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In Vitro Antifungal Activities and In Vivo Efficacies of 1,3-β-D-Glucan Synthesis Inhibitors L-671,329, L-646,991, Tetrahydroechinocandin B, and L-687,781, a Papulacandin

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The in vivo anti-*Candida* activities of 1,3-β-D-glucan synthesis inhibitors L-671,329, L-646,991 (cilofungin), L-687,901 (tetrahydroechinocandin B), and L-687,781 (a papulacandin analog) were evaluated by utilizing a murine model of disseminated candidiasis that has enhanced susceptibility to *Candida albicans* but increased sensitivity for discriminating antifungal efficacy. DBA/2 mice were challenged intravenously with 1×10^4 to 5×10^4 CFU of *C. albicans* MY1055 per mouse. Compounds were administered intraperitoneally at concentrations ranging from 1.25 to 10 mg/kg of body weight twice daily for 4 days. At 6 h and 1, 2, 3, 4, 7, and 9 days after challenge, five mice per group were sacrificed and their kidneys were homogenized and plated for enumeration of *Candida* organisms (CFU per gram). Progressiveness of response trends and no-statistical-significance-of-trend doses were derived to rank compound efficacy. 1,3-β-D-Glucan synthesis 50% inhibitory concentrations were determined by using a *C. albicans* (MY1208) membrane glucan assay. *Candida* and *Cryptococcus neoformans* MICs and minimal fungicidal concentrations were determined by broth microdilution. L-671,329, L-646,991, L-687,901, and L-687,781 showed similar 1,3-β-D-glucan activities, with 50% inhibitory concentrations of 0.64, 1.30, 0.85, and 0.16 μg/ml, respectively. Data from in vitro antifungal susceptibility studies showed that L-671,329, L-646,991, and L-687,901 had similar MICs ranging from 0.5 to 1.0 μg/ml, while L-687,781 showed slightly higher MICs of 1.0 to 2.0 μg/ml for *C. albicans* MY1055. Lipopeptide compounds were ineffective against *C. neoformans* strains. Results from in vivo experiments comparing significant trend and progressiveness in response analyses indicated that L-671,329 and L-646,991 were equipotent but slightly less active than L-687,901, while L-687,781 was ineffective at 10 mg/kg. Fungicidal activities of L-671,329, L-646,991, and L-687,901 were observed in vivo, with significant reduction in *Candida* CFU per gram of kidneys compared with those in sham-treated mice at doses of ≥2.5 mg/kg evident as early as 1 day after challenge.

In the last decade, the incidence of serious fungal infections has reached record levels. This increase is due primarily to the AIDS epidemic, an expanding number of immune deficiency syndromes, and modern medical techniques that predispose a much larger population to opportunistic fungal infections. The number of current therapies available to counter the mycoses has been developed at approximately the same rate since the inception of antifungal drug therapy. Amphotericin B (AMB), developed in the 1950s, still remains the drug of choice for most fungal diseases mainly because it is broad spectrum and fungicidal, but it is considered to be relatively toxic (36, 37). The broad-spectrum azole antifungal agents developed more recently, although considered to be safer and less toxic than AMB, are fungistatic, which has limited their utility in many clinical settings (28, 36). Many of these new antifungal agents are ineffective against deep-seated, life-threatening mycoses. Therefore, the critical need for new fungicidal agents which are safe and effective is evident.

Our efforts toward identification of a promising antifungal agent have focused on 1,3-β-D-glucan synthesis inhibitors. 1,3-β-D-Glucan has been identified as a crucial structural target present in the cell walls of many fungi such as *Candida albicans* (5, 6, 15, 25, 28, 32, 33, 34) and presumably in the cyst form of *Pneumocystis carinii*, since the cysts are sensitive to zymolyse in a lytic enzyme system containing 1,3-β-glucanohydrolyase (22, 30). Compounds from two chemically distinct structural classes, the lipopeptide echinocandinlike antifungal agents and papulacandins, have previously been shown to inhibit 1,3-β-D-glucan synthesis in vitro (1, 3, 7, 10, 24, 26, 29, 35, 42). The lipopeptides are cyclic hexapeptides containing fatty acyl side chains, and the papulacandins are lipid-linked saccharides. Both compound classes are fungicidal but exhibit a narrow antifungal spectrum in vitro, and some have demonstrated efficacy in vivo in conventional animal models of disseminated candidiasis and *Pneumocystis* (*carinii*) pneumonia (8, 11, 16–18, 30, 37, 39).

As difficult as it is to treat serious fungal diseases in humans, so too is it to discover, develop, and evaluate useful antifungal compounds by utilizing animal models that can predict antifungal efficacy in the clinic. Since in vitro susceptibility results (especially with azole antifungal agents) are known to have variable correlation with clinical efficacy (20), it becomes imperative to develop clinically relevant animal models that simulate the immunocompromised patient populations afflicted. Therefore, a mouse model utilizing congenitally immunodeficient DBA/2 mice, which is similar to one previously described by Hector and Yee (19), was developed. DBA/2 mice, deficient in complement component 5 (C5), show enhanced susceptibility to disseminated candidiasis yet are very responsive to therapy. This model, referred to as the target organ kidney assay (TOKA), can

