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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 L-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 (Cancidas; 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). Concomitantly, 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 antiyeast 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 (−)-(1*R*,2*S*)-2-amino-4-methylene-cyclopentane 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 $Na_2HPO_4 \cdot 2H_2O$ (1.4 g/liter) and NaH_2PO_4 (8.0 g/liter) or $Na_2HPO_4 \cdot 2H_2O$ (7.2 g/liter) and NaH_2PO_4 (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 isoleucine, leucine, or valine 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 (bioMérieux, Nuertlingen, Germany). Strains were preserved by being freeze-dried in milk powder (Struthmannn, 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 1×10^3 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 PLIVA in GLP quality. FLC was purchased from Chemoiberica, 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 according to CLSI standard M27-A [15]) was observed (fluconazole and icofungipen), while the MIC of amphotericin B was determined as a clear endpoint.

Tests were repeated at least five times on different days, using different preparations of the individual strains.

Animals. Six-week-old male, specific-pathogen-free, CFW1 mice and male, specific-pathogen-free, Sprague-Dawley rats (200 g body weight) were obtained from Harlan Winkelmann, Paderborn, Germany. Male Sprague-Dawley rats, 200 g body weight, were obtained from Zentral Institut fuer Versuchstiere, Hannover, Germany. Animals were adjusted to the housing conditions for 1 week prior to use. Water and food were given ad libitum. There were five animals per treatment group unless otherwise stated.

Infection models. The infection models are described in more detail elsewhere (27). Briefly, the inoculum for infection was prepared from 24-h cultures of isolate on malt extract agar slopes (Difco, Sparks, MD) at 28°C. Yeast cells were scraped off, diluted with phosphate buffer, and vortexed. The inoculum was adjusted by turbidometric analysis and confirmed by quantitative plating.

Infection was induced by injection of *Candida* suspension (3×10^5 or 1×10^6 CFU per mouse and 5×10^6 CFU per rat) via the lateral tail vein. The animals were observed for signs of disease and mortality after infection twice daily for 7 to 40 days, depending on the experiment. *Candida* infection in mice affected predominantly kidneys that showed extensive granulomatous nephritis. Intravenous infection of rats led to a more disseminated infection involving kidneys, lung, heart, brain, and liver, resulting in mortality within 5 to 7 days. Following an observation period, surviving animals were sacrificed by inhalative exposure to CO_2.

Various *C. albicans* strains were checked for pathogenicity in mice. Strains causing high mortality of 80 to 100% after intravenous injection at 1×10^6 or 3×10^5 were used. Survival curves (Kaplan-Meier plots) were generated using the GraphPad software package Prism, California.

Efficacy of icofungipen in systemic infection with FLC-resistant yeast. Clinical isolates were obtained from various hospitals in Germany and were tested in vitro for susceptibility to FLC, as described above. Strains exhibiting an MIC toward FLC of ≤4 mg/liter were considered susceptible, whereas those with an MIC of ≥64 mg/liter were classified as resistant. Subsequently, strains were tested for pathogenicity in mice. About 75% of all strains caused a lethal infection in the model, and no differences (ratio of pathogenic versus nonpathogenic strains) were observed between FLC-sensitive and FLC-resistant strains. In total, 27 FLC-susceptible and 27 FLC-resistant strains causing 80 to 100% mortality in the systemic mouse infection model were identified.

Treatment. Icofungipen, free base or hydrochloride salt, was dissolved in a solution containing 5% glucose and 0.2% agar (Sigma, St. Louis, MO). It was given orally twice daily (b.i.d.) by gavage at various doses and time intervals. Unless otherwise stated, treatment was initiated 30 min after infection. Control animals received vehicle.

Statistical analysis. Statistical analysis was performed using GraphPad Prism Software version 4.0 (Graph Pad Software Inc., San Diego, CA) as indicated in the individual figures.

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/well). Testing in RPMI 1640 medium in these experiments was done according to the CLSI guideline M27-A (15). MICs for amphotericin B and FLC were within the recommended CLSI quality control range of 0.5 to 2 and 0.25 to 1 μg/ml, respectively.

