Efficacy, Plasma Pharmacokinetics, and Safety of Icofungipen, an Inhibitor of *Candida* Isoleucyl-tRNA Synthetase, in Treatment of Experimental Disseminated Candidiasis in Persistently Neutropenic Rabbits

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Icofungipen (formerly PLD-118) is a synthetic derivative of the naturally occurring β -amino acid cispentacin that blocks isoleucyl-tRNA synthetase, resulting in the inhibition of protein synthesis and growth of fungal cells. We investigated the efficacy, plasma pharmacokinetics, and safety of icofungipen in escalating dosages for the treatment of experimental subacute disseminated candidiasis in persistently neutropenic rabbits. Icofungipen was administered for 10 days starting 24 h after the intravenous inoculation of 10^3 *Candida albicans* blastoconidia. Study groups consisted of rabbits treated with icofungipen at 4 (ICO-4), 10 (ICO-10), and 25 (ICO-25) mg/kg of body weight/day in two divided dosages, rabbits treated with fluconazole at 10 mg/kg/day, rabbits treated with amphotericin B at 1 mg/kg/day, and untreated controls. Levels of icofungipen in plasma were derivatized by phthalaldehyde and quantified by high-performance liquid chromatography with fluorescence detection. Rabbits treated with ICO-10 ($P < 0.01$) and ICO-25 ($P < 0.001$) showed significant dosage-dependent tissue clearance of *C. albicans* from the liver, spleen, kidney, brain, vitreous, vena cava, and lung in comparison to untreated controls. ICO-25 cleared *C. albicans* from all tissues and had activity comparable to that of amphotericin B versus untreated controls ($P < 0.001$). Pharmacokinetics of icofungipen in plasma approximated a dose-dependent relationship of the maximum concentration of drug in serum and the area under the concentration-time curve. There was no significant elevation of the levels of hepatic transaminases, alkaline phosphatase, bilirubin, urea nitrogen, or creatinine in icofungipen-treated rabbits. Icofungipen followed dose-dependent pharmacokinetics and was effective in the treatment of experimental disseminated candidiasis, including central nervous system infection, in persistently neutropenic rabbits.

Disseminated candidiasis is an important cause of nosocomial fungal infection in immunocompromised patients. *Candida* species now constitute the fourth-most-common pathogen in nosocomial infections (1, 5, 7, 10, 11, 16, 17, 21, 23, 26, 27, 30). Conventional amphotericin B and its lipid formulations are mainstays of therapy for serious *Candida* infections; however, clinical usage is limited by its infusion-related nephrotoxicity and high acquisition cost (22). Triazole compounds are another option for the treatment of disseminated candidiasis; however, the emergence of resistance to antifungal triazoles may limit their utility (29). Echinocandins are also effective against disseminated candidiasis; however, they are limited to parenteral routes of administration (3, 4, 9, 12, 13, 24, 25, 31). There is, therefore, a continuing need for new classes of antifungal compounds that have potent antifungal activity, improved safety, and flexibility in formulation.

Icofungipen (formerly PLD-118 or BAY-10-8888) is a new oral and parenteral antifungal compound with anti-*Candida*

activity. As a synthetic derivative of the naturally occurring β -amino acid cispentacin, icofungipen has a novel mechanism of action that is thought to arise from the inhibition of isoleucyl-tRNA synthetase, protein biosynthesis, and fungal cell growth (6, 18, 38, 39). In vitro studies have demonstrated the antifungal activity of icofungipen against *Candida albicans*, including azole-resistant strains (A. Hasenoeherl, M. Skerlev, N. Marsic, W. Schoenfeld, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 2143, 2001), and non-*albicans* *Candida* spp. (W. Schoenfeld, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 812, 2002). In vivo studies also have demonstrated the antifungal activity of icofungipen in animal models of disseminated candidiasis (W. Schoenfeld, J. Mittendorf, A. Schmidt, U. Geschke, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 2144, 2001; A. Hasenoeherl, N. Marsic, K. Orescovic, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1229, 2003) and of fluconazole-resistant oropharyngeal candidiasis (V. Petraitis, R. Petraitiene, A. Kelaher, A. Sarafandi, T. Sein, R. L. Schaefele, A. Francesconi, J. Bacher, T. J. Walsh, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 188, 2002).

Little is known, however, about the in vivo activity of icofungipen in the treatment of disseminated candidiasis in per-

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sistently neutropenic hosts. We therefore investigated the antifungal efficacy, plasma pharmacokinetics, and safety of icofungipen in a persistently neutropenic rabbit model of disseminated candidiasis.

MATERIALS AND METHODS

Test isolate. The clinical isolate *C. albicans* NIH 8621 (ATCC MYA-1237) was originally obtained from a granulocytopenic patient with autopsy-proven disseminated candidiasis and used for all experiments. The isolate was subcultured from a frozen stock culture (stored at -80°C on potato dextrose agar slants) on Sabouraud dextrose agar (SGA) plates, incubated at 37°C for 24 h, and maintained during the course of the experiments at 4°C .

Antifungal susceptibility testing. The MICs of each antifungal agent for *C. albicans* were determined by the approved broth microdilution method of the National Committee for Clinical Laboratory Standards reference M27-A2 or adapting modifications of the method (19).

