Introduction
Cryptococcal meningitis occurs in approximately 8–30% of the patients with the acquired immune deficiency syndrome (AIDS). Despite antifungal therapy, mortality remains high (6–29%). A study in AIDS patients with cryptococcal meningitis suggested that fluconazole was not as effective as amphotericin B. It is thus now recommended that treatment should be started with amphotericin B and flucytosine and then switched to fluconazole. Fluconazole is still often used as first-line therapy because of frequent toxic effects due to amphotericin B. In experimental models of cryptococcosis, fluconazole was effective when administered early after inoculation. In the murine model of locally established cryptococcal meningitis, Ding et al. reported recently that the range of effective dose combinations of fluconazole and flucytosine reduced progressively as the severity of infection increased. It is not known whether fluconazole remains effective in a model of sustained disseminated cryptococcosis (i.e. in a situation resembling natural infection in humans). In addition to antifungal therapy, cranial hypertension should be treated as a high opening pressure of the cerebrospinal fluid (CSF) at the time of the diagnosis has been associated with delayed sterilization of the CSF and reduced survival. Short-term steroid therapy is prescribed as an adjuvant measure for this reason. Although such a regimen has been successfully used in children with bacterial meningitis, a clear demonstration of its efficacy for cranial hypertension due to cryptococcosis is still lacking. Whether steroids affect fungal growth or interfere with the pharmacokinetics and efficacy of fluconazole, as has been reported for some antibiotics, is unknown.

The aims of this study were: (i) to assess the impact of fluconazole treatment timing on efficacy in disseminated murine cryptococcosis; (ii) to determine the influence, if any, of short-term dexamethasone, a steroid with marked antiinflammatory properties, on the mycological outcome and on pharmacokinetics of fluconazole; and (iii) to compare the performance of HPLC and a bioassay in the measurement of fluconazole concentrations in plasma and tissues.

Fluconazole, with or without dexamethasone for experimental cryptococcosis: impact of treatment timing

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The time of initiation of fluconazole treatment with or without dexamethasone, and the impact on mycological outcome and drug pharmacokinetics were assessed in a murine model of disseminated cryptococcosis. Non-infected mice and mice with disseminated cryptococcosis were given saline, dexamethasone, or fluconazole ± dexamethasone, 1 or 8 days after infection. CFUs were counted in tissues, and fluconazole concentrations were determined in plasma and tissues by HPLC and a bioassay. Despite fluconazole tissue and plasma concentrations which were above the minimal inhibitory concentration, the numbers of CFUs in brain and lung tissues were reduced after early \((P = 0.002\) and 0.04, respectively), but not after late fluconazole treatment. The administration of dexamethasone did not have a deleterious effect on the number of CFUs, fluconazole pharmacokinetics or antifungal activity. In conclusion, the size of the fungal burden influences the effective level of fluconazole activity in lung and brain. These results strongly suggest that potential antifungal agents should be studied following both early and late administration in experimental cryptococcosis.
Materials and methods

Infecting organism

Cryptococcus neoformans (# IP 1222.80) maintained by monthly transfer on Sabouraud agar slants was used as the infecting organism. The minimum inhibitory concentration (MIC) of fluconazole, (determined by a broth microdilution method) for this organism, was 1 mg/L. The inoculum was prepared by growing the organism in yeast nitrogen base broth, supplemented with 2% glucose (Difco Laboratories, Detroit, MI, USA) for 18 h on a rotary shaker at 30°C. The yeast cells were washed in sterile saline (NaCl) and then counted with a Malassez haemocytometer. The precise number of viable cells was determined by duplicate plate counts on Sabouraud–chloramphenicol agar.

Mice were inoculated intravenously in the lateral tail vein with $2.6 \times 10^5$ yeast cells/mouse.

Animals

Male outbred OF1 mice (Ifa Credo, l’Arbresle, France) (mean body weight 22 g) were housed 5–7/cage in our animal facilities and received food and water ad libitum. Animal experimentation guidelines were followed in all studies.

Drugs

Fluconazole, obtained as a powder from Pfizer Central Research (Sandwich, Kent, UK), was dissolved in sterile distilled water (DW) to obtain 5 mg/kg (fluconazole 5) or 15 mg/kg (fluconazole 15) in a 0.2-mL volume. A fresh solution was prepared daily and administered by gavage every 8 h, taking into account fluconazole pharmacokinetics in mice ($t_{1/2}$ of 4.8 h). Dexamethasone, obtained as a 4 g/L solution from Merck Sharp and Dohme-Chibret Laboratories (Riom, France), was diluted in NaCl to obtain 0.15 mg/kg in a 0.2 mL volume. The drug was injected (ip) every 8 h, precisely 30 min before fluconazole administration. Diluents of each drug were administered in the same volume (DW po and NaCl ip). Treatments were administered for 3 days (nine injections).