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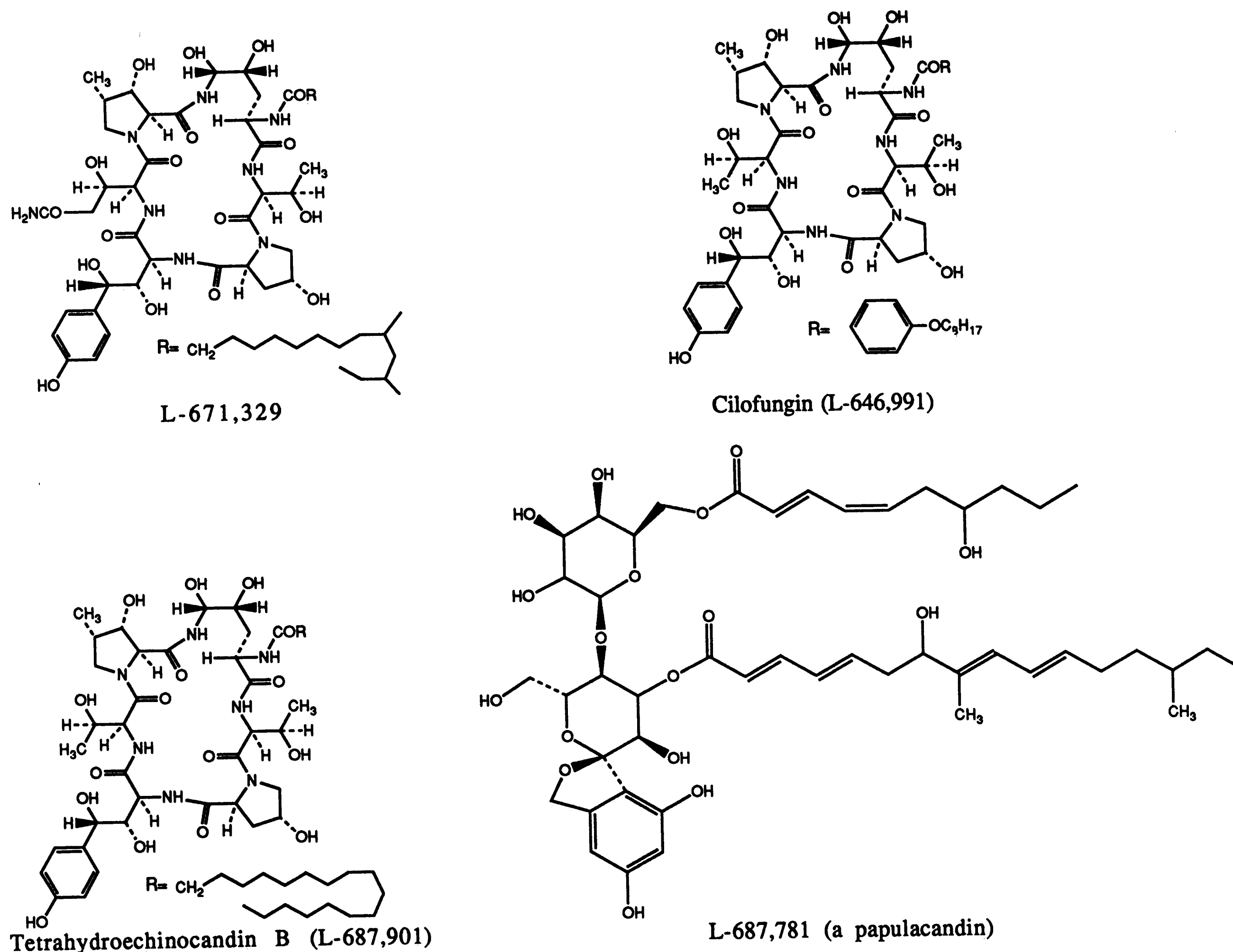

FIG. 1. Chemical structures of 1,3-β-D-glucan inhibitors.

discriminate fungistatic from fungicidal agents in vivo and was used to examine 1,3-β-D-glucan synthesis inhibitors, including lipopeptides L-671,329, L-646,991 (cilofungin), and L-687,901 (tetrahydroechinocandin B) and a novel papulacandin, L-687,781, to determine their in vivo efficacies. 1,3-β-D-Glucan synthesis 50% inhibitory concentrations (IC_{50}) were established by using a *C. albicans* (MY1208) protoplast membrane glucan assay. Compounds were also evaluated against a panel of clinically relevant and animal-virulent fungi with established antifungal susceptibility patterns to determine MICs and minimum fungicidal concentrations (MFCs).

MATERIALS AND METHODS

Antifungal compounds. Figure 1 shows the chemical structures of the three lipopeptide compounds, L-671,329, L-646,991 (cilofungin), and L-687,901 (tetrahydroechinocandin B), and of a novel papulacandin, L-687,781, used in these studies. These compounds were produced by scientists at Merck, Sharp and Dohme Research Laboratories, Rahway, N.J., and were shown by high-performance liquid chromatography to be >95% pure (31, 39, 41). AMB (Fungizone), E. R. Squibb & Sons, Inc., Princeton, N.J., was purchased from Bell Medical Supply, Edison, N.J. Fluconazole (FCZ) was obtained from Pfizer Central Research, Groton, Conn. Ketoconazole (KTZ) and itraconazole (ITZ) were obtained from Janssen Biochimica, Beerse, Belgium.

1,3-β-D-Glucan synthesis assay. A glucan synthase assay was established by using *C. albicans* protoplast membranes to evaluate compounds for in vitro 1,3-β-D-glucan synthesis inhibition as described previously (30). The 1,3-β-D-glucan synthesis assay utilized was a modification of a procedure previously described by Cabib and Kang for *Saccharomyces cerevisiae* (4). Briefly, protoplasts of *C. albicans* MY1208, isolated in mid-log phase, were prepared as described by Taft et al. (35), except that 1.2 M glycerol was substituted for 1.2 M sorbitol to facilitate preparation of protoplast membrane fractions, since glycerol has viscosity properties considerably lower than those of sorbitol. Protoplasts were washed three additional times after Novozyme treatment, and aliquots of washed protoplasts were stored in 1.2 M glycerol-phosphate-buffered saline at −80°C. The glucan synthase system was prepared prior to use by sonication, centrifugation, and resuspension in storage buffer. Determinations for all test compounds were performed in triplicate. The 1,3-β-D-glucan synthesis IC_{50} was defined as the concentration at which the compound inhibits formation of 50% of trichloroacetic acid-precipitable polysaccharides gener-

TABLE 1. MICs and MFCs of L-671,329, cilofungin, tetrahydroechinocandin B, a papulacandin, and AMB for yeast isolates

Fungus	MIC/MFC (μg/ml) of:				
	L-671,329	L-646,991 (cilofungin)	L-687,901 (tetrahydroechinocandin B)	L-687,781 (papulacandin)	L-588,872 (AMB)
C. albicans					
MY1055	1/0.12	1/0.5	0.25/0.5	1/1	1/1
MY1585	1/0.12	1/1	1/1	2/1	4/2
MY1208	2/2	1/0.5	1/0.5	2/1	2/2
MY1028	1/0.5	0.5/0.5	1/0.25	2/2	1/1
MY1750	1/0.25	1/1	1/0.5	1/1	2/2
MY1783	0.5/0.25	1/0.5	2/1	2/2	2/2
C. tropicalis MY1012	0.25/0.12	1/0.5	1/0.5	2/2	4/4
C. parapsilosis					
MY1009	8/8	32/32	1/4	2/2	4/4
MY1010	4/4	16/16	4/4	1/1	2/2
C. guilliermondii MY1019	128/32	128/>128	>128/32	>128/>128	2/2
C. neoformans					
MY1051	>128/>128	>128/>128	>128/>128	>128/>128	1/1
MY1146	>128/>128	>128/>128	>128/>128	>128/>128	1/1

ated by a membrane preparation from *C. albicans* protoplasts.