Hasenoehrl et al. reported that, because of its mode of action (active uptake via the isoleucyl transporter into yeasts and subsequent protein biosynthesis inhibition), icofungipen (PLIVA Pharmaceutical Industry, Inc., Zagreb, Croatia) requires a defined medium composition with minimal chain amino acids to achieve reproducible results (Hasenoehrl et al., 41st ICAAC). This property is reminiscent of measuring MICs of flucytosine in defined medium to minimize exposure to excess pyrimidines that may falsely elevate flucytosine MICs. The most consistent activity of icofungipen against *C. albicans* isolates was observed in yeast nitrogen base medium. Similar conclusions were reached by Ruhnke et al. (M. Ruhnke, C. Radecke, and D. Westphal, Abstr. 44th Intersci. Conf. Antimicrob. Agents and Chemother., abstr. 843, 2004). Thus, yeast nitrogen broth-1% glucose medium (YNG; K-D Medical, Inc., Columbia, Md.) (0.67% yeast nitrogen base [Difco], 1% glucose [pH 7.0]) or YNG medium supplemented with serum was used as the growth medium for determination of the MICs of icofungipen and as a diluent for icofungipen in vitro studies (T. Galic, G. Ergovic, S. Plesko, K. Oreskovic, M. Kolega, W. Schoenfeld, and R. Antolovic, Abstr. 44th Intersci. Conf. Antimicrob. Agents and Chemother., abstr. 844, 2004). The inoculum was prepared by selecting several colonies from 24-h-old cultures of *Candida albicans* grown on SGA plates. Colonies were suspended in sterile saline and adjusted to a 0.5 McFarland turbidity (approximately 1×10^6 to 5×10^6 CFU/ml using spectrophotometric methods) and then diluted to a final inoculum of approximately 1×10^3 CFU/ml (approximately 200 CFU/well). The exact inoculum used was verified by quantitative subcultures on SGA plates.

A stock solution of icofungipen was prepared in 1% glucose YNG medium or YNG medium supplemented with 5% rabbit serum with a starting concentration of 64 $\mu\text{g/ml}$ (51.2 μl of the stock solution in 1,948.8 μl of the medium = 128 $\mu\text{g/ml}$, which would be diluted in a microplate with an equal volume of fungal inoculum). Microplate wells were filled with serial twofold dilutions of icofungipen (50 μl) with a concentration range of 64 $\mu\text{g/ml}$ to 0.125 $\mu\text{g/ml}$. Columns 2 to 12 were filled with 50 μl of fungal inoculum (final volume is 100 μl per well). Microtiter plates were incubated at 35°C for 24 and 48 h according to *Candida* growth rate.

The MIC was defined as the lowest well displaying 50% inhibition (MIC-2) with icofungipen compared to the drug-free control well. Visual readings were performed, and MICs were determined in three or more experiments. Preliminary studies indicated that the reproducibility of the endpoint was greater at MIC-2 than at MIC-0 (optically clear). MICs were determined in six experiments and, at MIC-2, were found to be 0.5 $\mu\text{g/ml}$ each time. When read at MIC-0, the median MIC was 2.0 $\mu\text{g/ml}$ (range, 1.0 $\mu\text{g/ml}$ to 4.0 $\mu\text{g/ml}$). No significant trailing was observed under these growth conditions.

The MIC of fluconazole (Pfizer Inc., Groton, Conn.) was determined in RPMI 1640 (BioWhittaker, Walkerville, Md.). Antibiotic medium 3 (NIH Media Unit, Bethesda, Md.) was used for determination of the MICs of amphotericin B (Fungizone; Apothecon Bristol-Myers Squibb Company, Princeton, N.J.). The starting inoculum of *C. albicans* was approximately 10^3 CFU/ml. The MIC endpoints of fluconazole were read as the lowest concentration at which a prominent decrease in turbidity (MIC-2) relative to the turbidity of the growth control well was observed. Amphotericin B MIC results were read at MIC-0 (100% inhibition of growth [first clear well]). One drug-free and one yeast-free well were included in each plate as controls.

Time-kill studies. (i) Inoculum preparation. To characterize the in vitro pharmacodynamics and potential fungicidal activity of icofungipen, amphotericin B, and fluconazole, time-kill assays were performed against the *C. albicans* isolate using previously described methods (24, 34, 35). The inoculum for the time-kill assays was prepared by subculturing the *C. albicans* isolate from a frozen stock

culture (stored at -80°C on potato dextrose agar slants) onto an SGA plate and incubating the isolate for 24 h to 48 h at 37°C . Fungal suspensions were prepared by inoculating three to five colonies into a starter broth of 50 ml of Emmon's modified Sabouraud glucose broth (SGB; K-D Medical, Inc., Columbia, Md.) and incubating the colonies at 37°C for 24 h in a shaking water bath. The suspension was washed three times with normal saline, resuspended, counted with a hemacytometer, and confirmed with quantitative cultures. Concentrations of approximately 1.0×10^3 CFU/ml of *C. albicans* were used for time-kill assays for all compounds.

(ii) Drug concentrations and media. One milliliter of *C. albicans* suspension for the time-kill assay of amphotericin B was transferred into 50 ml of fresh antibiotic medium 3 broth in each 250-ml Erlenmeyer flask containing growth control and amphotericin B (0.1, 0.5, and 1.0 $\mu\text{g/ml}$). The same method was applied to icofungipen (4, 8, 16, and 64 $\mu\text{g/ml}$) and fluconazole (4, 8, 16, and 64 $\mu\text{g/ml}$), with the exception of using YNG broth. The flasks were incubated at 37°C for 16 h in a shaking water bath to generate logarithmic-phase growth.