Several complex (YPD, LB, YPG, Sabouraud, and RPMI 1640) and two chemically defined (YNGW and YNG) media were tested. Under these experimental conditions, only testing in YPG 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 icofungipen were significantly lower if chemically defined media (YNGW and YNG) were used.

For further testing, therefore, YNG was chosen. We also tested whether MICs were altered by branched-chain amino acids (isoleucine, leucine, and valine) known to interfere with the uptake of icofungipen (31, 32). MICs increased in the presence of isoleucine, leucine, and valine (Table 2), particularly with isoleucine, which increased the mean MIC after 24 h from 2 to 256 μg/ml.

(ii) Influence of pH. Susceptibility testing at different pH values was studied repeatedly on three different *C. albicans* strains in YNG medium. Strains were chosen on the basis of their susceptibility to icofungipen in YNG medium at pH 7 and differed in their susceptibilities at 24 h. *C. albicans* PSCF 0440 was the most sensitive strain (MIC, 0.5 to 4 μg/ml), ATCC 90028 showed moderate susceptibility (MIC, 4 to 8 μg/ml), and PSCF 0085 had the highest MICs (8 to 64 μg/ml). Incremental changes of 0.5 pH units of the YNG medium between 4.0 and 8.0 did not significantly influence the in vitro activity of standard antifungal compounds (data not shown). Apart from an increase in MIC at pH <5 or pH ≥7 after 48 h of incubation (about two- to fourfold), no significant differences were found for icofungipen. Testing was therefore performed at pH 6 to 7 after 24 h of incubation.

(iii) Influence of inoculum size. Inoculum sizes of three different strains of *C. albicans* influenced the MICs of both icofungipen and several azole standards (Table 3) in the same way, irrespective of the method of inoculum preparation (stationary, logarithmic liquid, or plate culture) (data not shown). As expected, sensitivity to amphotericin B was not affected

TABLE 1. Influence of growth medium composition on MICs of tested antifungals[a]

Medium	*C. albicans* strain	MIC (μg/ml) for:					
		Amphotericin B		Fluconazole		Icofungipen	
		24 h	48 h	24 h	48 h	24 h	48 h
YNGW	ATCC 90028	0.5	1	0.5	0.5	8	8
	PSCF 0440	0.5	1	0.25	0.5	4	4
	PSCF 0085	0.25	0.5	0.25	0.5	4	32
YNG	ATCC 90028	1	1	0.5	0.5	8	8
	PSCF 0440	1	2	0.5	0.5	4	4
	PSCF 0085	0.5	1	0.5	0.5	8	32
YPD	ATCC 90028	1	1	0.5	1	>64	>64
	PSCF 0440	1	2	1	1	64	>64
	PSCF 0085	0.5	1	1	1	>64	>64
LB	ATCC 90028	0.125	0.5	0.5	0.25	>64	>64
	PSCF 0440	0.125	0.5	0.5	0.5	16	32
	PSCF 0085	1	1	0.5	0.5	>64	>64
YPG	ATCC 90028	2	4	0.5	0.5	64	>64
	PSCF 0440	2	4	0.5	2	32	64
	PSCF 0085	1	2	0.5	0.5	>64	>64
Sabouraud	ATCC 90028	0.5	0.5	0.5	1	64	>64
	PSCF 0440	0.5	1	1	1	16	32
	PSCF 0085	0.5	0.5	1	1	>64	>64
RPMI 1640	ATCC 90028	0.5	0.5	0.5	0.5	64	>64
	PSCF 0440	0.25	1	0.5	0.5	16	32
	PSCF 0085	0.5	0.5	0.25	0.25	32	>64

[a] 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).