Broth microdilution assay for MIC and MFC determinations. Media. Yeast nitrogen base (Difco) with 1% dextrose and Sabouraud dextrose agar (SDA) were used for MIC and MFC determinations.

Compounds. Test compounds were solubilized in 10% dimethyl sulfoxide at 2,560 μg/ml. AMB was prepared according to manufacturer's instructions and diluted to 2,560 μg/ml in distilled water. The compounds were then diluted to 256 μg/ml in yeast nitrogen base with 1% dextrose and were serially diluted twofold, yielding final drug concentrations ranging from 128 to 0.06 μg/ml. All tests were performed in duplicate.

Organisms. Yeasts included in the panel (Table 1) were selected because of their resistance or susceptibility to known antifungal agents, virulence, source, and clinical importance. Long-term stock cultures were maintained in water, and working stocks were maintained on SDA and transferred monthly.

Inoculum. Working stocks were used to prepare 4-h log-phase broth cultures that were then diluted to a turbidity equivalent to a 0.5 McFarland standard measured with a spectrophotometer set at 530 nm. This yielded a cell concentration of 1×10^6 to 5×10^6 CFU/ml. Microplates (with 96 wells) containing test compounds were inoculated with an MIC-2000 (Dynatech), which delivers 1.5 μl per well, yielding a final inoculum of 1.5×10^3 to 7.5×10^3 cells per well. Drug-free growth control wells were included. Microplates were incubated at 35°C.

MIC and MFC interpretations. Results were recorded as growth or no growth after 24 h for *Candida* spp. and 48 h for *Cryptococcus* spp. The MIC was defined as the lowest concentration of drug showing no visible growth. After MIC data were recorded, plates were shaken on a Sarstedt TPM2 shaker to resuspend the cells and a MIC-2000 inoculator was used to transfer a 1.5-μl sample from each well in the microplate to a single-well tray containing SDA. Inoculated trays were incubated at 35°C, and results were recorded at 24 or 48 h. The MFC was defined as the lowest concentration of drug showing no growth or fewer than four colonies per spot. The sensitivity for determination of the MFC when using a 1.5-μl sample has been calculated to fall between 83 and 99% killing, which is different from the traditional 99.9% killing.

Selection of a *C. albicans*-susceptible mouse strain. Mice. C5-deficient DBA/2 (Taconic Farms, Germantown, N.Y.), outbred CD-1 (Charles Rivers, Wilmington, Del.), and CD-1-cyclophosphamide (CY) immunosuppressed, congenitally immunodeficient N:NIH(S)III (*nu/nu*, *bg/bg*, XID, triple-immunodeficient; Massachusetts General Hospital, Boston, Mass.), C57BL/6 (+/*bg*) heterozygous, and C57BL/6 (*bg/bg*) beige (University of Wisconsin Gnotobiotic Laboratory, Madison, Wis.), female mice weighing 17 to 20 g were used for 50% lethal dose (LD_{50}) determinations. CY treatment consisted of injection of mice with a single 300-mg/kg of body weight intraperitoneal dose administered 5 days prior to initiation of infection.

Infection. *C. albicans* MY1055, a human clinical pathogenic isolate originally obtained from Williamsburg Community Hospital, Williamsburg, Va. (MCV 7.270), was used. Growth from an overnight SDA culture was suspended in sterile saline, and cell concentrations were determined by counting in a hemacytometer. Viable count was confirmed by serial 10-fold dilution and plating the inoculum on SDA. Five mice per group were injected intravenously in their lateral tail veins with 0.2 ml of serial 10-fold-diluted cell suspensions. Final inocula ranged from 10^1 to 10^7 cells per mouse, depending on the particular mouse strain that was challenged.

TOKA evaluation of in vivo efficacy. Mice. DBA/2 female mice weighing 17 to 20 g were used. This inbred strain of mouse is congenitally immunodeficient in C5.

Infection. A cell suspension of *C. albicans* MY1055 was adjusted to 3.75×10^5 cells per ml as described above. When 0.2 ml of this cell suspension was administered intravenously in the tail veins of mice, the final inoculum was 7.5×10^4 cells per mouse (approximately one 14-day LD_{50}).

Therapy. Compounds were tested at titrated concentrations (serial twofold dilutions) in this model. AMB was tested at concentrations ranging from 0.78 to 12.5 mg/kg administered orally once daily for 4 consecutive days. KTZ, FCZ, and ITZ were tested at 12.5 to 100 mg/kg administered orally once daily q.d. for 4 consecutive days. L-671,329, cilofungin, tetrahydroechinocandin B, and L-687,781 were administered intraperitoneally at concentrations ranging from 1.25 to 10 mg/kg twice daily for 4 consecutive days. Infected sham-treated mice were administered the appropriate solvent vehicles (desoxycholate for AMB; 0.2 N HCl for

KTZ; 10% dimethyl sulfoxide for ITZ, lipopeptides, L-687,781; and distilled water for FCZ) by following the same treatment schedules described for test compounds.

Quantitation and recovery of yeast cells. Five mice per treatment group and time point group were sacrificed by using carbon dioxide gas at 6 h and 1, 2, 3, 4, 7, and 9 days (considered full TOKA) or after 7 days (considered abbreviated TOKA) after initiation of intravenous infection. Paired kidneys were removed aseptically and weighed, and kidneys were placed in sterile Whirl-Pak bags (Nasco) containing 5 ml of sterile saline. The organs were homogenized in the bags (40) and serially diluted in saline, and aliquots were spread onto the surface of SDA plates. Plates were incubated at 35°C for 48 h, and yeast colonies were enumerated for determination of CFU per gram of kidneys. The limit of detection for this procedure is 50 CFU per pair of kidneys.