(iii) Sampling and quantitative subculture procedure. The growth suspensions were sampled at predetermined time points (0, 2, 4, 6, 12, and 24 h following the addition of the antifungal), and 100- μl aliquots were plated in dilutions of 10^{-2} , 10^{-3} , and 10^{-4} onto one SGA plate per aliquot. The colonies were counted after 48 h of incubation at 37°C . The lower limit of quantitation for the time-kill assay was 10 CFU/ml. Time-kill assays for all concentrations were performed in triplicate.

(iv) Time-kill plots and interpretation. The calculated number of CFU per milliliter was plotted for each time point. Fungicidal activity was defined as a ≥ 3 log₁₀ (99.9%) reduction in CFU/ml from the starting inoculum. Fungistatic activity was a $<99.9\%$ reduction in CFU/ml from the starting inoculum.

Inoculum. For preparation of the inoculum, three to five well-isolated colonies were sampled from freshly grown culture plates and suspended in 50 ml of Emmon's modified SGB (pH 7.0) in a 250-ml Erlenmeyer flask. The suspension was incubated in a gyratory water bath at 80 oscillations per min at 37°C for 18 h. The *Candida* suspension was then centrifuged at $3,000 \times g$ for 10 min and washed three times with sterile normal saline (Quality Biological, Inc., Gaithersburg, Md.). The concentration was adjusted by use of a hemacytometer and was confirmed by quantitative cultures of a 10-fold serial dilution. An inoculum of 10^3 blastoconidia suspended in a 5-ml volume of normal saline was slowly administered to each rabbit via the indwelling silastic central venous catheter on day 6 of the experiment. The inoculum size was confirmed by plating serial dilutions onto SGA plates. The pattern of infection of disseminated candidiasis permitted survival of nearly all rabbits throughout the experiment.

Animals. Female New Zealand White rabbits (Hazleton Research Products, Inc., Denver, Pa.) weighing 2.4 to 3.3 kg at the time of inoculation were used in experiments ($n = 45$). These studies were approved by the Animal Care and Use Committee of the National Cancer Institute. Rabbits were individually housed, maintained with water and standard rabbit feed ad libitum, and monitored under humane care and use in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and according to National Institutes of Health guidelines for animal care and in fulfillment of the guidelines of the National Research Council (20). Vascular access was established in each rabbit by the surgical placement of a silastic tunneled central venous catheter as previously described (33). The silastic catheter permitted nontraumatic venous access for repeated blood sampling for studies of biochemical and hematological parameters, plasma pharmacokinetics, and administration of parenteral agents. Serum samples were drawn from all rabbits at the initiation of immunosuppression, during the course of disseminated candidiasis, and before death. Rabbits were euthanized according to Animal Care and Use Committee-approved prespecified humane endpoints by intravenous (i.v.) administration of pentobarbital (65 mg of pentobarbital sodium/kg of body weight; pentobarbital sodium was in the form of 0.5 ml of Beuthanasia-D special [euthanasia solution]; Schering-Plough Animal Health Corp., Union, N.J.) at the end of each experiment, 24 h after administration of the last dose of study drug.

Immunosuppression and maintenance of neutropenia. Cytarabine (AraC, Cytosar-U; The Upjohn Company, Kalamazoo, Mich.) was administered i.v. for induction and maintenance of neutropenia. Profound neutropenia (a neutrophil concentration of <100 neutrophils/ μl) was achieved in the disseminated candidiasis model with an initial i.v. course of 440 mg of AraC per m^2 daily for 5 days before inoculation of the rabbits. A maintenance dose of 440 mg of AraC per m^2 was administered at 2-day intervals during the experiment.

All rabbits received ceftazidime (Glaxo Pharmaceuticals, Division of Glaxo, Inc., Research Triangle Park, N.C.) at a dose of 75 mg/kg i.v. twice daily, gentamicin (Elkins-Sinn, Inc., Cherry Hill, N.J.) at a dose of 5 mg/kg i.v. every other day, and vancomycin (Abbott Laboratories, North Chicago, Ill.) at a dose of 15 mg/kg i.v. daily from day 4 of chemotherapy for the prevention of oppor-

tunistic bacterial infections during neutropenia. To prevent antibiotic-associated diarrhea due to *Clostridium spiroforme*, all rabbits received 50 mg of vancomycin per liter of drinking water.

Total leukocyte counts and the percentages of neutrophils were monitored twice weekly with a Coulter Counter (Coulter Corporation, Miami, Fla.) and by use of peripheral blood smears and differential counts, respectively.