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

Amino acid	MIC (μg/ml) for amino acid at a concn (mM) of:							
	0		0.1		1		10	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Isoleucine	2	4	16	16	64	128	256	256
Leucine	2	4	8	8	32	32	64	128
Valine	2	4	8	8	8	32	64	128

[a] 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 3. Influence of inoculum size on MICs of tested antifungals[a]

Inoculum size (CFU/well)	*C. albicans* strain	MIC (μg/ml) for:					
		Amphotericin B		Fluconazole		Icofungipen	
		24 h	48 h	24 h	48 h	24 h	48 h
50	ATCC 90028	0.25	0.5	0.5	0.5	1	2
	PSCF 0440	0.25	0.5	0.5	0.5	0.5	2
	PSCF 0085	0.5	2	2	2	4	>64
100	ATCC 90028	0.5	0.5	0.5	0.5	1	4
	PSCF 0440	0.5	0.5	0.5	0.5	0.5	2
	PSCF 0085	1	2	2	2	4	>64
150	ATCC 90028	0.5	1	0.5	0.5	1	4
	PSCF 0440	0.5	1	0.5	1	1	2
	PSCF 0085	1	2	2	2	8	>64
200	ATCC 90028	0.25	1	0.25	0.5	1	4
	PSCF 0440	0.5	1	0.5	1	1	4
	PSCF 0085	1	2	4	4	>64	>64
500	ATCC 90028	0.5	1	0.25	1	2	8
	PSCF 0440	0.5	1	0.5	2	1	8
	PSCF 0085	1	2	4	4	>64	>64
1,000	ATCC 90028	0.5	1	1	>64	8	>64
	PSCF 0440	0.5	1	1	>64	2	16
	PSCF 0085	1	2	4	>64	>64	>64
5,000	ATCC 90028	0.5	1	1	>64	16	>64
	PSCF 0440	0.5	1	2	>64	4	16
	PSCF 0085	1	2	4	>64	>64	>64
10,000	ATCC 90028	0.5	1	1	>64	16	>64
	PSCF 0440	0.5	1	2	>64	4	16
	PSCF 0085	1	2	8	>64	>64	>64

[a] MICs were determined in YNG medium after 24 or 48 h of incubation. Experiments were repeated five times independently, and representative experiments are 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 azole. Ninety percent of the strains of *C. albicans* showed susceptibility to icofungipen with MICs ranging from 8 to 32 μg/ml, giving an MIC_{90} of 32 μg/ml. The total range of MICs in this set of strains varied between 4 and >64 μg/ml. MIC_{90} values for amphotericin B and clotrimazole were 1 μg/ml and 0.5 μg/ml, respectively (Table 4).

TABLE 4. In vitro activity of icofungipen against 69 clinical isolates of *C. albicans*[a]

Compound	MIC (μg/ml)		
	50%	90%	Range
Icofungipen	16	32	4–>64
Clotrimazole	≤0.125	0.5	≤0.125–2
Amphotericin B	1	1	≤0.125–4

[a] 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_{80} of ≤4 mg/liter and the other with an MIC_{80} 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.

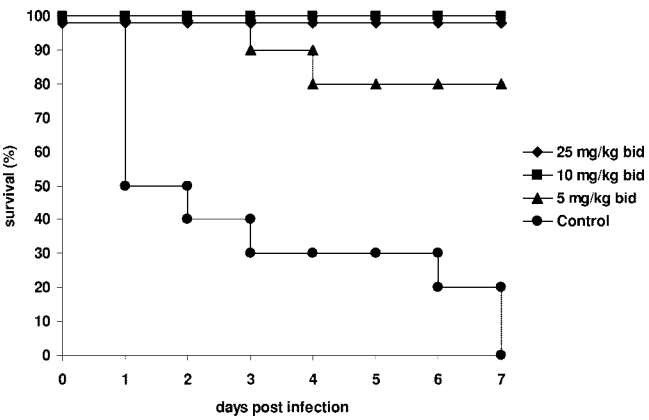

FIG. 1. Efficacy of icofungipen in the *C. albicans* lethal infection model in mice. Ten animals per group were infected with 10^6 yeasts (ATCC 200498) intravenously 30 min prior to the first dosing and treated with icofungipen orally b.i.d. at indicated dosages for 4 days. MIC of icofungipen, 1 μg/ml. All treated groups showed significant survival in comparison to controls (P values of at least <0.002, calculated according to survival curves and a chi-square test).

