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Efficacy of Itraconazole Solution in a Rabbit Model of Invasive Aspergillosis

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The efficacy of an itraconazole-cyclodextrin solution against *Aspergillus fumigatus* was assessed in an immunosuppressed, temporarily leukopenic rabbit model of invasive aspergillosis and compared with that of amphotericin B. Oral itraconazole solution at dosages of 20 and 40 mg/kg/day improved survival as compared with that of controls. Itraconazole (40 mg/kg/day) not only improved survival and reduced antigen levels but also significantly eradicated *A. fumigatus* from tissues and was as effective as amphotericin B in these studies. The higher dose of itraconazole produced higher levels in serum, which correlated with improved efficacy of the drug. This itraconazole-cyclodextrin solution was well absorbed and was effective in the treatment of experimental invasive aspergillosis; it demonstrates the potential of this class of agents in improving therapy for invasive aspergillosis.

Invasive aspergillosis is associated with significant morbidity and mortality despite therapy with amphotericin B (2, 5). Management of invasive aspergillosis is further complicated by toxicity associated with amphotericin B therapy (7). Itraconazole is a triazole antifungal compound with excellent in vivo and in vitro activity against *Aspergillus* spp. (3, 6, 19). Itraconazole has been successfully used to treat patients with invasive aspergillosis, with good outcomes achieved even in immunosuppressed patients (4). However, erratic absorption of itraconazole following oral administration has been reported for some patients and low serum drug levels have been associated with clinical failures of the drug (6). Adequate delivery of drug via oral administration is likely essential for successful therapy, particularly in immunosuppressed patients at highest risk for invasive aspergillosis. Absorption of itraconazole and related compounds like saperconazole have been shown to improve with the use of cyclodextrins, and improved absorption could result in increased therapeutic utility of these compounds (9, 10).

In this immunosuppressed model, rabbits are made leukopenic and are further immunocompromised with steroid therapy (14, 15). Extensive infection develops in liver, kidney, lung, and brain, which is similar to clinical dissemination of invasive aspergillosis (12, 14). Efficacy of therapy is assessed by mortality, semi-quantitative organ cultures, and *A. fumigatus* antigen measurement. In this study, we assessed the activity of a novel itraconazole-cyclodextrin solution as compared with that of amphotericin B in a lethal model of experimental infection.

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MATERIALS AND METHODS

Rabbit model. New Zealand White rabbits were immunosuppressed as previously described (14, 15) with a single dose of cyclophosphamide (200 mg) given intravenously on the first day of the model and triamcinolone acetonide (10 mg) (Westwood Pharmaceuticals, Buffalo, N.Y.), which was given subcutaneously each day. With this immunosuppressive regimen, the rabbits have reduced total leukocyte counts through day 7, as previously reported (15). Twenty-four hours after immunosuppression, groups of five to eight rabbits were challenged intravenously with a lethal inoculum of 10^6 *A. fumigatus* conidia. Antifungal therapy was given as described below. Each group contained at least one untreated control rabbit. Blood was obtained daily for determining total leukocyte counts and serum aspergillus antigen. Ceftazidime (200 mg) (SmithKline Beecham, Philadelphia, Pa.) was administered intramuscularly daily beginning on the day of challenge to prevent intercurrent bacterial infection.

Amphotericin B (Fungizone; Bristol-Myers Squibb, Princeton, N.J.) or itraconazole-cyclodextrin solution (provided by Janssen Research Foundation, Beerse, Belgium) therapy was begun 24 h after challenge (10). The itraconazole-cyclodextrin solution was administered orally via gastric gavage tube (American Pharmaseal Company, Valencia, Calif.) at doses of 20 or 40 mg/kg/day for 5 days. Amphotericin B was diluted with 5% dextrose in sterile water at a ratio of 1 mg of drug to 10 ml of diluent and was given intravenously over 30 min through a lateral ear vein at a dose of 1.5 mg/kg/day for 5 days.

