In Vitro Activity and In Vivo Efficacy of Icofungipen (PLD-118), a Novel Oral Antifungal Agent, against the Pathogenic Yeast Candida albicans

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Icofungipen (PLD-118) is the representative of a novel class of antifungals, beta amino acids, active against Candida species. It has been taken through phase II clinical trials. The compound actively accumulates in yeast, competitively inhibiting isoleucyl-tRNA synthetase and consequently disrupting protein biosynthesis. As a result, in vitro activity can be studied only in chemically defined growth media without free amino acids that would compete with the uptake of the compound. The MIC of icofungipen was reproducibly measured in a microdilution assay using yeast nitrogen base medium at pH 6 to 7 after 24 h of incubation at 30 to 37°C using an inoculum of 50 to 100 CFU/well. The MICs for 69 Candida albicans strains ranged from 4 to 32 μg/ml. This modest in vitro activity contrasts with the strong in vivo efficacy in C. albicans infection. This was demonstrated in a lethal model of C. albicans infection in mice and rats in which icofungipen showed dose-dependent protection at oral doses of 10 to 20 mg/kg of body weight per day in mice and 2 to 10 mg/kg/day in rats. The in vivo efficacy was also demonstrated against C. albicans isolates with low susceptibility to fluconazole, indicating activity against azole-resistant strains. The efficacy of icofungipen in mice and rats was not influenced by concomitant administration of equimolar amounts of t-isoleucine, which was shown to antagonize its antifungal activity in vitro. Icofungipen shows nearly complete oral bioavailability in a variety of species, and its in vivo efficacy indicates its potential for the oral treatment of yeast infections.

Only a very few compound classes are currently available for systemic treatment of Candida infections (5, 7, 23). Azoles (e.g., fluconazole [FLC], itraconazole, and voriconazole) can be administered orally and parenterally, while amphotericin B and the recently introduced echinocandin, caspofungin (Candidas; Merck Sharp and Dome), are given only intravenously; amphotericin B was approved recently for inhalative treatment of bronchopulmonary infection by Aspergillus fumigatus. New clinical developments have been made only in the area of azoles (e.g., voriconazole, posaconazole, and ravuconazole) and echinocandins (e.g., anidulafungin and micafungin) (6, 8, 10, 19). On the other hand, FLC-resistant Candida albicans strains are observed frequently in AIDS patients who require long-term treatment and/or prophylaxis (1, 3, 18, 21–26). Comitantly, the occurrence and spread of primary FLC-resistant Candida species (e.g., C. krusei) have been reported. These trends increase the need for an effective alternative antifungal agent, especially for oral treatment and prophylaxis of yeast infections.

In 1989, Konishi et al. isolated from Bacillus cereus cispentacin, a natural cyclic beta amino acid with significant antifeedant activity (9, 12) and in vivo efficacy after oral dosing (12, 16, 17). Subsequently, in an effort to identify novel orally available and safe antifungal compounds, cyclic beta amino acids were studied by Bayer AG. During this research, the (−)-(1R,2S)-3-aminomethylcyclopentane carboxylic acid was identified and analyzed in more detail (13). The compound, previously known as BAY 10-8888, was licensed to GlaxoSmithKline Research Centre Zagreb Ltd. (formerly PLIVA) and renamed PLD-118; its generic name is icofungipen.

Icofungipen is a beta amino acid, which differs in chemistry, biology, and mechanism of action from other antifungal compound classes. Its mechanism of action is based on inhibition of isoleucyl-tRNA synthetase, intracellular inhibitory concentrations at the target site being achieved by active accumulation in susceptible fungi (31, 32). In this report, we describe (i) the basic in vitro activity of icofungipen against C. albicans and (ii) its in vivo efficacy in various models of fungal infection after oral dosing.