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

Isolates (no. of isolates)[a]	Icofungipen dose (mg/kg)[b]	No. (%) of strains associated with indicated survival rate (%)[c]		
		>80	51–80	31–50
All isolates (89)	5	34 (38)	55 (66)	
Isolates with survival of <80% at 5 mg/kg icofungipen (55)	10	33 (60)	15 (27)	7 (13)

[a] Clinical isolates of *C. albicans* causing >80% mortality in untreated animals.
[b] The stated dose was given orally twice daily over 4 days, starting 0.5 h after infection.
[c] Animals (10 per isolate) were infected intravenously, and survival was monitored for 10 days.

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.

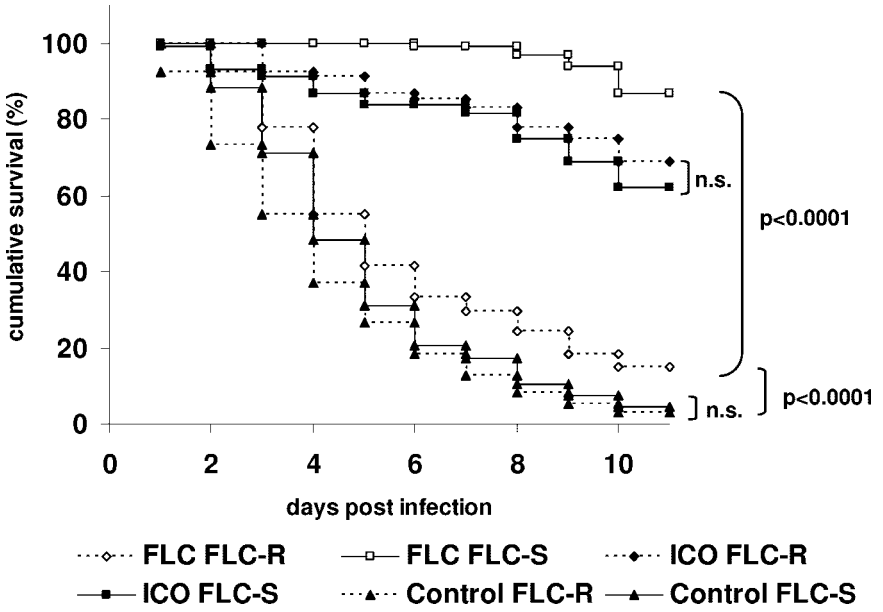

FIG. 2. Efficacy of icofungipen (ICO) and FLC in the lethal *C. albicans* infection model in mice. Mice were infected with FLC-susceptible (FLC-S; MIC, ≤4 mg/liter) and FLC-resistant (FLC-R; MIC, ≥64 mg/liter) strains. Cumulative survival is shown for the two isolate and treatment groups. Each treatment arm represents the survival data from infection experiments with 27 different clinical isolates; group size was 5 mice per strain (total number of mice per treatment arm, 135). Icofungipen (10 mg/kg twice daily) and FLC (2 mg/kg/day) were given orally twice daily for 4 days. n.s., not significant. *P* values were calculated according to survival curves and a chi-square test.

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.

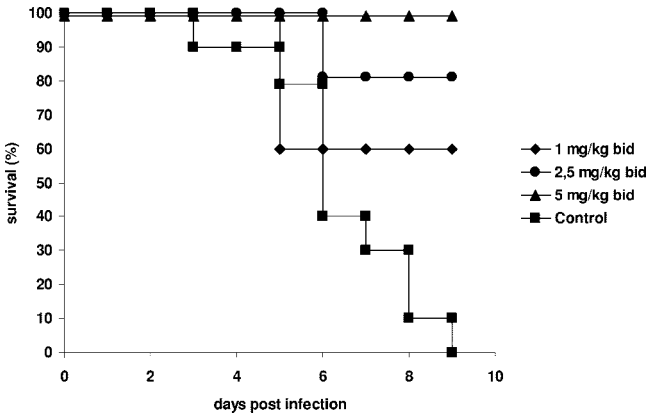

FIG. 3. Efficacy of icofungipen in a lethal systemic *C. albicans* (ATCC 200498) infection in rats. Icofungipen was given at 1, 2.5, and 5 mg/kg orally b.i.d. for 5 days (days 0 to 4), with intravenous infection of *C. albicans* at day 0. Rats ($n = 10$ per treatment arm) were observed for up to 40 days, and no further changes in survival in the treated groups were observed (data not shown). All treated groups showed significant survival in comparison to controls (*P* values of at least <0.02, calculated according to survival curves and a chi-square test).

