vitro6 and in vivo models.7,11 Recent evidence indicates
that the antifungal mechanism of sertraline is probably
through perturbation of translation and inhibition of fungal
protein synthesis.7

The present study provides evidence of the potent anti-
cryptococcal activity of sertraline in vitro and in vivo, of-
fering a potential option for the management of human
cryptococcosis. At the same time, it is the first report of
therapeutic efficacy of sertraline in a murine model of C.
neoformans meningoencephalitis, which is the most com-
mon clinical presentation of the disease. Further random-
ized clinical trials need to be performed to demonstrate the
relevance of sertraline as an antifungal agent for meningal
cryptococcosis.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are
responsible for the content and the writing of the article.

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