MATERIALS AND METHODS

Media. YNG, YPG, YNGW, YPD, LB, Sabouraud dextrose, and RPMI 1640 media were used and prepared as described elsewhere (4, 24, 30). YNG medium was buffered to pH 6 or pH 7 using Soerensen buffer containing Na₂HPO₄ · 2H₂O (14 g/liter) and NaH₂PO₄ (8.0 g/liter) or Na₂HPO₄ · 2H₂O (7.2 g/liter) and NaH₂PO₄ (3.6 g/liter) (Merck, Germany). Under all conditions in which these buffers were used, no change in incubation pH was observed. To evaluate the influence of amino acids, YNG was supplemented with leucine, valine, or alanine at a final concentration of 0.1 mM, 1 mM, or 10 mM. RPMI 1640 powdered medium was prepared according to the CLSI (formerly NCCLS) standard M27-A (15). YNG media were adjusted to a given pH (4.0 to 8.0) by the addition of NaOH or HCl.

Strains. A control strain was obtained from the American Type Culture Collection (C. albicans ATCC 90028). Clinical specimens were isolated from various tissues (blood, mucosal surfaces, and skin) at the University Hospital (KBC), Zagreb, Croatia, and from various clinical centers in Germany.

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Clinical isolates of *C. albicans* were identified by chlamydospore formation on rice agar (Merck, Darmstadt, Germany). Strains unable to form chlamydospores were biochemically identified using the API ID 32C system (bioMerieux, Nuerlingen, Germany). Strains were preserved by being freeze-dried in milk powder (Struthmann, Kleve, Germany) and stored at 4°C.

Isolates were stored at –70°C in a Cryobank (Mast Diagnostica, Germany) and cultured on Sabouraud agar at 35°C prior to being tested.

A clinical isolate of *C. albicans*, BSMY 212 (C. albicans strain, deposited at ATCC as ATCC 200498), from the Bayer strain collection was used to establish basic parameters of infection and efficacy. In addition, various clinical isolates were used as well as strains from the ATCC (Bethesda, Maryland), as indicated.

**Inoculum preparation.** *Candida* species were grown overnight at 37°C in YPG medium without shaking. The overnight suspension was centrifuged at 1,000 × g for 10 min. The supernatant was decanted, and the pellet was resuspended in phosphate buffer, pH 8. Using a McFarland standard protocol, optical density was adjusted to approximately 10^7 CFU/ml. Dilutions of fungal suspensions were made, as required, in YNG medium. A final inoculum of 1 × 10^9 CFU/ml (resulting in 100 CFU/microtiter well) was used. Inoculum size was checked in every experiment by plating 10 μl of the suspension on Sabouraud 2% dextrose agar plates. Alternatively, inoculum was prepared by direct colony suspension from a 24-h Sabouraud 2% dextrose agar plate incubated at 37°C.

For inoculum consisting of logarithmic-phase cells, one to two colonies were resuspended in 5 ml of YPG and incubated for 2 h at 37°C with moderate shaking. From this culture, 1 ml was added to 50 ml of YNG and further incubated for 16 h.

**Compound preparation.** Icofungipen was prepared at PLYIA in GLP quality. FLC was purchased from Chemocberica, Spain, while clotrimazole, amphotericin B, miconazole, nystatin, and flucytosine were obtained from USP. Icofungipen was dissolved in phosphate buffer as a stock solution of 5 mg/ml. All other substances were dissolved in N,N-dimethylformamide, and final working solutions were prepared in YNG medium.

**Broth microdilution assay.** The highest concentration in the broth microdilution assay (96-well plates; Greiner, Germany) was 64 μg/ml, and twofold dilutions were used subsequently. The total volume was 100 μl/well incubated at the indicated temperature. Optical density was determined at 590 nm using a spectrophotometer (SpectraFluor Plus; Tecan, Switzerland). MICs were determined as the lowest concentration at which a prominent decrease in turbidity (score 2 or higher) was observed in the test wells as compared with the drug-free control well (50 to 100 CFU/ml). For determining the MICs of Icofungipen using broth microdilution, YNG medium was used.

**Infection models.** The infection models are described in more detail elsewhere (27). Briefly, the inoculum for infection was prepared from 24-h cultures of logarithmic-phase cells known to interfere with the uptake of ifofungipen. Inoculum sizes of three strains of *C. albicans* strains were used. Inoculum sizes of three strains of *C. albicans* strains were used depending on the strain tested and the desired outcome. Inoculum sizes of three strains of *C. albicans* strains were used depending on the strain tested and the desired outcome. Inoculum sizes of three strains of *C. albicans* strains were used depending on the strain tested and the desired outcome.