Organ cultures. Cultures and histopathological evaluations were performed at time of autopsy or when the animals were sacrificed (24 to 48 h after completion of therapy in the treated rabbits). Rabbits were sacrificed, following anesthesia with ketamine (35 mg/kg) (Bristol Laboratories, Syracuse, N.Y.) and xylazine (10 mg/kg) (Mobay Corp., Shawnee, Kans.), by lethal exsanguination. Cultures were taken by placing minced organ samples directly on blood agar and on Sabouraud dextrose agar plates. Samples were considered positive when two or more colonies of *A. fumigatus* was present on ≥1 g of minced organ tissues plated directly on Sabouraud dextrose and blood agar plates or when semi-quantitative cultures of tissue homogenates contained 20 or more CFU/g of tissue (13, 15). The tissue burden of *A. fumigatus* was evaluated with a modification (13) of the semi-quantitative culture technique of Graybill and Kaster (8). Samples of liver, kidney, lung, and brain were manually

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TABLE 1. Itraconazole levels in serum as measured by bioassay in immunosuppressed rabbits

Dose (mg/kg/day)	n	No. of doses	Time (h)	Mean ± SE level (μg/ml) in serum (range)
40	3	1	1	4.8 ± 0.3 (3.8–4.8)
	5	1	3	7.4 ± 0.7 (4.9–8.8)
	5	1	22	2.0 ± 0.7 (<0.5–4.3)
	7	3	4	9.1 ± 1.2 (5.5–13.8)
	7	5	22	2.6 ± 0.9 (0.7–7.5)
20	5	1	4	2.7 ± 0.2 (5.5–13.8)
	5	1	22	<0.5 (<0.5–1.5)
	5	3	5	3.6 ± 0.5 (2.5–4.9)

chopped, weighed, diluted 1:10 (wt/vol) with sterile saline, and homogenized for 25 s with an electric tissue homogenizer (TRI-R Instruments, Rockville Centre, N.Y.). Then, 1.0 and 0.1 ml of each organ homogenate were plated in duplicate on Sabouraud dextrose and blood agar. Plates were incubated for 48 h at 37°C, and colonies were counted. These methods, combined, detect from 2 to 20,000 CFU/g of tissue. For each animal, only a single kidney or lung was sampled at random.

Drug levels. Levels of itraconazole in serum were measured by bioassay using modifications of previously reported techniques (1). Briefly, *Candida kefyr* ATCC 46764 was grown for 3 to 7 days on Sabouraud dextrose agar at 25°C. Two colonies were added to yeast nitrogen base (YNB) (Difco, Detroit, Mich.) glucose broth and incubated for 4 to 5 h at 37°C. The test organism was adjusted to the no. 2 McFarland standard, and 0.5 ml was added to 35 ml of melted and cooled YNB agar. The inoculated media was poured into plastic plates (150 by 15 mm), and wells were cut in the solidified medium with a 7-mm punch. A standard curve was made by using known concentrations of itraconazole dissolved in PEG-400 (Union Carbide, Danbury, Conn.). Samples and standards were placed in duplicate 50-μl aliquots into wells on each of two plates, and plates were incubated at 37°C for 24 h. Inhibition zones were measured, and sample zones were compared to standards.

Inhibition ELISA for serum aspergillus antigen. The procedures for the inhibition enzyme-linked immunosorbent assay (ELISA) and for its required antigen and antibody preparation were performed as previously reported (14, 15, 17).

Statistical analysis. The Fisher exact test and the Wilcoxon rank sum test were used where appropriate. Statistical significance was defined as $P < 0.05$, adjusting for multiple dose comparisons. Specifically, six drug group comparisons were made for each organ evaluated so that the level of significance was defined as $P < 0.008$.

RESULTS

Mean peak levels in serum by bioassay are shown in Table 1. Mean peak levels in serum at 3 to 4 h after the first dose of itraconazole (20 and 40 mg/kg/day) were 2.7 ± 0.2 and 7.4 ± 0.7 μg/ml, respectively. Trough levels in serum after the first dose of therapy were below the limits of detection of this assay (<0.5 μg/ml) for itraconazole (20 mg/kg/day) as compared with 2.0 ± 0.7 μg/ml for itraconazole (40 mg/kg/day). A 23 to 33% increase in peak levels was seen following three doses of therapy.