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

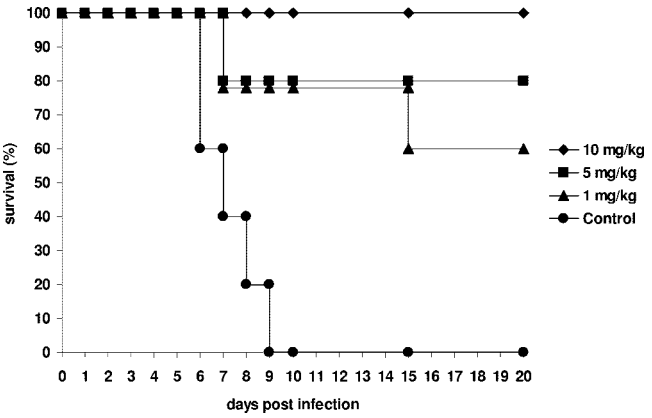

FIG. 4. Efficacy of a single oral dose of icofungipen in rats with a lethal systemic *C. albicans* (ATCC 200498) infection ($n = 5$). Icofungipen was given 30 min after the intravenous inoculation of *C. albicans* (5×10^6 CFU).

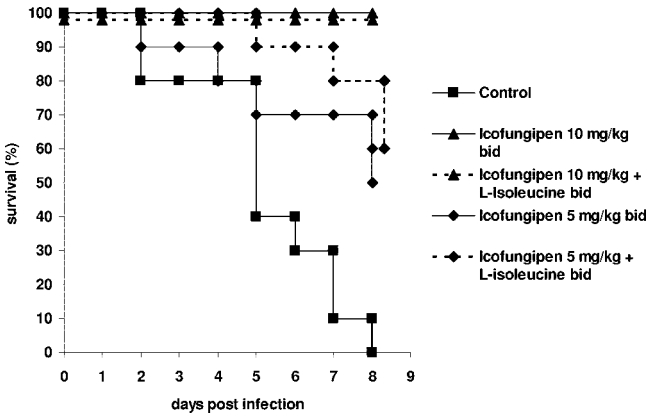

FIG. 5. Influence of L-isoleucine on the in vivo efficacy of icofungipen in the *C. albicans* (ATCC 200498) mouse infection model. CFW1 mice ($n = 10$) were infected intravenously and treated orally b.i.d. for 4 days. Icofungipen was given alone or was mixed prior to the dosing with an equimolar amount 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.

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.

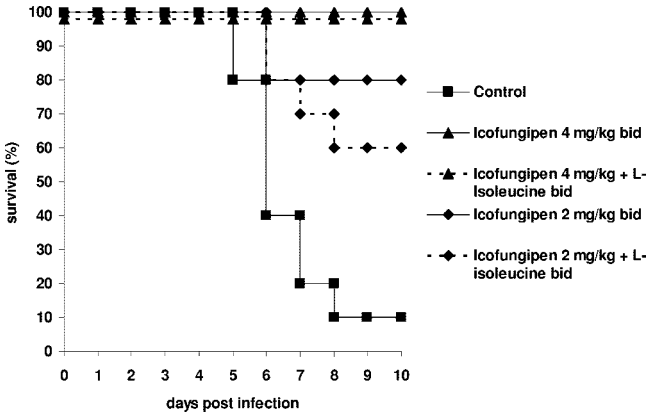

FIG. 6. Influence of L-isoleucine on the in vivo efficacy of icofungipen in the *C. albicans* (ATCC 200498) rat infection model. Rats ($n = 10$) were infected intravenously and treated orally b.i.d. for 4 days. Icofungipen was given alone or mixed prior to the dosing with an equimolar amount of L-isoleucine.