Antifungal compounds and treatment groups. The treatment groups in the model of disseminated candidiasis consisted of untreated control animals and animals treated with icofungipen, amphotericin B, or fluconazole. Therapy was initiated 24 h postinoculation and continued throughout the course of the experiments for 10 days. Icofungipen was provided by Pliva Pharmaceutical Industry, Inc., as a powder for parenteral administration. Icofungipen was dissolved in 0.9% saline and administered i.v. at dosages of 2 (ICO-4), 5 (ICO-10), and 12.5 (ICO-25) mg/kg twice daily. There were 9 rabbits in each icofungipen dose group. Deoxycholate amphotericin B was resuspended in sterile water, maintained at 4°C, and diluted at a 1:5 ratio with sterile 5% dextrose (Abbott Labs, North Chicago, Ill.) to achieve a final concentration of 1 mg/ml, immediately prior to use, according to the manufacturer's instructions. Amphotericin B was administered i.v. at 1 mg/kg/day slowly (0.1 ml every 15 s) once daily ($n = 6$). Fluconazole was administered i.v. at 10 mg/kg once daily ($n = 6$).

Assessment of in vivo antifungal efficacy. Antifungal activity in the model of disseminated candidiasis was determined by quantitative clearance of *C. albicans* from tissue. Representative sections of liver, spleen, kidney, lung, vena cava anterior, and brain were weighed, and each tissue sample was then homogenized (Stomacher 80; Tekmar Corp., Cincinnati, Ohio) in sterile reinforced polyethylene bags (Tekmar Corp., Cincinnati, Ohio) with sterile 0.9% saline for 30 s (32).

Antifungal activity in treatment of *Candida* infection of the eyes was also assessed. The globes of the eyes were carefully dissected using an aseptic technique. The removed globe was transferred to a sterile petri dish (Falcon; Becton Dickinson Labware, Becton Dickinson and Co., Franklin Lakes, NJ). The sclera was incised with sharp scissors at the posterior pole, and 0.3 to 0.4 ml of vitreous humor was slowly aspirated into a sterile tuberculin syringe. The specimens of vitreous humor from both globes were pooled together.

Each tissue homogenate or vitreous humor specimen was serially diluted 10^{-1} to 10^{-4} in sterile 0.9% saline. Aliquots (100 μ l) of undiluted tissue homogenate or vitreous humor and of each dilution were separately plated onto Emmon's modified SGA containing chloramphenicol and gentamicin. Culture plates were incubated at 37°C for 24 h, after which CFU were counted and the number of CFU/g of tissue was calculated for each organ. Carryover of the drug was controlled by serial dilution and by streaking a small-volume (100 μ l) aliquot onto a large volume of agar (1 full agar plate per 100- μ l aliquot) (15). The method was sensitive enough to detect ≥ 10 CFU/g. The culture-negative plates were counted as 0 CFU/g. Data were graphed as the $\log_{10}$ (CFU/g) means $\pm$ standard errors of the means (SEM).

Histopathological analysis. Representative sections of kidney, liver, spleen, and brain were prepared for histologic studies. Tissue specimens were excised and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and then stained with either periodic acid-Schiff or Grocott-Gomori methenamine-silver stain. Tissues were microscopically examined for structural changes in *C. albicans* and for visual correlation with microbiological clearance.

In vitro morphological studies. *Candida albicans* was grown at 37°C for 24 h in a gyratory water bath in SGB. The suspension was washed two times with normal saline, resuspended, and counted with a hemacytometer. A concentration of approximately 10^6 CFU/ml of *C. albicans* stock suspension was prepared. The *C. albicans* stock suspension and YNB 1% glucose medium (K-D Medical, Inc., Columbia, Md.) were combined in a 1:10 suspension to obtain a final concentration of 10^5 CFU/ml in 100 ml and then subsequently diluted to 10^4 - and 10^3 -CFU/ml suspensions. Then 9.9 ml of each of these suspensions was aliquoted into 15-ml conical tubes labeled 0, 0.25, 0.5, 1, 2, 4.8, 16, 32, and 64 μ g/ml. Icofungipen was weighed and prepared to obtain a 6.4-mg/ml stock concentration in a final volume of 5 ml using 1% YNB as solvent. Twofold serial dilutions were performed from the icofungipen stock solution to create drug concentrations of 0.25 μ g/ml with 1% YNB. A 0.1-ml quantity of the drug solution was added to the corresponding 15-ml conical tube based on particular concentration. Then the conical tube was vortexed, and 3 ml of each mixture was placed in 24-well flat-bottom plates. Plates were incubated at 37°C for 24 h. The wells of the plates were examined under a high-power field using inverted microscopy. The plates were then spun at 3,000 rpm for 10 minutes at room temperature. The supernatant was decanted, and the pellet was resuspended in 0.5 ml of normal saline.

Six drops of resuspended pellet were placed on labeled slides that were placed in cytocentrifuge cups. A cytocentrifuge was performed in a Cytospin 2 (Shandon Thermo Electron, Inc., Pittsburgh, Pa.) instrument where the material was cen-

FIG. 1. Time-kill curve plots of icofungipen (ICO), fluconazole (FLC), and amphotericin B (DAMB) against *C. albicans*. Icofungipen concentrations of 4, 8, 16, and 64 μ g/ml, fluconazole concentrations of 4, 8, 16, and 64 μ g/ml, and amphotericin B concentrations of 0.1, 0.5, and 1 μ g/ml were studied in relation to a growth control. Data plotted are the means $\pm$ SEM of the results from three separate experiments for each growth curve. As the SEM was small for several time points, the error bars may not always be apparent in the time-kill curves.

trifuged at 75 rpm with low acceleration for 10 min. After centrifugation, the slides were Gram stained and read under a 40 $\times$ oil immersion lens.

