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Pharmacokinetics/pharmacodynamic correlations of fluconazole in murine model of cryptococcosis

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ABSTRACT

The emergence of fluconazole-resistant *Cryptococcus gattii* is a global concern, since this azole is the main antifungal used worldwide to treat patients with cryptococcosis. Although pharmacokinetic (PK) and pharmacodynamic (PD) indices are useful predictive factors for therapeutic outcomes, there is a scarcity of data regarding PK/PD analysis of antifungals in cryptococcosis caused by resistant strains. In this study, PK/PD parameters were determined in a murine model of cryptococcosis caused by resistant *C. gattii*. We developed and validated a suitable liquid chromatography-electrospray ionization tandem mass spectrometry method for PK studies of fluconazole in the serum, lungs, and brain of uninfected mice. Mice were infected with susceptible or resistant *C. gattii*, and the effects of different doses of fluconazole on the pulmonary and central nervous system fungal burden were determined. The peak levels in the serum, lungs, and brain were achieved within 0.5 h. The AUC/MIC index (area under the curve/minimum inhibitory concentration) was associated with the outcome of anti-cryptococcal therapy. Interestingly, the maximum concentration of fluconazole in the brain was lower than the MIC for both strains. In addition, the treatment of mice infected with the resistant strain was ineffective even when high doses of fluconazole were used or when amphotericin B was tested, confirming the cross-resistance between these drugs. Altogether, our novel data provide the correlation of PK/PD parameters with antifungal therapy during cryptococcosis caused by resistant *C. gattii*.

1. Introduction

Cryptococcosis caused by *Cryptococcus gattii* has emerged as an important cause of morbidity and mortality in healthy individuals. The lesions caused by this fungal pathogen generally occur in the lungs and the central nervous system (CNS) and manifest as pulmonary disease and meningoencephalitis, respectively. The limited number of antifungal drugs available to treat patients with cryptococcosis is a global concern, since this disease is lethal when untreated and tends to be recalcitrant to treatment, even when antifungals are used correctly (Chen et al., 2012; Chen et al., 2014; Franco-Paredes et al., 2015; Gullo et al., 2013; Smith et al., 2014).

Fluconazole is the most widely used antifungal worldwide, and there are countries where this triazole is the only available drug for treating *Cryptococcus* spp. infections (Sudan et al., 2013). Fluconazole has low toxicity, an excellent safety profile, a long half-life, and linear pharmacokinetics (PK). Furthermore, fluconazole is unaffected by food or gastric acidity and penetrates well into the CNS, but the emergence of fluconazole-resistant *C. gattii* (Matos et al., 2012; Pfaller et al., 2009) is limiting its usage. In addition, amphotericin B, the main polyene used to treat meningoencephalitis, exhibits dose-dependent nephrotoxicity, and possible cross-resistance with fluconazole, which may limit the use of this agent (Laniado-Laborin and Cabrales-Vargas, 2009).

The evaluation of the pharmacokinetics (PK) and pharmacodynamics (PD) of fluconazole may provide useful data regarding the outcome of cryptococcosis treatment. PK/PD indices describe the relationships between the minimum inhibitory concentration (MIC) and the peak concentration (C_{max}/MIC), area under the curve (AUC/MIC), and the percentage of time that drug concentrations exceed the MIC (T > MIC), yielding predictive data about the therapy (Andes, 2006).

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However, the PD data of fluconazole use in fluconazole-resistant *C. gattii* treatment are scarce. Moreover, there are few studies focused on the *in vivo-in vitro* correlation of cryptococcosis caused by *C. gattii* (Mendes et al., 2010).

The aim of this study was to evaluate the correlation between PK/PD modeling and antifungal resistance of *C. gattii* in a murine model of cryptococcosis. We developed and validated a liquid chromatography-electrospray ionization tandem mass spectrometry method (LC-ESI-MS/MS) for the determination of fluconazole concentrations in the serum, lungs, and brain of mice; the data were applied for PK/PD analyses and correlated with the outcome of diseases caused by susceptible or resistant *C. gattii*. Overall, our data suggest that PK/PD indices have an important predictive value of treatment outcome and that dose adjustment should be applied for cryptococcosis caused by resistant *C. gattii*.

2. Material and methods

2.1. C. gattii and antifungal drug susceptibility testing

We tested two strains of *C. gattii* from the culture collection of the Laboratório de Micologia/Universidade Federal de Minas Gerais, Brazil: L27/01 and L27/01$_F$ (mutant with resistance to fluconazole). The induction and maintenance of resistance to fluconazole were performed according to methods described in our previous study (Santos et al., 2014). Briefly, the L27/01 strain of *C. gattii* was cultivated in high fluconazole concentrations and developed resistance, even when cultivated in drug-free media more than 100 times. This phenotype was well characterized in our previous study (Santos et al., 2014). The MICs for fluconazole and amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) of L27/01 and L27/01$_F$ were determined by the microdilution method proposed by the Clinical and Laboratory Standards Institute (CLSI) M27-A3 method (CLSI, 2008) in three independent experiments performed in duplicate (Ferreira et al., 2013; Ferreira et al., 2015; Santos et al., 2012).