**Results**

Parameters influencing in vitro susceptibility testing. (i) **Influence of growth medium composition.** Results of repeated testing of three *C. albicans* strains in seven different growth media are shown in Table 1. The standard microdilution broth method was used, with the lowest possible inoculum still growing sufficiently in the drug-free control well (50 to 100 CFU/ml) after 24 versus 48 h in all complex media, regardless of the read-out time. However, MICs for ifofungipen were significantly lower in YNGW and YNG media were used. Under these experimental conditions, only in YNGW medium increased MICs for amphotericin B above those in RPMI 1640 medium. Icofungipen showed relatively high MICs (16 to >64 μg/ml) after 24 versus 48 h in all complex media, regardless of the read-out time. However, MICs for ifofungipen were significantly lower if chemically defined media (YNGW and YNG) were used.

**Inoculum size.** Inoculum sizes of three strains of *C. albicans* strains were used depending on the strain tested and the desired outcome. Inoculum sizes of three strains of *C. albicans* strains were used depending on the strain tested and the desired outcome. Inoculum sizes of three strains of *C. albicans* strains were used depending on the strain tested and the desired outcome. Inoculum sizes of three strains of *C. albicans* strains were used depending on the strain tested and the desired outcome. Inoculum sizes of three strains of *C. albicans* strains were used depending on the strain tested and the desired outcome. Inoculum sizes of three strains of *C. albicans* strains were used depending on the strain tested and the desired outcome.
TABLE 1. Influence of growth medium composition on MICs of tested antifungals

<table>
<thead>
<tr>
<th>Medium</th>
<th>C. albicans strain</th>
<th>MIC (µg/ml) for:</th>
<th>24 h</th>
<th>48 h</th>
<th>24 h</th>
<th>48 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>YNGW</td>
<td>ATCC 90028</td>
<td>Amphotericin B</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluconazole</td>
<td>0.125</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Icofungipen</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>YNG</td>
<td>ATCC 90028</td>
<td>Amphotericin B</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluconazole</td>
<td>1</td>
<td>2</td>
<td>0.5</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Icofungipen</td>
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<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>YPD</td>
<td>ATCC 90028</td>
<td>Amphotericin B</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluconazole</td>
<td>2</td>
<td>4</td>
<td>0.5</td>
<td>0.5</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Icofungipen</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>LB</td>
<td>ATCC 90028</td>
<td>Amphotericin B</td>
<td>0.125</td>
<td>0.5</td>
<td>0.5</td>
<td>0.25</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluconazole</td>
<td>0.125</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Icofungipen</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>YPG</td>
<td>ATCC 90028</td>
<td>Amphotericin B</td>
<td>2</td>
<td>4</td>
<td>0.5</td>
<td>0.5</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluconazole</td>
<td>2</td>
<td>4</td>
<td>0.5</td>
<td>2</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Icofungipen</td>
<td>1</td>
<td>2</td>
<td>0.5</td>
<td>0.5</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Sabouraud</td>
<td>ATCC 90028</td>
<td>Amphotericin B</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluconazole</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Icofungipen</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>ATCC 90028</td>
<td>Amphotericin B</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluconazole</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Icofungipen</td>
<td>0.5</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
<td>32</td>
<td>64</td>
</tr>
</tbody>
</table>

* MICs were determined after 24 or 48 h of incubation. Experiments were repeated five times independently, and representative experiments are shown; 64 µg/ml for each antifungal was the highest concentration chosen in the experiments.

while FLC showed lower susceptibility with increasing inoculum size. Starting at 200 CFU/well and becoming pronounced at >1,000 CFU/well, sensitivity toward icofungipen was markedly reduced with increasing inoculum size. Slight differences were observed between the three strains tested. For example, C. albicans PSCF 0085 was more sensitive to changes in inoculum size than the other strains. Based on these findings, the inoculum size used for further testing was limited to 50 to 100 CFU/well.

(iv) Influence of temperature of incubation. No difference was observed in growth of yeast either in the absence or in the presence of any antifungal substance between 30 and 37°C (data not shown).