Pharmacokinetic studies. The pharmacokinetics of icofungipen in plasma was investigated in six infected animals in each dosage cohort. Plasma sampling was performed on day 5 of antifungal therapy. Blood samples were drawn immediately after dosing and then at 0.17, 0.5, 1, 2, 4, 6, and 12 h postdosing in heparinized syringes. The plasma was immediately separated by centrifugation, and samples were stored at -80°C until assayed.

Concentrations of icofungipen in plasma were determined after protein precipitation with methanol (1:2.5 [vol/vol]) and derivatization with *o*-phthalaldehyde (OPA) by a reversed-phase high-performance liquid chromatography. Sample preparation was performed by adding 500 μ l of methanol to 200 μ l of matrix, vortexing, and centrifuging for 10 min at $6,000 \times g$. An aliquot of 50 μ l of the supernatant was filled into autosampler vials. Fifty microliters of fresh OPA solution was added from a reservoir and mixed for 1 min before 30 μ l of the resulting solution was injected onto the high-performance liquid chromatography column. For preparation of the OPA reagent, 50 mg of phthalaldehyde was dissolved in 1 ml of ethanol in a 25-ml volumetric flask and the flask was filled up to the mark with 0.1 M borate buffer (pH 9). Twenty microliters of 2-mercaptoethanol was then added. The mobile phase consisted of acetonitrile-methanol-0.01 M phosphate buffer (3.50:1.25:5.25 [vol/vol]), and the flow rate was 1.1 ml/min. Separation was achieved using a C_{18} analytical column (Nucleosil, 125 by 4.6 mm inner diameter, 5- μ m particle size; Thomson Liquid Chromatography) maintained at 40°C. Icofungipen was detected by fluorescence detection at 340-nm excitation and 430-nm emission. Quantitation was based on the peak height concentration response of the reference standard, prepared in either normal rabbit plasma. Ten-point standard curves (range of concentrations, 0.05 to 10 μ g/ml) were linear with r^2 values greater than 0.994. Samples containing concentrations exceeding the upper limit of the standard curve were assayed

FIG. 2. Response of disseminated candidiasis in persistently neutropenic rabbits to antifungal therapy measured by mean log (CFU/g) concentration of organism in the liver, spleen, and kidney in untreated controls ($n = 6$); rabbits treated with icofungipen at 4 mg/kg/day ($n = 9$) (ICO-4), 10 mg/kg/day ($n = 9$) (ICO-10), or 25 mg/kg/day ($n = 9$) (ICO-25); rabbits treated with fluconazole at 10 mg/kg/day ($n = 6$) (FLC); and rabbits treated with amphotericin B at 1 mg/kg/day ($n = 6$) (DAMB). Values are given as means $\pm$ SEM. †, $P < 0.01$; ¶, $P < 0.001$, in comparison to untreated controls using ANOVA with Bonferroni's correction for multiple comparisons.

after dilution with the mobile phase after determination of over-curve concentration-response linearity. The lower limit of quantitation was 0.100 $\mu\text{g/ml}$ in plasma. Accuracies were within $\pm 14\%$, and intra- and interday variabilities (precision) were $< 12\%$. At the lower limit of quantitation, accuracies and precision were within 11% and 12%, respectively.

Pharmacokinetic parameters for icofungipen were determined by model-independent analysis. The following pharmacokinetic parameters were determined: maximum concentrations in plasma (C_{max}), concentrations at 12 h after dosing (C_{min}), the area under the plasma concentration-time curve (AUC) from 0 to 12 h (AUC_{0-12}), calculated by trapezoidal estimation, the area under the plasma concentration-time curve from 0 h to infinity ($\text{AUC}_{0-\infty}$), and dose linearity, determined by comparison of the mean dose-normalized calculated $\text{AUC}_{0-\infty}$. Plasma drug clearance, apparent volume of distribution at steady state, and half-life were calculated by using standard equations (WinNonlin, version 4.0.1; Pharsight Corporation) (8). Statistical comparisons across dosage cohorts were made using analysis of variance (ANOVA).

Toxicity studies. Chemical determinations of potassium, aspartyl aminotransaminase, alanine aminotransaminase, creatinine, alkaline phosphatase, and total bilirubin concentrations in serum were performed by the Department of Laboratory Medicine in the NIH Warren Grant Magnuson Clinical Center with the penultimate sample drawn from each rabbit.

Statistical analysis. Comparisons between groups were performed by using ANOVA with Dunn's correction for multiple comparisons or the Mann-Whitney U test, as appropriate. All P values were two sided, and a P value of < 0.05 was considered significant. Values are expressed as means and SEMs. Statistical comparisons of pharmacokinetic parameters across dosage cohorts were made by using ANOVA.

RESULTS

Antifungal susceptibility testing. The MIC-2 (50% reduction) of icofungipen for *C. albicans* in YNG medium was 0.5 $\mu\text{g/ml}$. The MIC of fluconazole in RPMI 1640 was 0.25 $\mu\text{g/ml}$. The MICs of amphotericin B in antibiotic medium 3 were 0.125 to 0.5 $\mu\text{g/ml}$.