Therapeutic regimens

Two sets of experiments were performed: experiment A was a pharmacokinetic study in non-infected mice. Four groups of five or six non-infected mice were treated with fluconazole 5 + NaCl, fluconazole 5 + dexamethasone, dexamethasone + DW or DW + NaCl. Experiment B combined a comparison of pharmacokinetics in non-infected versus infected mice, and a mycological study. Treatment with fluconazole 15 was started at day one (early) or day eight (late) after inoculation, as summarized in Table I. Non-infected mice were treated at the same times. Before the last fluconazole administration, blood was drawn (approximately 150 μL by puncture of the retroorbital sinus) from non-infected mice (experiments A and B, groups 1 and 2) to determine trough fluconazole levels by bioassay.

After completion of treatments, exactly 1 h after the last fluconazole administration, mice were anaesthetized by chloroform inhalation. Blood was collected by heart puncture, centrifuged and plasma samples individually aliquoted and frozen at −80°C (HPLC assay) or −20°C (bioassay). Lungs and brains were aseptically removed, divided into two portions and weighed. The largest part was stored frozen at −80°C until processing for HPLC. The second part was ground in 250 or 500 μL of sterile DW. The

<table>
<thead>
<tr>
<th>Group no.</th>
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homogenate was used to perform cfu counts and its supernatant immediately frozen at –20°C for subsequent bioassay dosage of fluconazole.

**Fungal burden**

Ten-fold serial dilutions of tissue homogenates were plated (100 μL) in duplicate on Sabouraud–chloramphenicol agar and colony counts determined after incubation at 27°C for 2 days. Results are expressed as log$_{10}$ cfu/g of tissue ± standard error of the mean (S.E.M.). The threshold of detection was approximately 20 cfu/organ. For the apparently sterilized organs, a value of 0 was arbitrarily assigned.

**Fluconazole concentration measurement**

**HPLC.** After thawing, the tissue samples were homogenized in 0.5 mL of sterile DW. Plasma and tissue samples were blindly assayed for fluconazole by HPLC using the method recommended by Pfizer. Briefly, fluconazole was extracted with ethylacetate and separated by reversed-phase liquid chromatography. Fluconazole and the internal standard were detected by UV spectrophotometry at 260 nm. The limit of detection was 0.15 mg/L. Interrun coefficients of variation of the method were 12% for 0.25 mg/L, 5% for 2 mg/L and 3% for 20 mg/L. The range of assay linearity was 0.15–20 mg/L. Calibration curves were determined by linear regression of peak height versus plasma standard concentrations (use of peak area instead of detection was approximately 20 cfu/organ. For the apparently sterilized organs, a value of 0 was arbitrarily assigned.

**Fluconazole bioassay.** Fluconazole was measured in plasma and supernatants of tissue homogenates, as described previously. Samples were assayed blindly. Preliminary experiments showed that the standard curves obtained when known concentrations of fluconazole in lung or brain homogenates or in serum (data not shown) were assayed gave similar results. Thus, concentrations in all samples, including supernatants of tissue homogenates were determined using the standard curve obtained by assaying known concentrations of fluconazole in normal mouse serum. Control specimens of plasma or tissue supernatants from mice receiving dexamethasone alone had no antifungal activity (data not shown). Inhibition zone diameter was plotted against log$_{10}$ fluconazole concentration. Unknown concentrations were calculated by extrapolating from the standard curve. Calibration curves were not forced through the origin. The coefficient of variation was shown to be approximately 10%. Results are expressed in μg/g of organ using the following formula: $C \times V/W$, where $C$ refers to the concentration measured in mg/L, $W$ to the weight in g, and $V$ the homogenization volume in mL.

**Statistical analysis**

Statistical analyses were performed using Abacus Concepts, Statview II (Abacus Concepts, Inc., Berkeley, CA, USA). The organs in which a significant treatment effect on a given day was observed (as determined by the non parametric Kruskal–Wallis test) were further analysed by the non parametric Mann–Whitney test to determine the comparative effects of the treatment regimen. A similar analysis was performed to determine the effect of treatment timing, dexamethasone combination or infection on fluconazole concentrations. Comparisons of tissue/plasma concentration ratios were made using the paired Student’s t test after analysis of variance (ANOVA). Values obtained for concentrations of fluconazole, determined by HPLC and bioassay, were compared using orthogonal regression analysis. The level of significance was 0.05.