The survival of rabbits treated with itraconazole, amphotericin B, and controls are shown in Fig. 1. Survival was

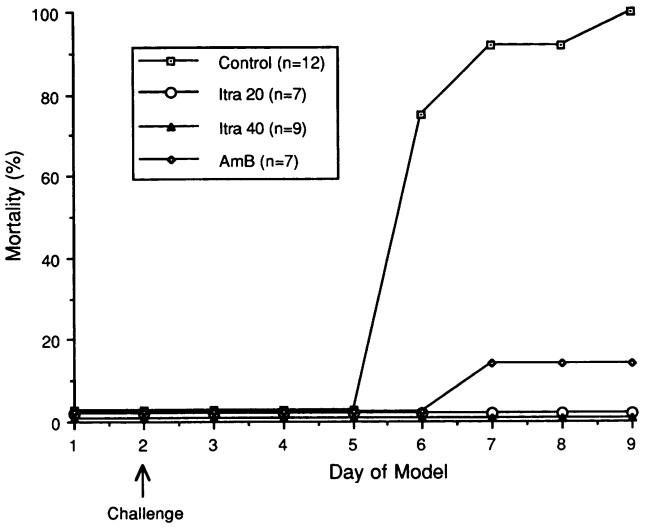

FIG. 1. Cumulative mortality of rabbits treated with itraconazole (Itra) and amphotericin B (AmB). Rabbits were challenged on day 2. Controls received no antifungal therapy. Treatment for rabbits consisted of AmB (1.5 mg/kg/day) or Itra (20 or 40 mg/kg/day) initiated 24 h after challenge and continued for 5 days.

significantly prolonged by itraconazole (20 and 40 mg/kg/day) and amphotericin B ($P < 0.001$). Mortality occurred in 12 of 12 untreated controls by day 9 of the model as compared to 0 of 7 and 0 of 9 rabbits treated with itraconazole (20 and 40 mg/kg/day, respectively) and 1 of 7 treated with amphotericin B.

Semi-quantitative culture results of organ cultures are shown in Table 2. Extensive infection occurred in the liver, lung, and kidney tissues of all untreated controls. Itraconazole at 40 mg/kg/day and amphotericin B significantly reduced the tissue burden in liver, lung, and kidney as compared with controls; results for itraconazole (40 mg/kg/day) and amphotericin B were not significantly different from each other. Itraconazole (20 mg/kg/day) significantly reduced tissue counts in liver and kidney but not lung or brain tissues as compared with controls. In contrast, itraconazole (40 mg/kg/day) and amphotericin B reduced tissue counts 100- to more than 1,000-fold as compared with controls.

The number of infected organs in treated animals and untreated controls is shown in Table 3. Although itraconazole (20 mg/kg/day) significantly reduced the tissue burden of *A. fumigatus*, liver in three of seven, lung in six of seven, and kidney in six of seven rabbits remained infected, as were all liver, lung, and kidney tissues from untreated controls. In

TABLE 2. Semi-quantitative organ cultures of rabbits treated with antifungal therapy begun 24 h after challenge

Group[a] (n)	Colony counts (mean $\log_{10}$ CFU/g of tissue ± SE)[b]			
	Liver	Kidney	Lung	Brain
Control (12)	3.7 ± 0.1	3.3 ± 0.1	2.9 ± 0.1	1.1 ± 0.3
Itra 20 (7)	1.3 ± 0.6†	1.9 ± 0.4†	2.5 ± 0.4	0.4 ± 0.4
Itra 40 (9)	<0.3*	0.6 ± 0.4*	0.4 ± 0.2*‡	<0.3
AmB 1.5 (7)	0.3 ± 0.3*	<0.3*‡	0.9 ± 0.2‡	0.8 ± 0.5

[a] Groups were untreated (control) or treated with itraconazole (Itra) at 20 or 40 mg/kg/day or with amphotericin B (AmB) at 1.5 mg/kg/day.

[b] $P < 0.001$ (*) and < 0.008 (†) versus controls and < 0.008 versus Itra 20 (‡) by Wilcoxon rank sum.

TABLE 3. Organ cultures of temporarily immunosuppressed rabbits

Group[a] (n)	No. of positive cultures/no. of rabbits cultured[b]			
	Liver	Kidney	Lung	Brain
Control (12)	12/12	12/12	12/12	6/12
Itra 20 (7)	3/7†	6/7	6/7	1/7
Itra 40 (9)	0/9*	2/9*	2/9*	0/9†
AmB 1.5 (7)	1/7*	0/7*‡	3/7†	2/7

[a] See Table 2, footnote *a*.
[b] $P < 0.001$ (*) and < 0.008 (†) versus controls and < 0.002 versus Itra 20 (‡) by the Fisher exact test.