Time-kill assays. Time-kill curves for icofungipen, fluconazole, and amphotericin B are presented in Fig. 1. There was a concentration-dependent inhibition of growth by icofungipen against *C. albicans*. Growth of *C. albicans* was inhibited by icofungipen at concentrations between 8 and 64 $\mu\text{g/ml}$ at 12 h. Slight growth was observed at icofungipen concentrations between 16 and 64 $\mu\text{g/ml}$ at 24 h. Culture of organisms recovered from wells containing icofungipen and demonstrating regrowth at 24 h revealed no changes in MICs, suggesting that deterioration of drug in vitro or emergence of subpopulations with inducible drug resistance may account for this effect.

Marked concentration-dependent fungicidal activity was observed with amphotericin B. A decrease in *C. albicans* growth $> 99.9\%$ was observed at amphotericin B concentrations of 0.5 and 1 $\mu\text{g/ml}$.

FIG. 3. Response of disseminated candidiasis in persistently neutropenic rabbits to antifungal therapy measured by mean log (CFU/g) concentration of organism in the lung, brain, vena cava, vitreous humor in untreated controls ($n = 6$); rabbits treated with icofungipen at 4 mg/kg/day ($n = 9$) (ICO-4), 10 mg/kg/day ($n = 9$) (ICO-10), 25 mg/kg/day ($n = 9$) (ICO-25); rabbits treated with fluconazole at 10 mg/kg/day ($n = 6$) (FLC); and rabbits treated with amphotericin B at 1 mg/kg/day ($n = 6$) (DAMB). Values are given as means $\pm$ SEM. *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$, in comparison to untreated controls using ANOVA with Bonferroni's correction for multiple comparisons.

Antifungal therapy. Icofungipen demonstrated a significant dosage-dependent antifungal effect in the treatment of disseminated candidiasis across all icofungipen dosage groups (ICO-10 and ICO-25). Rabbits treated with ICO-25 and amphotericin B demonstrated the greatest degree of eradication of *C. albicans* from the liver ($P < 0.001$), spleen ($P < 0.001$), kidney ($P < 0.001$), lung ($P < 0.001$), brain ($P < 0.001$), vena cava ($P < 0.01$), and vitreous humor ($P < 0.01$) compared to untreated controls (Fig. 2 and 3). ICO-10-treated rabbits also demonstrated a significant reduction or clearance of *C. albicans* in the liver ($P < 0.01$), spleen ($P < 0.01$), kidney ($P < 0.01$), lung ($P < 0.001$), brain ($P < 0.001$), and vena cava ($P < 0.01$) compared to that of untreated controls. ICO-4-treated rabbits demonstrated a significant reduction of *C. albicans* in the brain only ($P < 0.01$). Fluconazole-treated rabbits demonstrated a significant reduction and clearance of organisms in the liver ($P < 0.001$), spleen ($P < 0.001$), kidney ($P < 0.01$), lung ($P < 0.01$), brain ($P < 0.001$), vena cava ($P < 0.01$), and vitreous humor ($P < 0.01$) compared to the untreated control rabbits.

Histopathology. There was a dosage-dependent effect of fewer lesions per low-power field ($\times 40$) detected in tissues.

Lesions were markedly reduced in tissues from untreated controls compared to ICO-4-treated rabbits, barely detectable in tissues from ICO-10-treated animals, and nonexistent in tissues from ICO-25-treated rabbits. At high magnification ($\times 400$ and $\times 1,000$), inspection of the lesions demonstrated a dosage-dependent effect on the structures of hyphae, pseudohyphae, and blastoconidia. Hyphae and pseudohyphae were disrupted, truncated, and distorted in the ICO-4 group (Fig. 4A and B). These morphological changes were even more apparent in the few residual lesions in tissues of ICO-10-treated rabbits where hyphal cells were attenuated in some areas in association with distended yeast-like structures in other areas (Fig. 4A and C).

In vitro morphological studies. In vitro studies did not demonstrate the dose-dependent distortion of hyphal structures observed in vivo. Instead, there appeared to be a dose-dependent decrease in budding of yeasts.

Pharmacokinetics. The observed plasma concentration-versus-time profiles of icofungipen following administration of 2, 5, and 12.5 mg/kg twice daily (BID) are depicted in Fig. 5. The corresponding pharmacokinetic parameters are listed in Table 1.

Dosages of 2, 5, and 12.5 mg/kg BID resulted in escalating

FIG. 4. Dosage-dependent antifungal effect on microscopic morphology of the *Candida* cell structure from icofungipen-treated rabbits. Panels A to C demonstrate a transition from predominantly hyphae and pseudohyphae in untreated controls to disrupted pseudohyphal elements of *Candida albicans*. (A) Untreated controls. Magnification, $\times 368$ (periodic acid-Schiff stain [PAS]). (B) ICO-4. Magnifications, $\times 368$ and $\times 920$ (PAS, inset). (C) ICO-10. Magnifications, $\times 368$ and $\times 920$ (PAS, inset).

FIG. 5. Concentration of icofungipen in plasma of rabbits with disseminated candidiasis after multiple dosages of 2, 5, and 12.5 mg/kg BID for 5 days.

mean peak levels in plasma that ranged from 23.09 ± 4.86 to 54.58 ± 4.56 µg/ml. Icofungipen was eliminated with mean β clearances ranging from 0.19 to 0.42 liter/h/kg. The compound exhibited a volume of distribution approximating that of total body water that was independent of the dosage. Across the investigated dosage range, icofungipen demonstrated nonlinear plasma pharmacokinetics, as evidenced by a dose-dependent increase in plasma clearance and a decrease in the dose-normalized AUC_{0-12} .