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 influenced by the concentration of amino acids present in all complex 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 relatively low concentrations of amino acids (methionine, histidine, 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 icofungipen, leads to a dose-dependent increase in the MIC of icofungipen (Table 2).

Inoculum size proved to be a critical parameter for measuring 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. albicans* (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 subinhibitory level under the conditions used.

In principle, the MICs of icofungipen for yeasts are dependent 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 synthetase, 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 vitro 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 reproducible method that correlates individual MIC levels with response rates in animal models and later clinical studies.

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 comparably 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 are tightly regulated in all species, and no disease in humans is known in which isoleucine levels are specially affected, except a very rare hereditary disorder, maple syrup disease (14). In this disease, metabolism of branched amino acids is affected. In these patients, an increase in leucine is observed, which in turn may result in isoleucine deficiency. However, concomitant dietary uptake or the use of balanced amino acid solutions could eventually have an impact on the antifungal efficacy of the 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

1. **Asai, K., N. Tsuchimori, K. Okonogi, J. R. Perfect, O. Gotoh, and Y. Yoshida.** 1999. Formation of azole-resistant *Candida albicans* by mutation of sterol 14-demethylase P450. Antimicrob. Agents Chemother. **43:**1163–1169.
2. **Brockmeyer, N. H., M. Ruhnke, K. Oreskovic, and J. Peterson.** 2004. A phase II study of icofungipen (PLD-118) in the treatment of oropharyngeal candidiasis (OPC) in HIV-positive patients, p. 418. Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother. American Society for Microbiology, Washington, D.C., 30 October to 2 November 2004.
3. **Cartledge, J. D., J. Midgley, and B. G. Gazzard.** 1997. Clinically significant azole cross-resistance in *Candida* isolates from HIV-positive patients with oral candidosis. AIDS **11:**1839–1844.
4. **Chapin, K. C., and P. R. Murray.** 1999. Media, p. 1687–1707. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 7th ed. American Society for Microbiology, Washington, D.C.
5. **Dismukes, W. E.** 2000. Introduction to antifungal drugs. Clin. Infect. Dis. **30:**653–657.
6. **Ernst, E. J.** 2001. Investigational antifungal agents. Pharmacotherapy **21:** 165S–174S.
7. **Georgopapadaku, N. H.** 1998. Antifungals: mechanism of action and resistance, established and novel drugs. Curr. Opin. Microbiol. **1:**547–557.
8. **Hossain, M., and M. Ghannoum.** 2000. New investigational antifungal agents for treating invasive fungal infections. Expert Opin. Investig. Drugs **9:**1797–1813.
9. **Iwamoto, T., E. Tsujii, M. Ezaki, A. Fujie, S. Hashimoto, M. Okuhara, M. Kohsaka, and H. Imanaka.** 1990. FR109615, a new antifungal antibiotic from *Streptomyces setonii*. Taxonomy, fermentation, isolation, physico-chemical properties and biological activity. J. Antibiot. **43:**1–7.
10. **Johnson, M. D., and J. R. Perfect.** 2003. Caspofungin: First approved agent in a new class of antifungals. Expert Opin. Pharmacother. **4:**807–823.
11. **Klepser, M. E., E. J. Ernst, R. E. Lewis, M. E. Ernst, and M. A. Pfaller.** 1998. Influence of test conditions on antifungal time-kill curve results: proposal for standardized methods. Antimicrob. Agents Chemother. **42:**1207–1212.
12. **Konishi, M., M. Nishio, K. Saltoh, T. Miyaki, T. Oki, and H. Kawaguchi.** 1989. Cispentacin, a new antifungal antibiotic. I. Production, isolation, physico-chemical properties and structure. J. Antibiot. **42:**1749–1755.
13. **Mittendorf, J., F. Kunisch, M. Matzke, H.-C. Militzer, A. Schmidt, and W. Schoenfeld.** 2003. Novel antifungal beta-amino acids: synthesis and activity against *Candida albicans*. Bioorg. Med. Chem. Lett. **13:**433–436.
14. **Morton, D. H., K. A. Strauss, D. L. Robinson, G. Puffenberger, and R. I. Kelley.** 2002. Diagnosis and treatment of maple syrup disease: a study of 36 patients. Pediatrics **109:**999–1008.
15. **National Committee for Clinical Laboratory Standards.** 1997. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.
16. **Ohki, H., Y. Inamoto, K. Kawabata, T. Kamimura, and K. Sakane.** 1991. Synthesis and antifungal activity of FR109615 analogs. J. Antibiot. **44:**546–549.
17. **Oki, T., M. Hirano, K. Tomatsu, K. Numata, and H. Kamei.** 1989. Cispentacin, a new antifungal antibiotic. II. *In vitro* and *in vivo* antifungal activities J. Antibiot. **42:**1756–1762.
18. **Pappas, P. G., J. H. Rex, J. Lee, R. J. Hamill, R. A. Larsen, W. Powderly, C. A. Kauffman, N. Hyslop, J. E. Mangino, S. Chapman, H. W. Horowitz, J. E. Edwards, W. E. Dismukes, and the NIAID Mycoses Study Group.** 2003. A prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. Clin. Infect. Dis. **37:**634–643.
19. **Perfect, J. R.** 2002. New antifungal agents. Transpl. Infect. Dis. **4:**52–61.
20. **Petraitis, V., R. Petraitiene, A. M. Kelaher, A. A. Sarafandi, T. Sein, D. Mickiene, J. Bacher, A. H. Groll, and T. J. Walsh.** 2004. Efficacy of PLD-118, a novel inhibitor of *Candida* isoleucyl-tRNA synthetase, against experimental oropharyngeal and esophageal candidiasis caused by fluconazole-resistant *C. albicans*. Antimicrob. Agents Chemother. **48:**3959–39567.