contrast, itraconazole (40 mg/kg/day) significantly eradicated *A. fumigatus* from tissues as compared with controls. Liver in zero of nine ($P < 0.001$ versus controls), lung in two of nine ($P < 0.001$ versus controls), and kidney in two of nine ($P < 0.001$ versus controls) remained positive for *A. fumigatus*. Amphotericin B also eradicated *A. fumigatus* from tissues, and its effect was not significantly different from that of itraconazole (40 mg/kg/day). No kidney tissues from rabbits receiving amphotericin B remained positive, but one of seven liver, three of seven lung, and two of seven brain tissues remained infected. In addition, brain tissues in only one of seven rabbits receiving itraconazole (20 mg/kg/day) and zero of nine treated with itraconazole (40 mg/kg/day) ($P < 0.008$ versus controls) were positive for *A. fumigatus*.

Only itraconazole at 40 mg/kg/day effectively eradicated *A. fumigatus* from tissues as compared with controls. All 12 controls had positive *A. fumigatus* cultures from at least one site, as did 7 of 7 treated with itraconazole (20 mg/kg/day) and 5 of 7 treated with amphotericin B. In contrast, only two of nine rabbits treated with itraconazole (40 mg/kg/day) had residual positive cultures for *A. fumigatus* ($P < 0.001$ versus controls).

These doses of itraconazole and amphotericin B significantly reduced *A. fumigatus* antigenemia as compared with untreated controls. The final antigen values measured in serum, drawn at time of sacrifice or in the last serum sample drawn prior to death, are shown in Table 4. All treatment groups had lower antigen levels than controls, which correlated with the reduced tissue burden of *A. fumigatus*. Antigen levels remained over 50 ng/ml in two of seven rabbits receiving itraconazole (20 mg/kg/day) and in two of seven rabbits receiving amphotericin B, as compared with none of the nine rabbits treated with itraconazole (40 mg/kg/day) ($P < 0.003$ versus controls).

DISCUSSION

Newer therapies for invasive aspergillosis are needed to improve the outcome of this infection in immunosuppressed patients (2, 5). The newer azoles offer the potential for reduced toxicity and improved efficacy as compared with amphotericin B (7). These newer agents offer several potential advantages in the treatment of invasive fungal infection, including oral as well as intravenous administration, reduced nephrotoxicity, and an improved therapeutic index as compared with amphotericin B.

TABLE 4. Final serum antigen levels in temporarily immunosuppressed rabbits

Group (n)[a]	No. of rabbits with antigen >50 ng/ml/no. tested[b]	Mean ± SE antigen value (ng/ml) (range)[b]
Control (12)	12/12	3,325 ± 608 (235–5,000)
Itra 20 (7)	2/7	87 ± 58† (<10–410)
Itra 40 (9)	0/9*	<10† (<10–16)
AmB 1.5 (7)	2/7	29 ± 10† (<10–78)

[a] See Table 2, footnote *a*.
[b] $P < 0.001$ versus controls by Fisher exact test (*) and < 0.003 versus controls by Wilcoxon rank sum (†).

Itraconazole has been shown to be highly effective in the treatment of human and experimental invasive aspergillosis (6). Even in immunosuppressed patients, itraconazole has been shown to successfully treat invasive aspergillosis (4). However, erratic absorption of itraconazole in some patients, particularly patients who are immunosuppressed, has been a limiting factor in the therapeutic efficacy of this compound (6). This study demonstrates the potential itraconazole has in treating invasive aspergillosis. However, the results of this study also show the importance of drug delivery, as lower levels of drug in serum afforded less effective eradication of *A. fumigatus* from tissues, which may be particularly important in immunosuppressed hosts.

Improving bioavailability of the lipophilic azoles such as itraconazole and saperconazole has been difficult. One approach to improving absorption of these compounds after oral administration has been to solubilize these azoles in compounds known as cyclodextrins (10). The β-cyclodextrins are naturally occurring cyclic oligosaccharides of seven glucose units and are produced as a product of the enzymatic degradation of starch by *Bacillus macerans* (10). The cyclodextrins are used as carrier molecules for highly lipophilic drugs, as their structures contain a hydrophobic interior and hydrophilic exterior. Thus, lipophilic compounds like itraconazole and other azoles such as saperconazole can then be delivered in high concentrations via an oral route (10, 13).