Safety. Rabbits treated with icofungipen and fluconazole and untreated controls had no detectable increase or decrease in the levels of creatinine, urea nitrogen, potassium, bilirubin, or hepatic transaminases in serum (Table 2). Rabbits treated with amphotericin B had significantly higher levels of creatinine and urea nitrogen in serum than did rabbits treated with icofungipen or fluconazole ($P < 0.001$).

DISCUSSION

This study demonstrated that icofungipen, a synthetic derivative of the naturally occurring cyclic β -amino acid cispentacin isolated from *Bacillus cereus* and *Streptomyces setonii*, has activity comparable to that of amphotericin B and fluconazole in the treatment of experimental subacute disseminated candidiasis in persistently neutropenic rabbits.

Icofungipen demonstrated antifungal activity in time-kill assays. The pattern of inhibition of icofungipen in time-kill assays more closely resembled that of fluconazole than that of amphotericin B. Although there was evidence of a concentration-dependent antifungal inhibition at 12 h, the in vivo effects of icofungipen, however, seemed more striking for dosage-dependent activity. However, as noted by Klepser and colleagues, the in vitro methods for time-kill fungicidal assays have not been standardized (14, 15). Perhaps further refinements of the time-kill assay method are warranted for molecules of the class of cispentacins.

Organisms cultured from wells containing icofungipen demonstrated regrowth at 24 h, suggesting either deterioration of compound or emergence of resistance. Although the MICs of these isolates were unchanged, we cannot exclude at this point

inducible resistance in time-kill assays. Further high-performance liquid chromatography studies are warranted to assess in vitro stability at 24 h of incubation.

Icofungipen demonstrated a dose-dependent in vivo antifungal effect in clearance of *Candida* from multiple tissues. Even at the lowest dosage tested of 2 mg/kg BID (ICO-4), a 1- to 3-log reduction was achieved. Although the differences between the means for the control and ICO-4 seem large, the significance of this difference is diminished by our conservative statistical approach using Bonferroni's correction. The response in the kidney is noteworthy given the common target of the kidney during the course of disseminated candidiasis. As icofungipen is known to be excreted by the urine, relatively high concentrations are likely achieved in both renal parenchyma and in urine to facilitate eradication of *Candida* from this tissue.

Icofungipen also demonstrated potent activity in the brain and eye. In the course of disseminated candidiasis, these are critical structures. *Candida* endophthalmitis is an important complication of candidemia and disseminated candidiasis. A new antifungal compound being developed for treatment of deeply invasive candidiasis should have documented activity in ocular infections. Activity was seen at the highest dosage of ICO-25 (12.5 mg/kg BID). Significant activity was also demonstrated in the brain at all dosages with apparent eradication at dosages of 5 mg/kg BID and 12.5 mg/kg BID (ICO-10 and ICO-25, respectively). *Candida* meningoencephalitis is a particularly severe problem in pediatric patients, especially in low-birth-weight infants in whom the frequency of central nervous system infection may be as high as 10 to 20%. In the pediatric population with *Candida* meningoencephalitis, clinically overt loss of developmental milestones and precipitation of seizures are common. Although the data demonstrated activity in the central nervous system, those findings are not definitive for treating *Candida* meningoencephalitis. We therefore recommend that further investigation is necessary to further characterize the pharmacokinetics and pharmacodynamics of icofungipen in the central nervous system.

There was also favorable activity of cispentacin molecules in treatment of the vascular thrombosis that occurred in association with the central venous catheter. Catheter-associated candidemia is increasingly recognized as a challenging infection (28, 36, 37). Antifungal activity at this site may help to

TABLE 1. Pharmacokinetic parameters of icofungipen in rabbits with disseminated candidiasis after multiple daily doses of 2, 5, and 12.5 BID for 5 days^a

Parameter	Result with icofungipen dose (mg/kg) of:			P value ^b
	2	5	12.5	
C_{max} (µg/ml)	23.09 ± 4.86	29.83 ± 3.33	54.58 ± 4.56	<0.001
C_{min} (µg/ml)	0.71 ± 0.17	0.88 ± 0.11	1.62 ± 0.18	0.002
AUC_{0-12} (µg·ml·h)	20.76 ± 2.42	35.96 ± 3.63	67.86 ± 4.71	<0.001
$AUC_{0-\infty}/dose$	5.85 ± 0.52	3.28 ± 0.25	2.45 ± 0.19	<0.001
V (liters/kg)	1.64 ± 0.28	1.80 ± 0.12	2.3 ± 0.15	0.032
CL (liters/h/kg)	0.19 ± 0.03	0.32 ± 0.03	0.42 ± 0.03	0.003

^a All values are expressed as means $\pm$ SEM of results for 6 rabbits per cohort. $AUC_{0-\infty}/dose$, dose-normalized area under the plasma concentration versus time curve from 0 h to infinity; V , apparent volume of distribution at steady state; CL, total plasma clearance.

^b Determined by ANOVA.