Statistical analysis used to rank compound efficacy. For each assay, a one-way analysis of variance was performed with $\log_{10}$ CFU per gram of kidneys to obtain a pooled measure of experimental variability. A statistical procedure developed by Tukey, Ciminera, and Heyse (38) that assesses the evidence of a trend in a response variable was used to analyze these data, since each compound was tested at a series of progressively higher dose concentrations. In this setting, "trend" was defined as a progressiveness of response, either increasing or decreasing, with increasing concentrations of the test compound with the sham treatment group included as a zero dose. Trend was assessed by using the usual regression slope of three candidate dose scalings (arithmetic, arithmetic-logarithmic, and ordinal) chosen because they span a wide range of expected biological response patterns. A slope along with the corresponding probability value that did not differ from zero was calculated for each dose scaling. If the minimum observed probability value was ≤0.05, the conclusion was that there was a progressiveness of response with increasing doses of the compound and also that the mean response to the highest dose was significantly different than the mean response to the sham treatment. Testing for trend proceeded sequentially by removing the highest dose each time a statistically significant trend (i.e., minimum $P \leq 0.05$) was observed. Testing was ended when the minimum probability value was higher than 0.05, and the associated high dose was referred to as the no-statistical-significance-of-trend (NOSTASOT) dose.

RESULTS

Inhibition of 1,3-β-D-glucan synthesis. Lipopeptide and papulacandin antibiotics are known to inhibit fungal cell walls (1, 3, 7, 10, 24, 26, 29, 35, 42), and our mode of action studies focused on 1,3-β-D-glucan synthesis inhibitors. When L-671,329, cilofungin, tetrahydroechinocandin B, and L-687,781 were added to membrane preparations from *C. albicans* incorporation of UDP-[^{14}C]glucose into trichloroacetic acid-precipitable polymer (identified by a Smith degradation for periodate-refactory, β-1,3-linked glucose units, which was at 75%) was significantly inhibited compared with that by a noninhibitor control. The level of inhibition with L-687,781 appeared slightly higher (IC_{50} = 0.16 ± 0.04 μM) than levels measured with L-671,329 (IC_{50} = 0.64 ± 0.05 μM), cilofungin (IC_{50} = 1.3 ± 0.10 μM), and tetrahydroechinocandin B (IC_{50} = 0.85 ± 0.25 μM).

In vitro antifungal activity. Table 1 shows the in vitro antifungal activities (MICs and MFCs) of lipopeptides L-671,329, cilofungin, and tetrahydroechinocandin B and

TABLE 2. LD_{50}s for *C. albicans* MY1055 in strains of inbred and outbred immunodeficient and immunocompromised mice

Mouse strain	Mean $\log_{10}$ CFU/mouse	Range (no. of tests)	CFU/mouse
N:NIH(S)III (triple deficient)	4.70	4.50–4.89 (2)	5.0×10^4
DBA/2 (C5 deficient)	4.68	4.51–4.86 (5)	7.3×10^4
CD-1 (CY treated)	4.91	4.86–5.04 (4)	8.1×10^4
C57BL/6 *bg/bg*	5.31	4.86–5.75 (2)	2.0×10^5
C57BL/6 +/*bg* (heterozygous)	5.50	5.50 (1)	3.2×10^5
CD-1 (outbred)	5.68	5.13–6.13 (4)	4.8×10^5

L-687,781 in the broth microdilution assay. The MICs and MFCs for lipopeptides and the papulacandin L-687,781 were comparable for the *Candida* species surveyed. Lipopeptide compounds exhibited fungicidal activities equal to or less than their MICs (≤0.06 to 2.0 μg/ml) for most *Candida* species but were ineffective against *Cryptococcus neoformans* strains. The MICs of test compounds for *Candida* species (except *Candida guilliermondii*) were within 1 to 2 (twofold) dilutions of each other and that of AMB. However, the MFCs of the papulacandin L-687,781 and AMB were higher (10-fold) for the *Candida* species than those of the lipopeptides, except in the case of *Candida parapsilosis*, against which L-687,781, L-671,329, and tetrahydroechinocandin B appeared to be equal to or slightly more active than cilofungin. It should be pointed out that the MICs in relation to MFCs may be misleading. Yeast cells from microtiter wells in which MIC determinations were conducted were examined microscopically. Those cells incubated with concentrations of compound above the MFC and in which turbidity was evident appeared to be lysed (9). Thus, the MIC may actually be lower than recorded, since the presence of nonviable cells may artifactually skew the MICs so that they appear to be higher. Only AMB appeared to be active against *C. guilliermondii* and *C. neoformans* strains, with MICs and MFCs of 1 to 2 μg/ml. The lipopeptides and the papulacandin L-687,781 were inactive in vitro against the *C. neoformans* strains tested.

A positive correlation of IC_{50}s to MFC in vitro antifungal activity for *Candida* species was not apparent when echinocandins were compared with the papulacandin. The papulacandin showed the lowest IC_{50}, 0.16 μM, but displayed higher MFCs for the *Candida* species than did echinocandins. L-671,329, cilofungin, and tetrahydroechinocandin B, with 1,3-β-D-glucan IC_{50}s 4- to almost 10-fold that of the papulacandin (0.64 to 1.3 μM), exhibited nearly equal MICs for *Candida* species but substantially lower MFCs than the papulacandin. On the other hand, L-671,329, cilofungin, and tetrahydroechinocandin B, all having 1,3-β-D-glucan IC_{50}s similar to each other, also showed comparable in vitro antifungal activities against *Candida* species.

