Fluconazole plus Cyclosporine: a Fungicidal Combination Effective against Experimental Endocarditis Due to Candida albicans

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Recent observations demonstrated that fluconazole plus cyclosporine (Cy) synergistically killed Candida albicans in vitro. This combination was tested in rats with C. albicans experimental endocarditis. The MICs of fluconazole and Cy for the test organism were 0.25 and >10 mg/liter, respectively. Rats were treated for 5 days with either Cy, amphotericin B, fluconazole, or fluconazole-Cy. Although used at high doses, the peak concentrations of fluconazole in the serum of rats (up to 4.5 mg/liter) were compatible with high-dose fluconazole therapy in humans. On the other hand, Cy concentrations in serum (up to 4.5 mg/liter) were greater than recommended therapeutic levels. Untreated rats demonstrated massive pseudomypohyal growth in both the vegetations and the kidneys. However, only the kidneys displayed concomitant polymorphonuclear infiltration. The therapeutic results reflected this dissociation. In the vegetations, only the fungicidal fluconazole-Cy combination significantly decreased fungal densities compared to all groups, including amphotericin B (P < 0.0001). In the kidneys, all regimens except the Cy regimen were effective, but fluconazole-Cy remained superior to amphotericin B and fluconazole alone in sterilizing the organs (P < 0.0001). While the mechanism responsible for the fluconazole-Cy interaction is hypothetical, this observation opens new perspectives for fungicidal combinations between azoles and other drugs.

Progress in the management of severely ill patients has been accomplished by an increase in the number of Candida infections (2). In profoundly immunocompromized individuals, and especially in neutropenic patients, the primary treatment of such infections is parenteral amphotericin B (Amb). This is one of the few compounds that has fungicidal activity in vitro and is still considered as a kind of a paradigm for antifungal therapy in vivo. Yet the pharmacodynamics of Amb remains poorly defined (13). Moreover, the conventional form of Amb is toxic, and its utilization is often limited by side effects. The lipid formulations of Amb are better tolerated but are much more expensive. Thus, the search for more convenient alternatives is warranted.

Azoles such as fluconazole show good activity against Candida albicans and have both an excellent oral bioavailability and a low toxicity (3). However, like the other compounds of this family, fluconazole is only fungistatic. Its efficacy relies on the presence of cellular host defenses such as polymorphonuclear cells (PMN), in the case of disseminated disease, and CD4 lymphocytes, in the case of mucosal infection (20). Therefore, while fluconazole is as effective as Amb against C. albicans in non-neutropenic patients (33), uncertainties still exist about its efficacy in the neutropenic host (7). Moreover, the increasing use of azoles has resulted in an epidemiological shift to Candida species with decreased susceptibility to this class of compounds (28), thus further underlying the need for more effective strategies.

To address this problem, we sought drug combinations that would increase the antifungal effect of fluconazole and tested their efficacy in rats with C. albicans experimental endocarditis. This model was particularly suitable because it presented the dual advantage of providing both a PMN-free infection system in the cardiac vegetation (4) and a PMN-rich system in the infected kidneys (32). Rats with experimental endocarditis were treated with fluconazole, cyclosporine (Cy), or the two drugs combined. This choice was based on a parallel in vitro study demonstrating that this combination was fungicidal and relied on the hypothesis that Cy might increase the intracellular fluconazole concentration by inhibiting intrinsic C. albicans efflux pumps (24). Amb was used as a control treatment.

MATERIALS AND METHODS

Microorganisms, growth conditions, and reagents. The laboratory strain C. albicans CAF2-1 (11) was used for both in vitro and in vivo experiments. C. albicans ATCC 90028 was used as a control for in vitro susceptibility tests (27). Unless otherwise stated, the organisms were grown at 35°C in Sabouraud liquid medium (Diagnostics Pasteur, Marnes La Coquette, France), in a shaking incubator at 200 rpm, or on Sabouraud agar plates. Stocks of the strains were kept at 70°C in liquid medium supplemented with 10% (vol/vol) glycerol.

Fluconazole was purchased from Pfizer AG (Zurich, Switzerland); Cy was purchased from Novartis Pharma (Basel, Switzerland); Amb-deoxycylolate was purchased from Brystol-Myers Squibb (Baar, Switzerland). All other chemicals were commercially available reagent-grade products.