In vitro testing of icofungipen against clinical isolates of C. albicans. Sixty-nine C. albicans strains collected from patients with cutaneous and/or mucocutaneous infections were included in the study to analyze the MIC distribution for clinical isolates. MIC testing (n = 10 per group) was done by broth microdilution assay using YNG medium at pH 7, an inoculum size of 50 to 100 CFU/mL, and 24 h of incubation. Results for icofungipen were compared with those for amphotericin B and clotrimazole, the most active azoles. Ninety percent of the strains of C. albicans showed susceptibility to icofungipen with MICs ranging from 8 to 32 µg/mL, giving an MIC₉₀ of 32 µg/mL. The total range of MICs in this set of strains varied between 4 and >64 µg/mL. MIC₉₀ values for amphotericin B and clotrimazole were 1 µg/mL and 0.5 µg/mL, respectively (Table 4).

TABLE 2. Influence of isoleucine, leucine, and valine on in vitro activity of icofungipen in YNG medium

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>MIC (µg/ml) for amino acid at a concen (mM) of:</th>
<th>24 h</th>
<th>48 h</th>
<th>24 h</th>
<th>48 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0.1</td>
<td>1</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
<td>48 h</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2</td>
<td>4</td>
<td>16</td>
<td>16</td>
<td>64</td>
<td>128</td>
<td>256</td>
</tr>
<tr>
<td>Leucine</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>32</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Valine</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>32</td>
<td>32</td>
<td>64</td>
</tr>
</tbody>
</table>

* MICs were determined after 24 or 48 h of incubation (C. albicans ATCC 90028). Experiments were repeated five times independently, and representative experiments are shown.
TABLE 4. In vitro activity of icofungipen against 69 clinical isolates of C. albicans

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (μg/ml)</th>
<th>50%</th>
<th>90%</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Icofungipen</td>
<td>16</td>
<td>32</td>
<td>4</td>
<td>4–64</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>≤0.125</td>
<td>0.5</td>
<td>≤0.125-2</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>≤0.125-4</td>
</tr>
</tbody>
</table>

* MICs were determined in YNG medium after 24 h of incubation.

In vivo efficacy of icofungipen in C. albicans infection in mice. A typical experiment demonstrating the efficacy of icofungipen in the mouse model is summarized in Fig. 1. Mice infected with C. albicans strain BSMY 212 (ATCC 200498) succumbed to their infection within 7 days. A dose-dependent protection was observed, with icofungipen giving 100% protection at 10 mg/kg of body weight b.i.d.

We analyzed the efficacy of the compound in vivo against a variety of clinical isolates obtained from different clinical centers, for which MICs ranged between 0.5 and 32 mg/liter. After identification to the species level, C. albicans strains were tested for pathogenicity and strains showing 80 to 100% mortality in the infection model were used in treatment studies. In a first series of infection experiments, all strains were exposed to 5 mg/kg icofungipen b.i.d. (10 animals per strain and dose); 38% of the strains (n = 34 isolates) were highly susceptible, i.e., survival was >80% (Table 5). In a second series, strains with lower survival rates of less than 80% (n = 55) at 5 mg/kg b.i.d. were exposed to a higher dose of 10 mg/kg b.i.d. All strains responded to therapy, with 75% of strains achieving more than 80% survival. There was no correlation with the in vitro susceptibility.

Subsequently, the in vivo efficacy of icofungipen against FLC-susceptible and FLC-resistant C. albicans strains was tested according to CLSI guidelines. Two groups were established, one consisting of strains with an MIC of ≤4 mg/liter and the other with an MIC of ≥64 mg/liter. There was no correlation between the MICs for FLC versus those for icofungipen. The mean MICs for icofungipen were broadly distributed between 1 and 32 mg/liter (data not shown). Subsequently, these strains were tested for pathogenicity in mice (each strain was tested in groups of five mice); only strains which gave 80 to 100% mortality were used in the subsequent efficacy trials. Pathogenic strains were tested in a group of 15 mice, 5 per treatment cohort (control, FLC, and icofungipen). Treatment was performed orally twice daily over 4 days. FLC was given at dose of 2 mg/kg/day and icofungipen at 20 mg/kg/day. At these dose levels in vitro, FLC-resistant and -susceptible strains could be clearly separated.