In vivo *Candida* susceptibility studies. In an effort to identify a mouse strain that was susceptible to a virulent *C. albicans* strain but also sensitive enough to respond to antifungal therapy, immunocompetent, immunosuppressed, and congenitally immunodeficient mice were surveyed. Data in Table 2 show LD_{50}s for immunocompetent and immunodeficient mice challenged intravenously with *C. albicans* MY1055. *C. albicans* MY1055 was found to be suitably virulent in outbred mice and in all other mouse strains surveyed in this study. DBA/2 mice were 1 $\log_{10}$ unit more susceptible to renal *C. albicans* infections than CD-1 mice

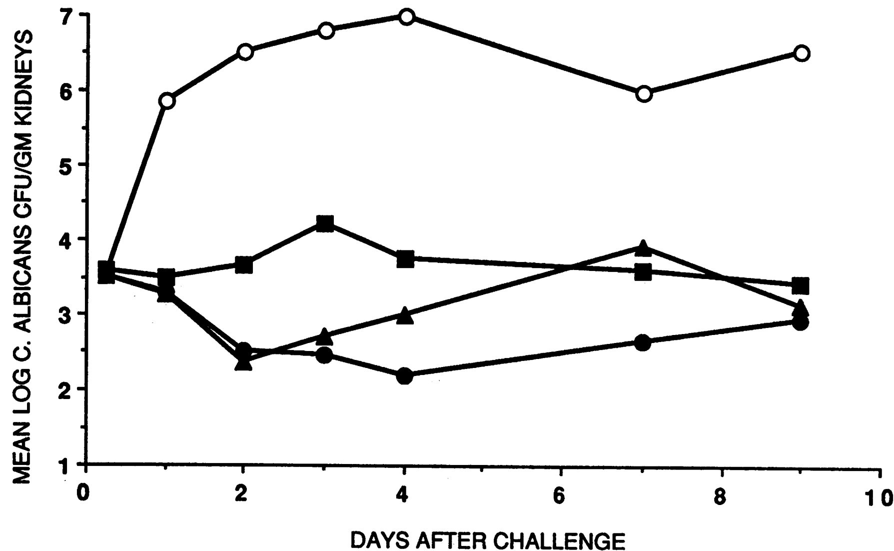

FIG. 2. Comparison of AMB (12.5 mg/kg) versus FCZ and KTZ (25 mg/kg) activities in the TOKA from 6 h to 9 days after challenge. Mice were treated twice daily for 4 days. Mean $\log_{10}$ *C. albicans* CFU per gram of kidneys from five mice at each time point were determined. Symbols: ○, sham treatment; ▲, FCZ (25 mg/kg); ●, AMB (12.5 mg/kg); ■, KTZ (25 mg/kg).

and were as susceptible to *C. albicans* MY1055 as CD-1 CY-treated and N:NIH(S)III mice, as indicated by lower LD_{50}s compared with those for C57BL/6 beige and +/*bg* heterozygous mice. Because of their heightened *C. albicans* susceptibility, well-defined genotype and phenotype, lack of artificially drug-induced immune suppression, and availability, DBA/2 inbred mice were employed to determine antifungal efficacy.

Evaluation of in vivo efficacies of AMB and azoles in the TOKA. Although significant ($P < 0.05$) reductions in *C. albicans* CFU per gram of kidneys compared with those of infected sham-treated mice were observed for the azoles and AMB at all levels tested, AMB reduced the mean *C. albicans* CFU per gram of kidneys about 5 log units during the first 4 days of treatment compared with those in sham-treated controls (Fig. 2). The azole antifungal agents KTZ and FCZ (at concentrations of 25 mg/kg) produced plateaued kinetics in which numbers of viable yeast cells recovered from kidneys were reduced but not eliminated, indicative of a fungistatic response in vivo (Fig. 2). During the first 3 days of treatment, FCZ exhibited activity comparable to that of AMB, but once treatment was terminated, the numbers of viable yeast cells recovered from kidneys were higher than in AMB-treated mice. Figure 3 shows results of studies in which AMB and azole antifungal agents FCZ, ITZ, and KTZ were evaluated at day 7 in the abbreviated TOKA. These azole antifungal agents were not evaluated at concentrations below 12.5 mg/kg in this study.

Evaluation of in vivo efficacies of L-671,329, cilofungin, tetrahydroechinocandin B, and a papulacandin in the TOKA. Figures 4 to 6 graphically compare the activities of L-671,329, cilofungin, tetrahydroechinocandin B, and the papulacandin L-687,781 when administered at concentrations ranging from 2.5 to 10 mg/kg in the full (9-day) TOKA. The echinocandin compounds L-671,329, cilofungin, and tetrahydroechinocandin B, administered at 5 mg/kg (Fig. 5) and 10 mg/kg (Fig. 6), display a fungicidal response similar to that of AMB (as in Fig. 2). Fungicidal activities of L-671,329, L-646,991, and L-687,901 were observed at doses of ≥2.50 mg/kg, with significant reduction in *Candida* CFU compared with those in sham-treated mice, evident as early as 1 day after challenge. Only tetrahydroechinocandin B was efficacious at 2.5 mg/kg (Fig. 4). None of the test compounds were effective at 1.25 mg/kg (data not shown). The papulacandin L-687,781 was ineffective at all concentrations tested in this model.

Data in Table 3 represent the mean $\log_{10}$ *C. albicans* CFU per gram of kidneys, significance of trend, and NOSTASOT doses at various times after challenge for infected drug-, and sham-treated controls. The echinocandin compounds were effective in reducing the numbers of *Candida* organisms in the kidneys of challenged mice, evidenced by significantly

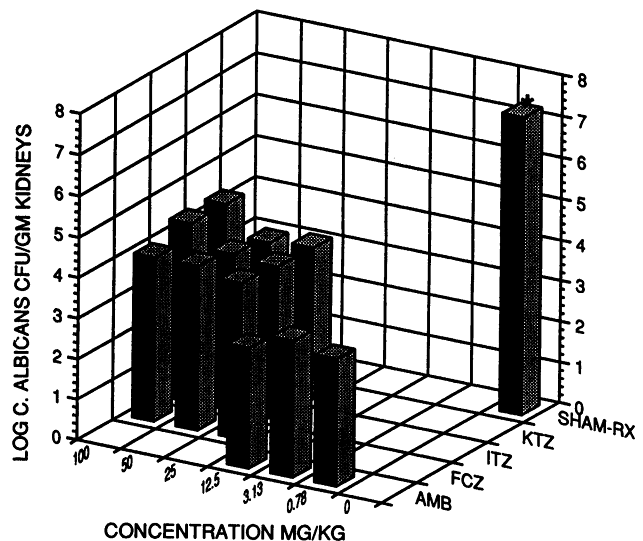

FIG. 3. AMB and azoles in the 7-day TOKA. *, significantly different from drug-treated groups ($P < 0.05$).