Susceptibility testing and time-kill experiments. The MICs were determined by a described broth microdilution method according to the NCCLS M 27-A standard (27). For time-kill curves, 10-ml tubes containing RPMI liquid medium, as recommended by the NCCLS M 27-A standard, were inoculated with ca. 10⁶ CFU of C. albicans CAF2-1 per ml (final concentration) from an overnight culture. Time-kill experiments with Amb were performed in antibiotic medium 3 (Difco Laboratories, Basel, Switzerland) supplemented with 2% glucose, as proposed by the NCCLS M 27-A standard. Drugs were added thereafter at concentrations achievable in the blood during treatment in experimental models. Fluconazole was used at 10 mg/liter (42), Cy was used at 0.625 mg/liter (6), and Amb was used at 0.5 mg/liter (10). Just before drug addition, as well as 12, 24, and 48 h later, samples were removed from the cultures, diluted, plated, and incubated 48 h before the colony count. A fungicidal effect was defined as a ≥99.9% decrease in viability compared to the original inoculum (25).

Production of endocarditis. Sterile aortic vegetations were produced in 200-g female Wistar rats (Iffa Credo, Lyon, France) as previously described (16). Fungal endocarditis was induced 24 h later by intravenous challenge of the animals with 6 × 10⁶ CFU C. albicans CAF2-1. To prepare the inocula, fresh Candida cultures were grown and followed by optical density (OD) measurements at a wavelength of 620 nm made with a spectrophotometer (SequanaTurner, Mountainville, Calif.). At an OD of 0.5, corresponding to ca. 1⁰ CFU/ml, cultures were diluted in physiological saline (at 4°C) before inoculation.
Inoculum sizes were controlled in parallel by colony count. The minimum inoculum infecting 90% of the animals (i.e., the 90% infectious dose [ID₉₀]), was ca. 3 × 10⁵ CFU, as established in preliminary studies. Control animals were sacrificed both at the start of treatment (12 h postchallenge) to evaluate the frequency and severity of infection at that moment and later (between 2 and 5 days) to monitor the natural course of the disease. Vegetations and kidneys were cultured as described below. In certain experiments the valve and kidneys of control animals were also processed for histology and stained with hematoxylin-eosin to examine fungal invasion and inflammatory infiltration in the tissues.

Antifungal treatment of experimental endocarditis. Treatment was started 12 h after fungal challenge and lasted for 5 days. The drugs were administered either experimentally (fluconazole and AmB) or subcutaneously (Cy). Because the primary aim of the experiments was to test the effect of fungicidal synergism in vivo, not to develop specific human therapy, large doses were used. Two regimens were tested. Regimen 1 consisted of a daily dose of 50 mg of fluconazole per kg, combined or not with 20 mg of Cy per kg. Regimen 2 consisted of a daily dose of 20 mg of fluconazole per kg, combined or not with 10 mg of Cy per kg. Controls receiving Cy alone at these same dosages were also included. AmB was given at a daily dose of 1 mg/kg as previously described (31, 44).

Drug concentrations in the plasma of rats. Plasma drug concentrations were determined on days 1 and 5 of therapy, as well as at the time of sacrifice. Drug levels were measured in groups of three rats and came from internal controls of therapeutic experiments, where adequate drug delivery was tested routinely. Blood was drawn by puncturing the peripheral sinuses of the animals at 1 or 6 h (peak concentrations of fluconazole and Cy, respectively) and at 24 h (trough concentrations) after drug administration. Fluconazole levels in the plasma were determined by a recently described bioassay using a hypersensitive C. albicans mutant (O. Marchetti, P. A. Majcherczyk, M. P. Glauser, D. Sanglard, J. Bille, and P. Moreillon, Program Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. J-145B, p. 494, 1998). Cy concentrations were determined in whole blood using a commercially available enzyme multiplied immunoassay (EMIT 2000 Cyclosporin Specific Assay; Behring, Duessingen, Germany). The limits of detection of the assays were ≤1 mg of fluconazole and 0.04 mg of Cy per liter. For both drugs the linearity of the standard curves was assessed by a regression coefficient of ≥0.99, and intra- and inter-run deviations or coefficients of variation were ≤15%. AmB levels in the plasma were not determined.

Evaluation of infection. Treated rats were killed 72 h after the last antifungal administration (i.e., 48 h after the trough drug level in the blood of the last dose) in order to ensure an optimal washout of the drug and to minimize the risk of antifungalcarryover onto the plates. Residual drug levels were also tested at that time. The vegetations and kidneys were dissected, weighed, homogenized in 1 and 2 ml of saline, respectively, and serially diluted before plating them for the colony counts. The numbers of colonies growing on the plates were determined after 72 h of incubation at 35°C. Fungal densities in the tissues were calculated by plating samples of the cultures on Sabouraud medium, and retested for fluconazole and AmB MICs by standard method. This test was not performed for Cy.