**Results**

**Evaluation of C. neoformans infection**

In this model of disseminated cryptococcosis, the infection progressed slowly between days four and 11 in the untreated control mice which gained weight (23 ± 2 g and 28 ± 2 g), despite significant increases in lung cfus ± S.E.M. (5.0 ± 0.6 and 7.3 ± 0.6; $P = 0.01$) and brain cfus ± S.E.M. (4.2 ± 0.1 versus 6.1 ± 0.2; $P = 0.001$). After early treatment with fluconazole, organs from two mice (two lungs, one brain) were apparently sterilized. The counts of cfus in brain ($P = 0.002$) and lung ($P = 0.04$) were significantly decreased in comparison with those of animals given NaCl alone. Late treatment failed to sterilize any of the organs and had little effect on cfu counts ($P > 0.05$, Kruskal–Wallis test) (Figure 1).

Dexamethasone alone did not modify the fungal burden in the organs, regardless of the time that treatment was started. In combination with fluconazole, early administration of dexamethasone was no better than fluconazole alone, whether sterilization was the end point (two lungs and one brain from three mice) or reduction in cfus in lung ($P = 0.009$) and brain ($P = 0.002$) (NaCl as control) (Figure 1). Delayed treatment with fluconazole + dexamethasone had no significant effects on cfu counts ($P > 0.05$, Kruskal–Wallis test). Lungs from infected mice treated 8 days after infection with dexamethasone + fluconazole weighed significantly less than those from control mice (121 ± 28 mg versus 223 ± 49 mg; $P < 0.05$), while dexamethasone alone (184 ± 38 mg) or fluconazole alone (173 ± 23 mg) had little effect.
Fluconazole pharmacokinetics

For animals receiving fluconazole alone, multiplying the dosage by three led to a three-fold increase in plasma concentrations, and at least a four-fold increase in the concentrations achieved in tissues. Fluconazole concentrations by HPLC were $3.1 \pm 0.6 \text{ mg/L}$ versus $9.4 \pm 2.4 \text{ mg/L}$ in the plasma, $4.1 \pm 1.1 \text{ versus } 16.3 \pm 3.7 \mu \text{g/g}$ in the brain and $8.8 \pm 2.9 \mu \text{g/g}$ versus $39.5 \pm 8.4 \mu \text{g/g}$ in the lungs of non-infected animals, receiving fluconazole 5 or early treatment with fluconazole 15, respectively. Fluconazole was not detectable in tissues by bioassay when animals had received $5 \text{ mg/kg tid}$ (experiment A). Detectable concentrations were measured in the brain (except in three specimens) when the dosage was increased to $15 \text{ mg/kg tid}$ (Table II), whereas they were still undetectable in most of the lung specimens (Figure 2). Trough levels, undetectable after treatment with fluconazole 5, were measurable after early treatment with fluconazole 15 ($1.5 \pm 0.6 \text{ mg/L}$) and were unaffected by combination with dexamethasone ($1.4 \pm 0.6 \text{ mg/L}$). Tissue/plasma fluconazole concentration ratios measured by HPLC in lung (mean ratio $\pm$ S.D.: $4.5 \pm 0.9$; range: $2.6–6.4$) and brain ($1.8 \pm 0.2$; $1.4–2.5$) ($P = 0.0001$) showed significant differences. Comparisons of fluconazole concentrations between groups (Table II) showed that HPLC-determined plasma, lung and brain concentrations were not influenced by infection, time of treatment initiation following inoculation or combination with dexamethasone ($P > 0.05$, Kruskal–Wallis test). Using bioassay, plasma fluconazole concentrations were significantly influenced by infection, only if fluconazole was administered alone early after infection ($P = 0.01$) or when

Figure 1. Effect of treatment with NaCl (■), dexamethasone (dexamethasone) (□), fluconazole alone (■) or in combination with dexamethasone (■) on fungal burden in lungs and brains of mice treated from day 1 to 4 (a) or day 8 to 11 (b) after infection with *C. neoformans* (mean ± S.E.M.). *, $P = 0.04$ versus NaCl (Mann–Whitney); †, $P = 0.009$ versus NaCl (Mann–Whitney); ‡, $P = 0.002$ versus NaCl (Mann–Whitney).