A total of 54 strains, 27 FLC-susceptible and 27 FLC-resistant strains, were used in the experiments. As can be seen from the Kaplan-Meier plot in Fig. 2, the cumulative mortality in the control group was almost 100%, regardless of the susceptibility of the causative pathogen to FLC. FLC was highly effective against susceptible strains but did not decrease mortality above that in vehicle controls when tested against strains with an MIC of ≥64 mg/liter. In contrast, icofungipen was effective against both FLC-susceptible and FLC-resistant C. albicans strains.

In vivo efficacy of icofungipen in C. albicans infection in rats. To demonstrate efficacy in rats, male Sprague-Dawley rats (n = 5) were infected by intravenous administration of 5 × 10^6 CFU per animal from a freshly prepared 24-h C. albicans culture. In this experiment, lethality was achieved within 10 days at 100%. Oral administration of icofungipen to rats (n = 10/group) at a dose of 5 mg/kg b.i.d. over 5 days resulted in 100% survival over a period of 40 days. At a dose of 2.5 mg/kg b.i.d., 80% survival was achieved (Fig. 3).

The experiments were repeated with four different C. albicans strains (ATCC 10261, 44373, 44505, and 62342). All strains led to a lethal infection under the experimental conditions within 3 to 10 days, whereas oral treatment with icofungipen for 10 mg/kg b.i.d. (five rats/group) resulted in 100% survival over 10 days (data not shown).

A single oral icofungipen dose, given simultaneously with infection, was also effective in preventing mortality. As can be seen from Fig. 4, dose-dependent protection was observed, achieving 100% survival at 10 mg/kg.

We subsequently analyzed the protective effect when initiating treatment 24 h after the infection. For this purpose, rats (n = 5/group) were challenged on day 0 and icofungipen was given at 1, 5, and 10 mg/kg b.i.d. over 5 days, starting on day 1.

TABLE 5. In vivo efficacy of icofungipen against clinical isolates of
C. albicans in a lethal model of infection in mice

<table>
<thead>
<tr>
<th>Isolates (no. of isolates)</th>
<th>Icofungipen dose (mg/kg)</th>
<th>No. (%) of strains associated with indicated survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All isolates (89)</td>
<td>5</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Isolates with survival of</td>
<td>34 (38)</td>
<td>51–80</td>
</tr>
<tr>
<td>&lt;80%</td>
<td>15 (27)</td>
<td>31–50</td>
</tr>
<tr>
<td>at 5 mg/kg icofungipen (55)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Clinical isolates of C. albicans causing >80% mortality in untreated animals.
* The stated dose was given orally twice daily over 4 days, starting 0.5 h after infection.
* Animals (10 per isolate) were infected intravenously, and survival was monitored for 10 days.
All animals in the control group died within 10 days, while at a dose of 10 mg/kg b.i.d., all icofungipen-treated animals survived during the 20-day observation period, even at the lowest dose of 1 mg/kg b.i.d., more than half of the animals survived (data not shown).

Influence of isoleucine dosing on efficacy of icofungipen. As demonstrated previously, the inhibition by icofungipen of isoleucyl-tRNA synthetase can be antagonized in vitro by the addition of L-isoleucine (29, 31). To test whether concomitant administration of L-isoleucine and icofungipen would antagonize the in vivo efficacy of icofungipen, L-isoleucine in equimolar mixtures with icofungipen was tested in the lethal model of C. albicans infection in mice and rats (10 animals/group). As can be seen from Fig. 5 and 6, no significant antagonism could be detected at any dose.

DISCUSSION

Our results demonstrate the in vitro activity and efficacy of icofungipen, a novel, orally available antifungal for the treatment of yeast infections. Icofungipen has a complex mode of action, antifungal properties being mediated by active transport into and accumulation by yeast cells, inhibition of isoleucyl-tRNA synthetase.
Chi-square test. Statistically significant, as calculated according to survival curves and a

differentiation of this mode of action, in vitro susceptibility assays were used as a starting point for developing clinically relevant
dosing regimens for treatment with icofungipen (11). Because of the complexity of its mode of action, it was important—for
in vitro susceptibility testing—to standardize incubation conditions, including medium composition and inoculum size; pH
and temperature had no clear effect. A challenge arose when we realized that the CLSI guidelines recommended for azoles
cannot be applied to icofungipen. The medium used in the reference method for azoles (RPMI 1640) (15) contains,
among other ingredients, amino acids (isoleucine, leucine, and valine) that counteract the antifungal activity of icofungipen.
Consequently, assay conditions had to be tailored specifically for icofungipen. It remains to be seen whether the resulting