2.2. Ethics statement

C57BL/6 male mice (21–24 g) obtained from Centro de Bioterismo (CEBIO) at Universidade Federal de Minas Gerais, Brazil, were used in this study. The mice had free access to food and water and were kept in a room that had a 12 h light/12 h dark cycle. All mice were housed in clean bedding (five mice per cage). The protocol of the animal studies was approved by the Comissão de Ética no Uso de Animais (CEUA) from Universidade Federal de Minas Gerais (Protocol 170/2011). All mice were monitored twice daily. All efforts were made to minimize suffering. Any mice that appeared moribund (*e.g.* intense piloerection, convulsions, lack of locomotor activity) were euthanized under anesthesia [intraperitoneal (i.p.) injection of ketamine hydrochloride (60 mg/kg) and xylazine (10 mg/kg) in sterile saline] by cervical dislocation by experienced animal handlers.

2.3. Measurement of fluconazole concentration in the serum, lungs, and brain

A LC-ESI-MS/MS method was developed and validated for fluconazole (Sigma-Aldrich) quantification in the serum, lungs, and brain of mice. Analyses were carried out on a Waters system (Milford, MA, USA), composed of a 1525 μ binary pump, a 2777 sample manager, a TCM/CHM column (Temperature Control Module/Column Heather Module) oven, and a Quattro LC triple quadrupole mass spectrometer equipped with an electrospray ion source. MassLynx v.4.1 software was used for data acquisition and analysis (Waters Corporation). Eight calibration standards (0.1, 1, 5, 10, 25, 50, 75, and 100 μg/ml) were prepared in untreated mice serum, and six concentrations (0.1, 1, 5, 10, 15, and 25 μg/ml) in untreated mice lung or brain samples. A 25 μl-aliquot of the internal standard solution (300 μg/ml ketoconazole in methanol) was added to 80 μl of serum, lung, or brain sample. After agitation, samples were extracted with 2 ml of ethyl acetate, methyl-*tert*-butyl ether, and dichloromethane (4:3:3 v/v) and centrifuged at 3400 rpm for 5 min at 4 °C. An aliquot of 1.6 ml of the organic layer was evaporated to dryness and reconstituted in the mobile phase (200 μl). This procedure also was applied to the samples obtained from mice used in the PK studies. A 20-μl aliquot was injected into the chromatography system. Chromatographic separation was performed on a ShinPack C18 column (100 × 4.6 mm i.d.; 5-μm particle size) from Shimadzu (Kyoto, Japan) maintained at 30 °C. The mobile phase consisted of 2 mM aqueous ammonium acetate containing 0.025% (v/v) formic acid and methanol (25:75 v/v), at a flow rate of 1 ml/min. Mass spectrometric detection was performed using electrospray ionization in positive mode. Ion signals were recorded by selective reaction monitoring (SRM) using the following transitions: m/z 307.1 → m/z 237.9 for fluconazole and m/z 531.2 → m/z 489.3 for ketoconazole (internal standard - IS). The method was properly validated for quantitation of fluconazole in the serum, lung, and brain.

Selectivity was evaluated by assaying blank samples of serum, lung and brain from different mice, who did not receive any drug. These samples were compared to those containing fluconazole or IS at the lower limit of quantitation (LLOQ).

Linearity was assessed by eight-point calibration curves in serum (0.1, 1, 5, 10, 25, 50, 75, and 100 μg/ml) and six-point calibration curves in lung and brain (0.1, 1, 5, 10, 15, and 25 μg/ml). Each point was assayed in duplicate, on 3 consecutive days. The curves were constructed by plotting the peak area ratio of fluconazole to the IS *versus* the concentration of fluconazole. The curves were evaluated by residuals and fitted by weighted quadratic regression (serum) or weighted linear regression (lung and brain). The LLOQ was established as the lowest concentration of calibration curve at which precision and accuracy was within 20%. In addition, the analyte responses at this concentration should be at least 5 times the baseline noise.

To evaluate the precision and accuracy of the method, replicates of QC samples at 3 concentration levels (0.3; 40 and 80 μg/ml for serum and 0.3; 10 and 20 μg/ml for lung and brain) were analyzed on different days. Intra-run and inter-run precisions were calculated and expressed as relative standard deviation (RSD%).

The extraction recovery of the method was determined by comparing the peak areas obtained from the matrix samples spiked with fluconazole with those of standard solutions in mobile phase at the same concentrations (0.3; 40 and 80 μg/ml for serum and 0.3; 10 and 20 μg/ml for lung and brain). The recovery of IS was determined in a similar way, at the working concentration.

The matrix effect was evaluated to verify whether the potential ion suppression or enhancement due to the co-elution matrix components existed in the analysis. The peak areas of fluconazole and IS from the spike-after-extraction samples were compared with those of the standard solutions in the mobile phase, at the same concentrations. This experiment was carried out at low and high QC concentrations of fluconazole and working concentration of IS.

2.4. PK determination

The validated method was used to determine the levels of fluconazole in the serum, lungs, and brain of uninfected mice. Animals were treated daily with 75 mg/kg fluconazole. Fluconazole (75 mg/kg/animal) was administered *per os* in 60 C57BL/6 male mice. At different time points (0, 0.17, 0.33, 0.50, 0.75, 1, 2, 3, 4, 6, 8, and 10 h), after fluconazole administration, a group of six animals was euthanized under anesthesia by cervical dislocation. The blood, lungs, and brain from each of these animals and from each animal in an additional group of six non-treated mice (control group) were collected. The blood was centrifuged immediately at 4000 rpm for 10 min, and the whole organ was completely ground with manual disperser and mixing (POLYTRON® PT 1200 E) in water during 90 s before centrifugation.