Selection for the emergence of fluconazole resistance in vivo. To test whether treatment failures might be due to in vivo resistance selection, 10 colonies randomly picked from the plates of each rat with positive valve cultures were pooled, grown in Sabouraud medium, and retested for fluconazole and AmB MICs by standard method. This test was not performed for Cy.

Statistical analysis. The viable counts in the organs of the different groups were compared by the Kruskal-Wallis one-way analysis of variance on ranks, followed by a pairwise method and by the Mann-Whitney test, as appropriate. The rates of organ sterilization were compared by the Fisher’s exact test. The tests were all two sided, and the significance level was always set at a P ≤ 0.05. The Bonferroni correction was used for multiple testing.

RESULTS

Antifungal susceptibility and time-kill curves. The MICs of fluconazole and Cy for the test organism were 0.25 and >10 mg/liter, respectively. Although Cy had no antifungal activity at this concentration, a parallel in vitro study indicated that even low concentrations of this compound could substantially increase the antifungal efficacy of fluconazole (24). This was best observed in the time-kill experiments presented in Fig. 1, performed with drug concentrations achievable in humans and rats (1, 23). It can be seen that while 10 mg of fluconazole per liter alone was only fungistatic and 0.625 mg of Cy per liter had no antifungal effect, combining the two compounds resulted in a progressive viability loss of the cultures that attained >3 log₁₀ CFU/ml after 48 h of drug exposure. The control treatment with 0.5 mg of AmB per liter was rapidly fungicidal.

Natural history of infection and histopathology of vegetations and kidneys. In the present experiments, all animals were inoculated with 6 × 10⁵ CFU of the test organism, i.e., two times greater than the ID₉₀, in order to ensure infection of the vegetations in all the animals. Figure 2A presents the fungal densities in the vegetations and kidneys both 12 h after inoculation and later (between 2 and 5 days after challenge) in the natural course of the infection. The infection progressed at both anatomical sites. In the vegetations, the median (interquartile range) log₁₀ CFU/g of tissue increased from 3.81 (3.52 to 4.24) at 12 h to a plateau of 6.38 (5.66 to 6.85) at 2 days or later (P < 0.0001). In the kidneys, the fungal densities increased from 5.08 (4.84 to 5.28) log₁₀ CFU/g to 12 h to a plateau of 5.48 (4.9 to 6.58) at 2 days or later (P < 0.05). However, although fungal densities in the vegetations and kidneys reached similar values during natural infection, the histopathological findings in these two tissues were quite different. In the vegetation (left panel of Fig. 2B), massive pseudohyphal growth and invasion by C. albicans was observed, but the lesions were essentially devoid of inflammatory cells. In the kidneys (right panel of Fig. 2B), on the other hand, multiple foci of fungal invasion could be seen, and all of them were surrounded by massive inflammatory infiltrates containing PMN. Thus, the two sites illustrated two distinct responses to infection in the same animal, mimicking both the neutrophilic host, in the vegetations, and the immunocompetent host, in the kidneys.

Pharmacokinetics of fluconazole and Cy in rats. Table 1 presents the drug concentrations measured in the serum of rats at days 1 and 5 of therapy in both the regimen 1 and the regimen 2 experiments. Fluconazole given alone did not accumulate over time. In contrast, Cy concentrations increased by two to three times over the treatment period and caused a parallel increase of one to five times in the fluconazole concentration at the end of treatment, when the two drugs were combined. As a result, the area under the curve (AUC) of fluconazole at day 5 had increased by 25 to 35% compared to that of fluconazole monotherapy (Table 1). However, although the drug levels in the blood were high, the fluconazole concentrations were still compatible with high-dose treatment in humans (1, 23). In contrast, Cy concentrations were up to 10
FIG. 2. Natural course of valve (left part) and kidney infection (right part) in animals challenged with twice the minimal inoculum producing endocarditis in 90% of animals with C. albicans CAF2-1. (A) Fungal density in organs in either the early-infection stage (12 h after inoculation) or the late-infection stage (≥2 days after inoculation). After 2 days, fungal densities reached a plateau and did not increase further. Each dot on the figure represents the fungal density in an individual animal. Horizontal bars represent the median values of the group. Statistically significant differences between groups are indicated by a P value of ≤0.05. (B) Histological aspect of both vegetation (left part) and kidney (right part) infection, as revealed by hematoxylin-eosin staining. While massive pseudohyphal fungal invasion was present in both tissues (C. albicans in figure), only kidneys were infiltrated with polymorphonuclear cells (PMN in figure).
times greater than the therapeutic concentrations in humans, which are between 0.1 and 0.4 mg/liter (45).