Figure 2. Correlations between fluconazole concentrations determined using the bioassay values plotted versus those measured by HPLC in (a) plasma (mg/L), (b) brain (µg/g) or (c) lung tissue (µg/g) (experiments A and B). Each dot represents an individual sample. For the organs in which fluconazole was not detectable by bioassay, the value arbitrarily assigned was $2 \mu \text{g/g}$. 
**Fluconazole treatment timing in cryptococcosis**

Table II. Mean ± s.d. plasma and tissue concentrations of fluconazole measured in mice inoculated or not with *C. neoformans* and treated from day 1 to 4 (early treatment) or day 8 to 11 (late treatment) with fluconazole (15 mg/kg tid) alone or combined with dexamethasone (0.15 mg/kg tid)

<table>
<thead>
<tr>
<th>Groups of mice</th>
<th>Number of mice</th>
<th>HPLC-determined fluconazole concentrations</th>
<th>Bioassay-determined fluconazole concentrations</th>
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<tr>
<td></td>
<td></td>
<td>plasma (mg/L) lung (μg/g) brain (μg/g)</td>
<td>plasma (mg/L) brain (μg/g)</td>
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<td>Early treatment</td>
<td></td>
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<td></td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>5</td>
<td>9.4 ± 2.4 39.5 ± 8.4 16.3 ± 3.7</td>
<td>7.7 ± 1.4d 9.8 ± 3.2</td>
</tr>
<tr>
<td>Group 2</td>
<td>5</td>
<td>8.8 ± 1.7 33.2 ± 6.5 13.8 ± 3.3</td>
<td>7.5 ± 1.3 10.5 ± 5.0</td>
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<tr>
<td>Group 5</td>
<td>7</td>
<td>9.8 ± 1.3 40.6 ± 3.2 18.9 ± 3.4</td>
<td>10 ± 1.1 14.4 ± 4.5c</td>
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<tr>
<td>Group 6</td>
<td>7</td>
<td>10.5 ± 1.5 45.9 ± 9.0 19.3 ± 3.5</td>
<td>9.5 ± 1.8 15.3 ± 6.5</td>
</tr>
<tr>
<td>Late treatment</td>
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<tr>
<td>Non-infected</td>
<td></td>
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<td></td>
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<tr>
<td>Group 7</td>
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<td>8.3 ± 1.6 38.2 ± 11.4 14.6 ± 3.1</td>
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<td>Group 8</td>
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<td>Group 11</td>
<td>6</td>
<td>9.3 ± 1 39.9 ± 7.6 18.3 ± 2.8</td>
<td>8.7 ± 1.3b 10.7 ± 1.7</td>
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<td>Group 12</td>
<td>7</td>
<td>8.1 ± 1.8 45 ± 10.6 14.6 ± 3</td>
<td>6.4 ± 1.1 10 ± 4.4d</td>
</tr>
</tbody>
</table>

*P = 0.01 versus infected mice treated with the same drug (Mann–Whitney).

Fluconazole concentration reported in one brain.

Fluconazole was not detectable in one brain.

ND, not determined.

the fluconazole + dexamethasone combination was compared with fluconazole alone administered to infected mice treated 8 days after infection (*P = 0.01*).

**Comparison between bioassay and high-pressure liquid chromatography for the measurement of fluconazole concentrations**

Comparison of plasma fluconazole concentrations measured using both assays were concordant as shown by their slope, which was not significantly different from 1, and an intercept which was not significantly different from 0. The correlation (*r*² = 0.506) was significant (*P < 0.0001*) (Figure 2). Similar analyses performed for brain fluconazole concentrations showed that both methods were correlated (*r*² = 0.59, *P < 0.0001), but not concordant (intercept different from 0). Since most of the bioassay values were missing, the correlation was not checked for the lungs.

**Discussion**

Animal experiments are both expensive and time-consuming, so investigators usually select the most convenient models. These are acute infections in which median survival times do not exceed 20 to 30 days, and early treatments are usually administered once or twice a day over a short time period.6–8,18,22,23 In this study, the main purpose was to investigate drug efficacy in the context of infection severity. An inoculum that generated a slowly progressing disseminated infection, with no death during the observation period was chosen. The severity of the infection by day 8 was arbitrarily taken as a reflection of the clinical picture in man. Our results clearly demonstrated a difference in in vivo antifungal efficacy dependent on the time of initiation of fluconazole treatment. When fluconazole was administered 24 h after the infecting dose, the degree of brain and lung infection was reduced and some organs apparently sterilized. At 8 days, the effect was much diminished.

The diminished efficacy of antifungal treatment, administered late after inoculation, could be attributed to several factors. The first could be inadequate dosing. A wide variety of treatment regimens have been reported in the literature.6–8 In the protocol selected here, animals were treated individually and the pharmokinetics of fluconazole treated 24 h after the infecting dose, the degree of brain and lung infection was reduced and some organs apparently sterilized. At 8 days, the effect was much diminished.