synthetase, and thus, blockade of protein biosynthesis. After verification of this mode of action, in vitro susceptibility assays
were used as a starting point for developing clinically relevant
dosing regimens for treatment with icofungipen (11). Because of the complexity of its mode of action, it was important—for
in vitro susceptibility testing—to standardize incubation conditions, including medium composition and inoculum size; pH
and temperature had no clear effect. A challenge arose when we realized that the CLSI guidelines recommended for azoles
cannot be applied to icofungipen. The medium used in the reference method for azoles (RPMI 1640) (15) contains,
among other ingredients, amino acids (isoleucine, leucine, and valine) that counteract the antifungal activity of icofungipen.
Consequently, assay conditions had to be tailored specifically for icofungipen. It remains to be seen whether the resulting

method can be transferred successfully to a standard mycology
lab for support of clinical therapy.

Ziegelbauer et al. found that the nitrogen source in the
growth medium affects the in vitro activity of icofungipen, as
judged by its influence on the uptake of the compound by yeast
cells (31, 32). Consequently, the activity of icofungipen in
chemically defined media (YNGW and YNG) was studied
since the same authors indicated that activity could be influ-
enced by the concentration of amino acids present in all com-
plex media. Inhibitory effects of branched amino acids added
to the YNG medium, as well as of some other amino acids,
such as methionine, were observed. To check whether rela-
tively low concentrations of amino acids (methionine, histi-
dine, and tryptophan) present in YNG medium could interfere
with the action of icofungipen, testing in the present study was
also done in YNGW medium (Table 1). This medium contains
the same nitrogen source as YNGW but lacks amino acids
completely. The results demonstrated that the concentrations
of amino acids present in YNG medium, 0.06 mM His, 0.098
mM Trp, and 0.13 mM Met, did not affect the in vitro activity
of icofungipen. However, the presence of three amino acids,
Val, Ile, and Leu, which use the same transporter as icofungi-
pen, leads to a dose-dependent increase in the MIC of icofun-
gipen (Table 2).

Inoculum size proved to be a critical parameter for measur-
ing the in vitro activity of icofungipen. The CLSI recommends
an inoculum size of 5 × 10^2 to 2.5 × 10^3 CFU per ml, which is
about 50 to 250 CFU/well in a final volume of 100 μl. We observed that 200 to 250 CFU/well decreased the inhibitory
activity of icofungipen against some clinical isolates of C. albi-
cans (Table 4). Therefore, the assay was designed to use an
inoculum of up to 150 CFU/well. This inoculum size gave rise
to an optical density at 590 nm of 0.5 to 1.0 after 24 h. This is
in accordance with the observation that most of the strains
grow sufficiently after 24 h (19). From the analysis of clinical
isolates, it is evident that most strains show the lowest MICs for
icofungipen after 24 h. The reason for this dependency of MIC
on the inoculum size remains to be elucidated. It is possible
that the strong cellular uptake of icofungipen may significantly
reduce the available concentration of icofungipen to a subin-
hibitory level under the conditions used.

In principle, the MICs of icofungipen for yeasts are depen-
dent on the activity and availability of the two targets, the
transporter for the compound and its intracellular target.
While it was shown that the intracellular concentration of the
tRNA synthetase influences the MICs of icofungipen (31), the
expression of the transporter presumably has a major impact
on the activity as well. For example, isoleucine inhibits the
effects of icofungipen on its final target, specific tRNA syn-
thetase, and it may also compete with the transporter for the
intracellular accumulation of icofungipen. Studies are in
progress to analyze the role of the transporter under various in
vivo conditions. Although in vitro testing in YNG medium
yielded reproducible MIC levels for icofungipen, the levels
were relatively high in comparison to those of other classes,
such as azoles, and did not correlate with the efficacy observed
in the animal models. Thus, experiments are currently being
performed to address this issue in order to develop a repro-
ducible method that correlates individual MIC levels with re-

dia. C. albicans

![Fig. 5](http://aac.asm.org/)

**FIG. 5.** Influence of L-isoleucine on the in vivo efficacy of icofungipen in the *C. albicans* (ATCC 200498) mouse infection model. CFW1 mice (*n* = 10) were infected intravenously and treated orally b.i.d. for 4 days. Icofungipen was given alone or mixed prior to the dosing with an equimolar amount of L-isoleucine. Differences between the icofungipen- and icofungipen-plus-isoleucine-treated animals were not statistically significant, as calculated according to survival curves and a chi-square test.