Due to the long half-life of the drugs, some residual levels of both fluconazole (up to 3 mg/liter) and Cy (up to 0.5 mg/liter) were detected in the plasma at the time of sacrifice. Nevertheless, these levels were unlikely to interfere with fungal growth on the agar plates, because all samples were diluted ≥100 times before being cultured for the colony count. This decreased the drug concentrations well below their MICs for the test organism. The concentrations of AmB in the serum were not determined because the pharmacodynamic parameters of this drug are still largely unknown (13).

Therapeutic results in vegetation. Figure 3A presents the fungal densities in the vegetation after 5 days of therapy compared to the fungal densities at treatment onset. Cy given alone and AmB did not decrease fungal counts in the vegetation and even allowed the organisms to grow in spite of therapy. Likewise, fluconazole monotherapy did not significantly reduce vegetation counts compared to those at treatment onset, and no difference between the efficacy of the “high-dose” regimen 1 and the lower-dose regimen 2 was detected (P > 0.05). Nevertheless, pooling the two fluconazole monotherapy experiments indicated that the drug did successfully treat 25 (40%) of the animals, a result that was significantly different from that obtained with untreated controls (P < 0.05).

In sharp contrast, treatment with either of the two fluconazole-Cy regimes demonstrated efficacy. When pooled together, the drug combination successfully treated 16 of 22 (73%) of the animals and was significantly better than fluconazole monotherapy when compared for both fungal densities (P < 0.0001) and sterile vegetation cultures (P < 0.05).

Because coadministration of Cy increased the AUC of fluconazole over time (Table 1), it was important to determine whether the better efficacy of the drug combination was really due to in vivo synergism or whether it merely resulted from fluconazole accumulation. The answer came from two observations. First, as mentioned above, the results of fluconazole monotherapy were similar between both the high-dose regimen 1 and the lower-dose regimen 2 (P > 0.05), indicating that at these high drug concentrations the treatment outcome was not dose dependent.

Second, comparison of fluconazole concentrations and AUCs in the serum in regimens 1 and 2 supported the superiority of the drug combination. Indeed, in high-dose regimen 1, fluconazole monotherapy produced peak levels and AUCs that were up to two times greater than those in the combination therapy of the lower-dose regimen 2. Yet the therapeutic results in this second group were significantly better than in the fluconazole monotherapy of regimen 1, both in terms of treatment success (3 of 12 [25%] rats successfully treated with high-dose fluconazole monotherapy in regimen 1 versus 8 of 12 [66%] in the lower-dose combination therapy of regimen 2; P < 0.05) and in terms of vegetation fungal densities (median = 5.7 log CFU/g of tissue [range, 2 to 7] after the high-dose fluconazole monotherapy of regimen 1 versus 2 log CFU/g [range 2 to 7] after the lower-dose combination treatment of regimen 2; P < 0.05). Thus, the best results were obtained with the lower fluconazole concentration provided that Cy was present in the regimen. Taken together, these observations highlighted the beneficial effect of the drug combination over that of high-dose fluconazole therapy and correlated with the in vitro synergism observed between both test drugs.

Therapeutic results in the kidneys. Figure 3B presents the fungal loads in the kidneys of the animals depicted in Fig. 3A. The general profile resembled that of the vegetation counts for both controls and rats treated with Cy alone. However, it was different for AmB and fluconazole monotherapy, which both significantly decreased kidney fungal titers compared to the untreated controls (P < 0.0001). Nevertheless, in spite of this decrease neither of these regimens successfully eradicated the infection, since all nine (100%) and all 26 (100%) rats treated with AmB and fluconazole, respectively, had positive kidney cultures. In contrast, fluconazole-Cy treatment sterilized the kidneys of 20 of 23 (87%) rats. This was significantly more effective than the two other antifungal regimens (P < 0.0001). Thus, the in vivo results supported the fungicidal synergism of the fluconazole-Cy combination observed in vitro.

Selection of resistance during in vivo therapy. Yeast cells recovered from all the valve homogenates from treatment failures were retested for their MIC of fluconazole and of AmB by standard methods. None of them had altered drug susceptibility compared to the parent strain.