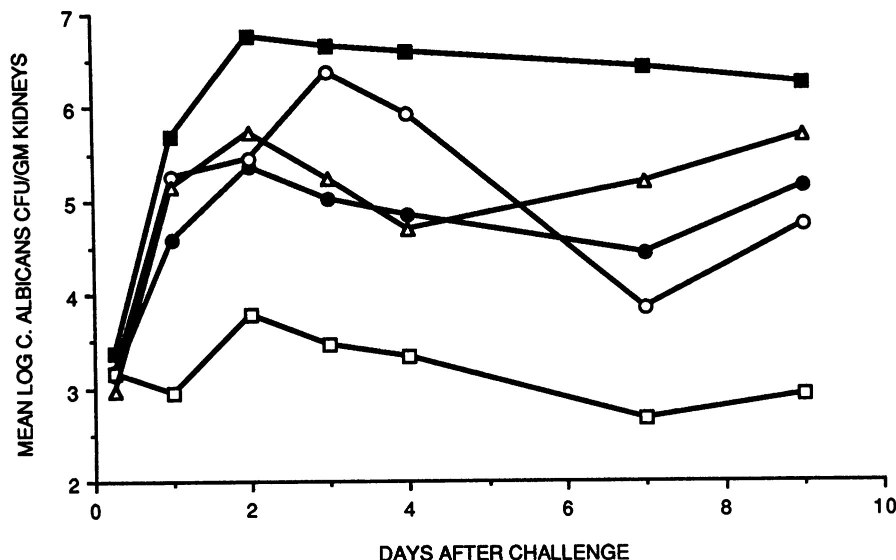

FIG. 4. Comparison of L-671,329, cilofungin, tetrahydroechinocandin B (L-687,901), and a papulacandin (L-687,781) administered at 2.5 mg/kg in the TOKA. Symbols: ■, sham treatment; ●, L-671,329; ○, cilofungin; □, L-687,901; △, L-687,781.

reduced mean $\log_{10}$ *C. albicans* CFU counts, while the papulacandin compound was ineffective in mouse kidney colony count reduction. NOSTASOT results indicated that the echinocandin compounds had comparable values (1.25 to 2.5 mg/kg), while the NOSTASOT values for the papulacandin compound were considerably higher (>10 mg/kg). By NOSTASOT analysis, tetrahydroechinocandin B was the most active compound in the TOKA, followed closely by L-671,329 and cilofungin.

Table 4 shows a comparison of percent reduction of *C. albicans* from infected sham-treated controls and *Candida* renal clearance (sterilization of kidneys of individual mice within groups) at times after challenge. These results parallel the trend analysis findings above. Tetrahydroechinocandin B was the most effective compound as indicated by the greatest percent reduction and percent renal clearance of *C. albicans*, followed closely by L-671,329 and cilofungin. Interestingly, tetrahydroechinocandin B was able to sterilize kidneys of mice (between one and four of five mice surveyed) within 1 to 2 days following challenge and treatment with concentrations as low as 2.5 mg/kg. Furthermore, tetrahydroechinocandin B treatment at 5 mg/kg (9 days after challenge) and 10 mg/kg (3 days after challenge) yielded sterile kidneys in five of five mice assayed by days 9 and 3 after challenge, respectively. On the other hand, L-671,329 and cilofungin required higher concentrations for longer

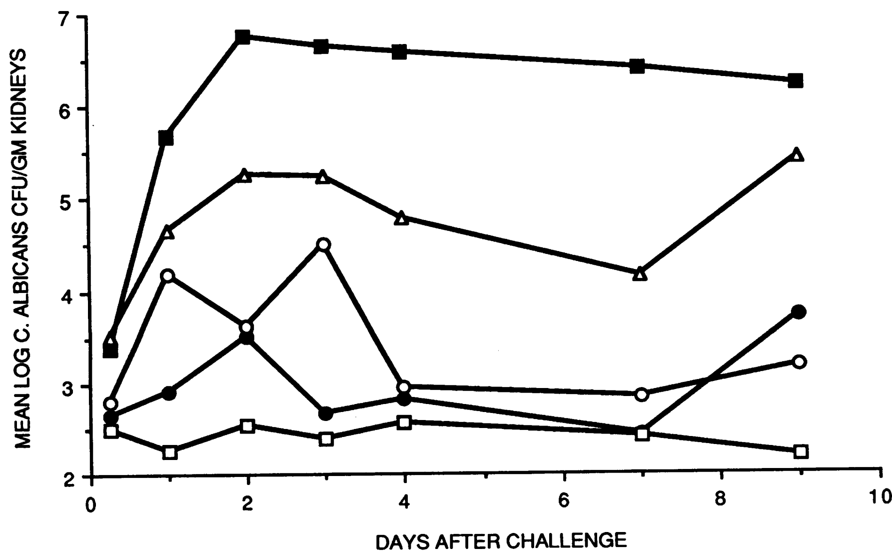

FIG. 5. Comparison of L-671,329, cilofungin, tetrahydroechinocandin B (L-687,901), and a papulacandin (L-687,781) administered at 5.0 mg/kg in the TOKA. Symbols are same as for Fig. 4.

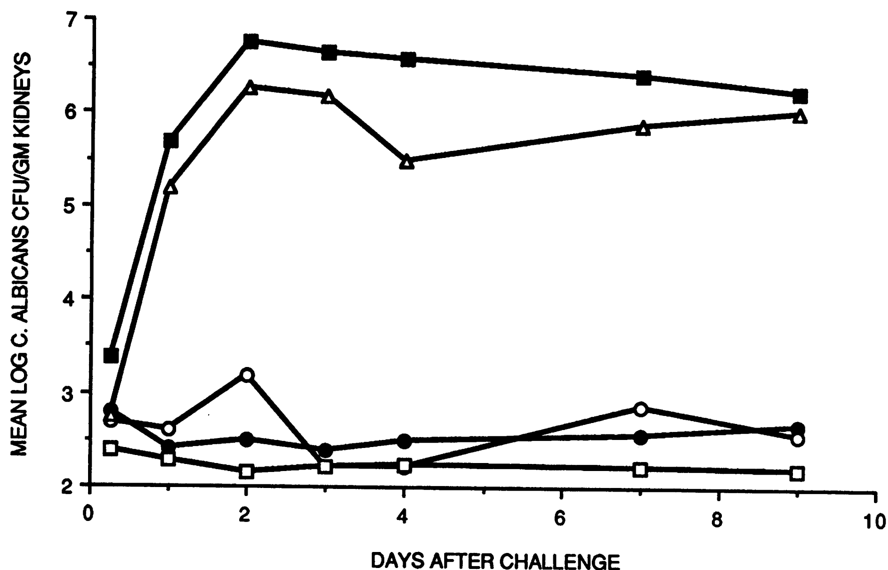

FIG. 6. Comparison of L-671,329, cilofungin, tetrahydroechinocandin B (L-687,901), and a papulacandin (L-687,781) administered at 10 mg/kg in the TOKA. Symbols are same as for Fig. 4.

periods of time to achieve comparable sterilization within kidneys of mice analyzed, while no sterilization was detected with the papulacandin compound.