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treatment. Although optimum methods of antifungal susceptibility testing (providing a reliable correlation between MIC and clinical outcome) are poorly defined, it is reasonable to think that fluconazole concentrations should be above the MIC both in plasma and in infected tissues. After only 3 days of treatment with 15 mg/kg tid, plasma fluconazole levels were approximately 10 mg/L, a concentration obtained in adults after a daily drug intake of 200 mg. All the concentrations measured in plasma and tissues were above the MIC. In another model, high fluconazole concentrations (>30 mg/L) in the CSF did not produce a local reduction in the number of cfus. In contrast, a strong correlation was observed by Velez et al. between the MIC for the infecting isolates and the outcome of murine infection as assessed by both cfu counts and survival. This result was obtained when the treatment was started 1 day after inoculation, and MICs were determined after 24 h of incubation; MIC and outcome were no longer correlated when the in-vitro incubation was prolonged. Doubtless numerous parameters influence the in-vivo activity of fluconazole.

The second explanation for the striking ineffectiveness of late fluconazole treatment could lie in a modification of the pharmacokinetics of the drug or of its antifungal activity. Since concentrations achieved both in plasma and tissues were unchanged despite progression of infection, it is more likely that the ineffectiveness of the drug was related to the difference in the fungal burden between days 4 and 11, or to the fungistatic nature of the drug. This poor response was observed, despite detection of fluconazole by bioassay, showing it to be present in a biologically active form not only in plasma, as previously demonstrated but also in tissues. The effect of fungal burden on antifungal efficacy shown here in vivo is well known in vitro, and is influenced by the fungicidal capacity of the drug. Using a different model, Ostrosky-Zeichner et al. showed that, even when administered 7 days after inoculation, amphotericin B, a fungicidal drug, lowered the numbers of cryptococci in the brain. Our results support those obtained already in humans. In AIDS patients, time to CSF sterilization was retarded and the percentage of early deaths was increased when fluconazole was used instead of amphotericin B for the primary treatment of cryptococcal meningitis. Thus, our data suggest that the time of treatment initiation (i.e. severity of the fungal infection) should be one of the parameters studied when determining the optimal regimen and comparing activity of new drugs with reference drugs in in-vivo experiments.

A further concern was the potentially deleterious effect(s) of dexamethasone on the course of the infection, on the antifungal activity of fluconazole or on its pharmacokinetics. None of these parameters was affected in this study, confirming a previous report which showed, in a different model of cryptococcal infection, that dexamethasone did not alter fungal burden in the brain. Dexamethasone did not modify the antifungal activity of fluconazole as measured by cfu counts in tissues, nor did it change that of amphotericin B in the study by Ostrosky-Zeichner et al. Previous studies dealing with antibacterial drugs showed decreased bacterial killing in the CSF or an increased rate of positive cultures when dexamethasone was combined with an antibiotic. Thus, these results do not provide evidence for any deleterious effect secondary to the short-term use of dexamethasone in this experimental model. A clear demonstration of the beneficial effect of dexamethasone for the treatment of cranial hypertension is still lacking in cryptococcal meningitis in humans, but it has been shown to reduce CSF pressure in other experimental or clinical settings. Our results do not suggest any contraindications for the short-term use of dexamethasone in the treatment of complications during acute cryptococcal meningitis.

HPLC and bioassay fluconazole determinations were correlated for plasma and brain. The correlations demonstrated are supported by data previously reported in human and rabbit plasma and CSF. The lower concentrations obtained by bioassay of plasma and tissues are supported by the data of Palou de Fernandez et al. They also reported lower fluconazole concentrations by bioassay than by gas chromatography in murine plasma. The fact that fluconazole was not detectable in most of the lung specimens by bioassay may be explained by a lower yield attributable to the different extraction procedures (weight of the organ, temperature). Since fluconazole is only weakly metabolized and the metabolites are inactive, both assays are appropriate for the measurement of fluconazole concentrations in plasma if assays are required to document non-compliance with treatment.

In these experiments, the brain/plasma concentration ratio was approximately 2, a figure close to that found in non-infected adults after 4 days of treatment with 400 mg/day (average ratio, 1.33; range, 0.70–2.39). In the mouse lungs, fluconazole concentrations were found to be twice those measured in the brain. Similar differences in tissue/plasma concentration ratios have been found in humans and rabbits, although individual tissue concentrations varied with the species studied and experimental design. Finally, no increases of fluconazole concentrations in the tissues of infected mice compared with those measured in non-infected tissues were observed.

In conclusion, this study demonstrates the influence of fungal burden on the in-vivo efficacy of fluconazole. The antifungal activity of new drugs should be assessed on experimental infections both of recent onset and longer duration.

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Fluconazole treatment timing in cryptococcosis

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