**DISCUSSION**

The present experiments confirmed in vivo the fungicidal activity of fluconazole combined with Cy recently observed in vitro (24). The rationale for using this particular drug associ-

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* Values represent the mean of three determinations for individual rats. Variations between animals were ±20%. Regimen 1, intraperitoneal administration of 50 mg of fluconazole (FLC) ± 20 mg of Cy per kg per day; regimen 2, intraperitoneal administration of 20 mg of fluconazole ± 10 mg of Cy per kg per day.

b The 24-h area under the serum concentration time curves (AUC0–24h in milligrams/hour/liter) of fluconazole were measured by the trapezoidal summation method.

Table 1. Serum pharmacokinetic parameters in animals treated with two different regimens of fluconazole and Cy
ation resulted from previous tests in which we screened a number of partner compounds for their potential synergism with fluconazole (24). The potential partner drugs were selected for their ability to interact with cell membranes and, possibly, to inhibit membrane transporters of the major facilitator and/or ABC transporter superfamilies (30).

In Candida spp. such transporters mediate active efflux of numerous molecules, including fluconazole. In drug-susceptible organisms, intrinsic basal expression of these transporters was shown to determine the MIC of the drug (36). In addition, overexpression of such determinants, including CDR1, CDR2, and CaMDR1, was shown to mediate azole resistance (37, 38). Thus, it was conceivable that certain membrane-active drugs might increase the efficacy of fluconazole not only in resistant Candida but also in susceptible ones by allowing accumulation of more of theazole inside of the yeast cell.

The endocarditis model was particularly well suited for validating this concept in vivo. Indeed, it allowed evaluating the therapeutic effect at two anatomic sites, which either lacked cellular host defense mechanisms or displayed massive PMN recruitment. In untreated animals, pseudohyphal growth and invasion by C. albicans were observed in both vegetations and kidneys, suggesting that fungal growth was rather similar at both sites. On the other hand, the therapeutic response was quite different in these two tissues. In the neutropenic environment of the vegetation, only the fungicidal fluconazole-Cy combination was effective. Since sterilization of such lesions is dependent on drug-induced killing, this was an expected result. Cy administered alone had no effect, thus confirming the lack of antifungal activity of this compound in this experimental setting. On the other hand, fluconazole monotherapy had some activity and sterilized the vegetations in 40% of the animals. Since abortive infections were observed in the natural course of endocarditis, it is possible that fluconazole administered alone eradicated such low-grade infections.

Less expected was the lack of effect of AmB in cardiac

FIG. 3. Therapeutic results in rats with experimental endocarditis. The figure presents the fungal densities in both the vegetations (A) and the kidneys (B) of rats sacrificed either at the beginning of therapy (control baseline) or after 5 days of treatment with various drug regimens. FLC, fluconazole. The figure dissociates between the results of therapeutic experiments performed with regimen 1 (closed symbols; results of two pooled experiments) and one experiment performed with regimen 2 (open symbols) (see Table 1). Since the therapeutic outcome was similar after either of these regimens, the results were pooled for statistical analysis. The horizontal bars indicate the median fungal densities in the organs of the pooled experiments. Each dot represents the fungal load in a single animal. Statistically significant differences between groups are indicated by a P value of ≤0.05.
lesions that contrasted with its very rapid fungal killing in vitro. This discrepancy most likely resulted from the complicated pharmacodynamic properties of this highly protein-bound compound, which requires prolonged treatment to achieve therapeutic concentrations of free drug in the deep layers of the valve vegetation (34). Alternatively, AmB might have been given at suboptimal dosages. However, although the serum levels were not tested, this possibility was contradicted by the fact that AmB was very effective at decreasing fungal densities in the kidneys. Moreover, previous studies using the same dosage of AmB in the endocarditis models also reported a very slow response of the infection (5, 31, 35, 43, 44).

In contrast to the vegetations, all of the regimens except Cy significantly decreased the fungal densities in the kidneys. This reflected the cooperation between PMN and antifungal drugs to eradicate the infecting organisms. An enhancement of the antifungal activity of fluconazole in the presence of phagocytes was previously reported (14). However, in spite of the improved efficacy of the other regimens, the fluconazole-Cy combination was nevertheless significantly more effective sterilizing kidneys, thereby demonstrating its superior therapeutic effect in both the vegetation and the kidneys.

The present experiments were performed with large drug doses and aimed at a proof of concept and are not therapeutic recommendations. Yet the results were obtained with fluconazole and a synergistic drug for treatment of experimental Candida albicans endocarditis in rabbits. Antimicrob. Agents Chemother. 40:263–269.