![Fig. 6](http://aac.asm.org/)

**FIG. 6.** Influence of L-isoleucine on the in vivo efficacy of icofungipen in the *C. albicans* (ATCC 200498) rat infection model. Rats (*n* = 10) were infected intravenously and treated orally b.i.d. for 4 days. Icofungipen was given alone or mixed prior to the dosing with an equimolar amount of L-isoleucine.
In models of lethal systemic *C. albicans* infection, icofungipen showed high efficacy in rats and mice, achieving 100% protection at oral doses of about 10 to 20 mg/kg/day in mice and about 10 mg/kg/day in rats. The higher efficacy in rats is most likely due to different pharmacokinetic behavior; the half-life of icofungipen in rats is significantly longer than that in mice, leading to higher systemic exposure in the rat (half-life in rats is 6 h versus 2.5 h in mice) (28).

In rats, the infection process is more generalized than in mice, in which almost exclusive trapping of *C. albicans* in the kidney occurs with subsequent kidney damage and lethal outcome. It is possible that icofungipen is highly effective in the mouse model since it is excreted via the urine (28). The excellent efficacy in the rat model, however, in which infectious foci could be identified in various organs, argues against this proposal (27). Icofungipen achieves homogenous tissue distribution, as evidenced by the distribution of radiolabeled compound (28). Good efficacy was also confirmed in immunosuppressed rabbits with systemic *C. albicans* infection (20). At present, the extent to which icofungipen is able to clear the infectious process is unclear. Further studies assessing the infectious burden in various organs are therefore necessary to evaluate the potency of the compound.

Of particular importance is the question of whether the compound is also active against FLC-resistant strains. We addressed this question in a series of in vivo experiments using a number of clinical isolates which (i) showed comparatively high pathogenicity and (ii) differed in their in vivo susceptibility to FLC. Using a moderate dose of 2 mg/kg/day icofungipen in mice, two groups of strains, susceptible and resistant, could be distinguished. Higher doses of FLC were partially able to overcome the resistance in vivo (data not shown). Under these conditions, icofungipen exerted similar activities against FLC-susceptible and -resistant strains. Thus, we confirmed that icofungipen is active against FLC-resistant strains, as could already be deduced from its different mode of action and the lack of substrate specificity toward efflux pumps (31, 32). Further evidence for activity of icofungipen against FLC-resistant strains is provided by its efficacy in a rabbit model of esophageal candidiasis using an FLC-resistant *C. albicans* strain (20).

Icofungipen acts by inhibition of intracellular isoleucine-tRNA synthetase. In vitro activity can be antagonized by the addition of L-isoleucine to the media. Isoleucine levels in blood and tRNA synthetase. In vitro activity can be antagonized by the addition of L-isoleucine to the media. Isoleucine levels in blood and cerebral candidiasis using an FLC-resistant compound. We thus had to determine whether the application of L-isoleucine together with icofungipen would counteract its antifungal efficacy. Neither in rats nor in mice could any effect on the efficacy of PLD-118 be observed at equimolar doses of icofungipen and L-isoleucine. Most likely, exogenously added L-isoleucine is taken up rapidly by cells and further integrated into proteins and thus cannot interact to a significant extent with fungal protein synthesis.

In summary, icofungipen shows high in vivo efficacy after oral dosing in lethal models of *Candida* infection. However, further studies are needed to correlate MICs with response rates in infection models.

Based on these data and the successful outcome of the phase I clinical study of humans (oral dosing), a phase II study of human immunodeficiency virus patients suffering from oropharyngeal candidiasis was performed and preliminary data were reported in 2004 (2).

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**REFERENCES**