DISCUSSION

C. albicans has been found to contain approximately 39% β-glucan in its cell wall (29). Actively metabolizing fungi sensitive to 1,3-β-D-glucan synthesis inhibition exhibit severe modifications of the cell wall and cytoplasmic membrane when exposed to glucan inhibitors (5). The lipopeptide compounds L-671,329, cilofungin, and tetrahydroechinocandin B and the papulacandin L-687,781 inhibit 1,3-β-D-glucan synthesis in an in vitro glucan assay, supporting the findings of others who have proposed inhibition of fungal cell wall synthesis as a mechanism of action for these compounds (23, 42). As described by others (17, 30), inhibition of fungal glucan is noncompetitive, and in our studies this was supported (data not shown), since IC_{50}s were found to be independent of substrate concentration (unpublished data). The 1,3-β-D-glucan synthesis levels of inhibition of the compounds examined in this study were not useful in predicting in vitro antifungal susceptibility or in vivo efficacy.

While the 1,3-β-D-glucan synthesis inhibitory activities (IC_{50}s) and in vitro antifungal activities (MICs and MFCs) of lipopeptide compounds were not useful in estimating efficacy in the TOKA, the results of these assays did predict the fungicidal responses observed. Although the spectrum of activity of 1,3-β-D-glucan inhibitors against fungi is considered narrow, the potencies (MICs and MFCs) of the lipopeptides against clinically relevant *C. albicans* and *Candida tropicalis* were significantly greater than that of AMB. The papulacandin L-687,781 demonstrated potency against *C. albicans* and *C. tropicalis* yeasts comparable to that of AMB. The lipopeptides and the papulacandin compound L-687,781 were relatively inactive against *C. guilliermondii* and *C. neoformans*, while L-671,329 appeared to be significantly more potent than the papulacandin L-687,781 against *C. tropicalis*. The reason for variable in vitro susceptibilities among the fungi to the 1,3-β-D-glucan synthesis inhibitors has not been definitively established. Explanations for variable responsiveness have been postulated to involve penetration of compound to the fungal cell wall glucan target, variable amount and type of glucan (1,3-β-D-glucan, 1,3-α-D-glucan, 1,6-β-D-glucan, etc.) present in yeast and filamentous cells, the metabolic state of the fungus, and perhaps an additional undefined mode of action or resistance mechanism. With regard to relative inactivities of 1,3-β-D-glucan synthesis inhibitors against *C. neoformans*, its cell wall composition has a much higher number of 1,6-β linkages (80%) which are highly branched compared with 1,3-β linkages (unpublished results). In contrast, nonglucan inhibitors AMB and azole antifungal agents are effective against *C. neoformans*, since the mechanism of action of the polyene AMB is disruption of membrane integrity after binding to membrane sterols and for the azoles it is inhibition of ergosterol biosynthesis, both leading to cytosolic leakage (2, 14, 21, 36).

The in vitro conditions in which antifungal agents are tested are known to dramatically influence resultant MICs and have at times been considered physiologically irrelevant (12, 13, 27). The methodology for testing antifungal agents in vitro is not yet standardized, and correlation to clinical outcome in vivo remains unestablished, especially for the azole class of antifungal agents (12, 13, 20). Additionally, standard animal models used to determine efficacies of antimicrobial agents in vivo are known to have limitations regarding their susceptibilities to opportunistic pathogens and sensitivities to antifungal treatment. Conventional animal models used for screening antifungal agents employing outbred mice may inappropriately represent the clinical situation in which patients exhibit immune deficiencies and become naturally predisposed to opportunists. Even with antifungal therapy, immunocompromised patients are unable to mount a normal immune response to counter fungal infections. Therefore, in vivo models utilizing immunodeficient animals susceptible to opportunistic fungal pathogens that respond to antifungal therapy and simulate human clinical responses have become prerequisite for the discovery, development, and evaluation of new antifungal agents.

TABLE 3. L-671,329, cilofungin, tetrahydroechinocandin B, and papulacandin activities in the TOKA

Compound	Dose (mg/kg)	Mean $\log_{10}$ *C. albicans* CFU/g of kidneys on day after challenge						
		0.25[a]	1	2	3	4	7	9
None	0	3.39	5.68	6.77	6.65	6.58	6.42	6.24
L-671,329	1.25	2.90	5.24	6.09	6.16	4.94	4.49[b]	5.76
	2.50	3.14	4.58	5.36[b]	5.01[b]	4.84	4.43[b]	5.14
	5.00	2.65	2.91[b]	3.52[b]	2.67[b]	2.82[b]	2.43[b]	3.74[b]
	10.00	2.79	2.42[b]	2.49[b]	2.38[b]	2.49[b]	2.56[b]	2.67[b]
SEM[c]		0.13	0.18	0.26	0.36	0.53	0.44	0.48
	NOSTASOT[d]	NC[e]	2.5	1.25	1.25	2.5	<1.25	2.5
Cilofungin (L-646,991)	1.25	3.17	5.64	6.57	5.39	5.90	5.20[b]	5.98
	2.50	3.18	5.26	5.45[b]	6.38	5.92	3.85[b]	4.73[b]
	5.00	2.80	4.18[b]	3.63[b]	4.50[b]	2.95[b]	2.85[b]	3.19[b]
	10.00	2.70	2.60[b]	3.19[b]	2.21[b]	2.21[b]	2.86[b]	2.56[b]
SEM		0.22	0.34	0.32	0.28	0.58	0.40	0.46
	NOSTASOT	NC	2.5	1.25	2.5	2.5	<1.25	1.25
Tetrahydroechinocandin B (L-687,901)	1.25	3.27	4.56[b]	5.39	4.46[b]	5.10[b]	3.60[b]	5.47
	2.50	3.16	2.94[b]	3.78[b]	3.46[b]	3.34[b]	2.67[b]	2.92[b]
	5.00	2.50	2.27[b]	2.55[b]	2.39[b]	2.56[b]	2.42[b]	2.20[b]
	10.00	2.39	2.28[b]	2.15[b]	2.21[b]	2.24[b]	2.22[b]	2.19[b]
SEM		0.12	0.17	0.32	0.48	0.29	0.43	0.57
	NOSTASOT	NC	<1.25	1.25	<1.25	<1.25	<1.25	1.25
Papulacandin (L-687,781)	1.25	2.92	5.17	5.70	5.54	5.17	5.20	4.89
	2.50	2.96	5.15	5.72	5.24	4.69	5.19	5.69
	5.00	3.51	4.67	5.26	5.25	4.79	4.16	5.45
	10.00	2.76	5.19	6.27	6.19	5.49	5.88	6.03
SEM		0.14	0.33	0.22	0.27	0.26	0.39	0.38
	NOSTASOT	NC	>10	>10	>10	>10	>10	>10