TABLE 2. Effects of different doses of icofungipen on various pharmacokinetic parameters in persistently neutropenic rabbits with disseminated candidiasis^a

Treatment group (n)	Serum creatinine (mg/dl)	BUN ^c (mg/dl)	Serum potassium (mmol/liter)	Serum ALT ^d (U/liter)	Serum AST ^e (U/liter)
Control (10)	0.79 ± 0.14	18.80 ± 1.27	4.33 ± 0.18	36.50 ± 9.59	25.40 ± 5.02
ICO-4 (5)	1.12 ± 0.07	16.80 ± 0.86	4.50 ± 0.19	16.60 ± 2.14	11.60 ± 1.96
ICO-10 (6)	1.23 ± 0.05	18.66 ± 2.84	4.68 ± 0.20	11.17 ± 1.05	9.66 ± 1.70
ICO-25 (6)	1.07 ± 0.04	19.33 ± 1.23	4.66 ± 0.11	17.83 ± 4.37	19.00 ± 9.45
FLC ^f (4)	1.15 ± 0.05	16.00 ± 2.12	4.80 ± 0.16	11.00 ± 0.91	8.50 ± 0.65
DAMB ^g (6)	4.62 ± 0.89 ^b	39.75 ± 3.24 ^b	4.35 ± 0.21	16.28 ± 3.35	12.42 ± 0.92

^a All values are given as means ± SEM.^b $P < 0.001$, determined by ANOVA.^c BUN, blood urea nitrogen.^d ALT, alanine aminotransferase.^e AST, aspartate aminotransferase.^f FLC, fluconazole.^g DAMB, amphotericin B.

eradicate foci of catheter-associated candidemia involving the great veins.

The structural changes evidenced histologically demonstrated a dose-dependent effect on *C. albicans*. As seen in Fig. 4A, the hyphae and pseudohyphae are long, slender, and characterized by numerous slender budding yeasts. By comparison, the few lesions that remained after 2 mg/kg BID of icofungipen demonstrated a marked disruption of the uniformity of hyphal structures, with disruption and truncation of pseudohyphae and hyphae. At the higher dosage, the paucity of remaining lesions and tissue demonstrated further disruption of fungal elements, resulting in distended pseudohyphae or enlarged yeast-like structures. These distorted structures are reminiscent of the effects of cell wall active agents such as echinocandins (2, 24). It is possible that the disruption of protein synthesis may result in alterations in cell wall structure as the result of loss of key transmembrane proteins, cell wall glycoproteins, or biosynthetic enzymes, such as chitin synthase and 1,3- β -glucan synthase. However, in vitro studies did not demonstrate the dose-dependent distortion of hyphal structures, suggesting that the in vivo histological findings may be due to combined contributions of cispentacin and innate host defense molecules. Further electron microscopic analyses of these effects are warranted.

The plasma pharmacokinetics of icofungipen demonstrated a dose-dependent but not a dose-proportional increase in the AUC and $C_{\max}$. With increased dosages, AUC, and $C_{\max}$, there was improved eradication of *Candida* from all investigated tissues. Moreover, as the dosage increased, clearance also significantly increased from 0.19 liter/h/kg to 0.42 liter/h/kg. This property is compatible with that of a small organic acid such as that of cispentacin. The kinetic profile appears to suggest a receptor-mediated clearance such that with increased concentration there is increased clearance at the receptor. Such receptors are likely at the level of the renal tubule where organic acids may be actively secreted. Further evaluation of these kinetic properties will allow a better understanding of renal clearance in single and multiple doses.

Following multiple dosing at the BID schedule, icofungipen exhibited no significant accumulation in plasma and linear disposition, as evidenced by a lack of significant differences between dose-normalized AUC and total clearance. The pharmacokinetics parameters of $C_{\max}$, $C_{\min}$, AUC_{0-12} , and $AUC_{0-\infty}$

are not clearly dose proportional. $AUC_{0-\infty}$ approaches dose proportionality with an almost twofold difference. However, dose proportionality seems to diminish for AUC_{0-12} and certainly diminishes for $C_{\max}$ and $C_{\min}$ between the 2- and 5-mg/kg dosage groups. This compares, however, with that of normal rabbits in which icofungipen was studied and found to be dose proportional across 2 mg/kg and 5 mg/kg as well as 12.5 and 25 mg/kg (A. H. Groll, D. Mickiene, V. Petraitis, R. Petraitiene, A. Sarafandi, A. Kelaheer, T. J. Walsh, Abstr. 44th Intersci. Conf. Antimicrob. Agents and Chemother., abstr. 237, 2004).

The data from normal and infected rabbits receiving icofungipen suggested that animals might clear higher concentrations of the compound more rapidly, hence resulting in a lower AUC. The data for the 5-mg/kg and 12-mg/kg dosage regimens in the infected animals are similar to those of healthy rabbits. However, $C_{\max}$, AUC_{0-12} , and $AUC_{0-\infty}$ of icofungipen at 2 mg/kg are somewhat higher in infected rabbits than in those of normal rabbits, suggesting perhaps some impaired clearance in infected animals. These higher levels may therefore account for the relevant similarities and lack of dose proportionalities between the 2-mg/kg and 5-mg/kg dosage levels in infected animals.

In summary, these findings established proof of principle that the cispentacin molecule icofungipen is active in the treatment of disseminated candidiasis in multiple tissue sites including those of the liver, spleen, kidney, central nervous system, and blood vessels. This antifungal activity, as well as the safety and kinetic profile, provides a foundation for investigation of this compound in immunocompromised patients with disseminated candidiasis.

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