21. **Revankar, S. G., O. P. Dib, W. R. Kirkpatrick, R. K. McAtee, A. W. Fothergill, M. G. Rinaldi, S. W. Redding, and T. F. Patterson.** 1998. Clinical evaluation and microbiology of oropharyngeal infection due to fluconazole-resistant *Candida* in human immunodeficiency virus-infected patients. Clin. Infect. Dis. **26:**960–963.
22. **Rex, J. H., M. G. Rinaldi, and M. A. Pfaller.** 1995. Resistance of *Candida* species to fluconazole. Antimicrob. Agents Chemother. **39:**1–8.
23. **Rex, J. H., T. J. Walsh, J. D. Sobel, S. G. Filler, P. G. Pappas, W. E. Dismukes, and J. E. Edwards.** 2000. Practice guidelines for the treatment of candidiasis. Clin. Infect. Dis. **30:**662–678.
24. **Sambrook, J., P. MacCallum, and D. W. Russell.** 2000. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Sanglard, D.** 2003. Clinical relevance of mechanisms of antifungal drug resistance in yeasts. Enferm. Infecc. Microbiol. Clin. **20:**462–469.
26. **Sanglard, D., and F. Odds.** 2002. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect. Dis. **2:**73–85.
27. **Schmidt, A., and U. Geschke.** 1996. Comparative virulence of Candida albicans strains in CFW1 mice and Sprague-Dawley rats. Mycoses **39:**157–160.
28. **Schoenfeld, W., and M. Parnham.** 2001. PLD-118, a novel antifungal for treatment of yeast infections: animal pharmacokinetics. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 2145. American Society for Microbiology, Washington. D.C.
29. **Seibold., M., and K. Tintelnot.** 2003. Susceptibility testing of fungi—current status and open questions. *In* E. Jucker (ed.), Antifungal agents—advances and problems. Birkhauser Verlag, Basel, Switzerland.
30. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1979. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Ziegelbauer, K.** 1998. Decreased accumulation or increased isoleucyl-tRNA synthetase activity confers resistance to the cyclic β-amino acid BAY 10-8888 in *Candida albicans* and *Candida tropicalis*. Antimicrob. Agents Chemother. **42:**1581–1586.
32. **Ziegelbauer, K., P. Babczinski, and W. Schoenfeld.** 1998. Molecular mode of action of the antifungal β-amino acid BAY 10-8888. Antimicrob. Agents Chemother. **42:**2197–2202.