[a] Significance of trend and progressiveness of response analysis was not determined at 6 h.
[b] A progressiveness of response trend was observed up to and including the indicated dose (38).
[c] SEMs are standard errors of the means for the preceding four pieces of data.
[d] NOSTASOT is the highest dose (milligram per kilogram) at which a statistically significant trend in reduction of mean $\log_{10}$ CFU per gram of kidneys not observed.
[e] NC, not calculated.

Congenitally immunodeficient DBA/2 mice (C5 deficient but without artificially induced suppression, e.g., pretreatments with immune suppressants such as cortisone, CY, or irradiation) were found to be highly susceptible to *C. albicans* systemic infections, as others have shown (19), and yet exhibited exceptional responsiveness to antifungal treatment and demonstrated sensitivity for discriminating fungistatic and fungicidal activities of compounds in vivo. Since *C. albicans* is naturally tropic to renal tissues, the kidneys allowed specific loci of infection (target organ). Infection was monitored to determine organ colonization and for evaluation of responsiveness to therapy throughout the course of infection but prior to mice becoming moribund. When viable *C. albicans* CFU per gram of kidneys were plotted over time, the fungistatic azole compounds KTZ, FCZ, and ITZ showed plateaued kinetics with no apparent sterilization of renal tissues. The fungicidal agents AMB and lipopeptide compounds exhibited both declining numbers of *C. albicans* CFU recovered over time and sterilization of renal tissues (at limits of detection) in groups of mice surveyed after treatment.

Statistical trend analysis, percent reduction of *C. albicans* compared with that in infected sham-treated control mice, and *C. albicans* renal clearance (sterilization) established that tetrahydroechinocandin B was the most efficacious compound in vivo, followed closely by L-671,329 and cilofungin, while the papulacandin compound L-687,781 was ineffective in this target organ assay. The papulacandin L-687,781 did exhibit in vivo activity in a rat model of *P. carinii* pneumonia (30, 39), and the lack of efficacy of L-687,781 against *C. albicans* in the TOKA remains unexplained. Since pharmacokinetics and protein binding studies have not been conducted with papulacandin L-687,781, whether these parameters may have contributed to its apparent inactivity remains to be determined. Interestingly, when tested in vitro against *C. albicans* MY1055, L-671,329 was the most potent compound (MFC, 0.12 μg/ml), while cilofungin, tetrahydroechinocandin B, the papulacandin L-687,781, and AMB were equally active against this strain (MFCs, 0.5 to 1.0 μg/ml). Although the papulacandin L-687,781 was between 4 and almost 10 times more potent than L-671,329, tetrahydroechinocandin B, and cilofungin in the glucan assay, L-687,781 was not active in vivo. However, it should be mentioned that the glucan used in the glucan inhibition assay was derived from a *C. albicans* strain (MY1208) different from the *C. albicans* strain (MY1055) used in the TOKA. Because in vitro susceptibility results sometimes show highly variable correlation with efficacy, antifungal agents should be tested in vivo and the TOKA may be considered a valuable and sensitive tool for evaluation of antifungal agents, especially when compounds cannot be discriminated from each other on the basis of in vitro susceptibility tests or 1,3-β-D-glucan inhibition assays, as our results have indicated. Other characteristics of com-

TABLE 4. Percent reduction and percent renal clearance of *C. albicans* in the TOKA

Compound	Dose (mg/kg)	% Reduction[a]/% clearance[b] on day after challenge						
		0.25[a]	1	2	3	4	7	9
None	0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
L-671,329	1.25	68/0	64/0	79/0	68/0	98/0	99/0	67/0
	2.50	44/0	92/0	96/0	98/0	98/0	99/0	92/0
	5.00	82/0	99/0	99/0	99/0	99/20	99/80	99/0
	10.00	75/0	99/20	99/40	99/40	99/60	99/80	99/100
Cilofungin (L-646,991)	1.25	40/0	9/0	37/0	95/0	79/0	94/0	45/0
	2.50	38/0	62/0	95/0	46/0	78/0	99/0	97/0
	5.00	74/0	97/0	99/0	99/0	99/20	99/40	99/20
	10.00	80/0	99/0	99/20	99/60	99/80	99/20	99/60
Tetrahydroechinocandin B (L-687,901)	1.25	24/0	92/0	96/0	99/0	97/0	99/40	83/20
	2.50	41/0	99/20	99/20	99/0	99/0	99/60	99/60
	5.00	87/80	99/60	99/60	99/60	99/60	99/80	99/100
	10.00	99/0	99/60	99/100	99/100	99/100	99/100	99/100
Papulacandin (L-687,781)	1.25	85/0	7/0	75/0	79/0	89/0	28/0	75/0
	2.50	84/0	11/0	74/0	89/0	96/0	29/0	−58/0
	5.00	−44/0	70/0	91/0	89/0	95/0	93/0	9/0
	10.00	90/0	2/0	9/0	5/0	79/0	−3/0	−3/0

[a] Percent reduction in recoverable yeast (CFU per gram of kidneys) at dose level from respective infected sham-treated control.
[b] Percent clearance represents percent of mice in sample group (five mice in group) with no detectable yeast recovered from kidneys. Limit of detection = 50 CFU/g of kidneys.

pounds such as pharmacokinetic parameters, bioavailability, excretion, metabolism, and protein binding also must be carefully considered when evaluating compound efficacy.

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