Hostetler and colleagues have shown that peak concentrations in serum (C_{max}) and areas under the time concentration curves from 0 to 24 h (AUC_{0-24}) were 24- and 121-fold higher, respectively, when itraconazole was administered after solubilization in hydroxy-β-cyclodextrin than in polyethylene glycol (10). As demonstrated by Hostetler and colleagues, although larger doses (100 and 200 mg/kg) of itraconazole produced C_{max} and AUC_{0-24} which were significantly higher than doses of 25 and 50 mg/kg, the differences were not linear as similar values were seen between the two larger doses. In addition, it was also noted that late levels (12 h) of both itraconazole and saperconazole were elevated, perhaps because of enterohepatic recirculation (10).

In this study, excellent bioavailability of itraconazole was seen with the itraconazole-cyclodextrin solution. Currently itraconazole is only commercially available in an oral formulation; novel approaches to improve solubility of those compounds, such as the use of cyclodextrins, may improve delivery of some azoles after oral administration and could improve the clinical efficacy of those drugs (9, 10).

Itraconazole was shown in higher doses to eradicate *A. fumigatus* from a significant number of the rabbits. Itraconazole has been shown to effectively reduce or eliminate *A. fumigatus* in experimental infections (11, 16, 18, 19). The results of this study show that reduction in the tissue burden of infection and eradication of the organism from tissues correlated directly with the levels achieved in serum from these immunosuppressed rabbits.

This lethal model is limited by the fact that organs from untreated controls were not cultured at the same time point as the treated rabbits. In other studies using a sublethal challenge, we have shown that untreated controls surviving until sacrifice have a tissue burden virtually identical to that

of controls cultured at autopsy (12). In addition, although animals were cultured 48 h after completion of antifungal therapy, residual drug in organs could have affected organ cultures. However, it is important that itraconazole not only reduced the tissue burden but also improved survival and reduced serum antigen values, which correlated with the reduced tissue burden of *A. fumigatus*.

In conclusion, itraconazole-cyclodextrin solution was well absorbed in this immunosuppressed rabbit model and effectively prolonged survival, significantly reduced antigenemia, and sterilized tissues. Improved efficacy occurred with improved delivery of the drug. These studies show that itraconazole may offer a significant improvement in the treatment of invasive aspergillosis.

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REFERENCES

1. **Bodet, C. A., J. H. Jorgensen, and D. J. Drutz.** 1985. Simplified bioassay method for measurement of flucytosine or ketoconazole. J. Clin. Microbiol. **22:**157–160.
2. **Bodey, G. P., and S. Vartivarian.** 1989. Aspergillosis. Eur. J. Clin. Microbiol. Infect. Dis. **5:**413–437.
3. **Denning, D. W., L. H. Hanson, A. M. Perlman, and D. A. Stevens.** 1992. In vitro susceptibility and synergy studies of *Aspergillus* species to conventional and new agents. Diagn. Microbiol. Infect. Dis. **15:**21–34.
4. **Denning, D. W., D. E. Stepan, K. G. Blume, and D. A. Stevens.** 1992. Control of invasive aspergillosis with oral itraconazole in a bone marrow transplant patient. J. Infect. **24:**73–79.
5. **Denning, D. W., and D. A. Stevens.** 1990. The treatment of invasive aspergillosis: surgery and antifungal therapy of 2,121 published cases. Rev. Infect. Dis. **12:**1147–1201.
6. **Denning, D. W., R. M. Tucker, L. H. Hansen, and D. A. Stevens.** 1989. Treatment of invasive aspergillosis with itraconazole. Am. J. Med. **86:**791–800.
7. **Graybill, J. R.** 1989. New antifungal agents. Eur. J. Clin. Microbiol. Infect. Dis. **5:**402–412.
8. **Graybill, J. R., and S. R. Kaster.** 1984. Experimental murine aspergillosis: comparison of amphotericin B and a new polyene antifungal drug, SCH 28191. Am. Rev. Respir. Dis. **129:**292–295.
9. **Hanson, L. H., K. V. Clemons, D. W. Denning, and D. A. Stevens.** 1991. Efficacy of saperconazole in systemic murine aspergillosis, abstr. PS3.65, p. 370. Program Abstr. XI Congr. Int. Soc. Hum. Anim. Mycol.
10. **Hostetler, J. S., L. H. Hanson, and D. A. Stevens.** 1992. Effect of cyclodextrin on the pharmacology of antifungal oral azoles. Antimicrob. Agents Chemother. **36:**477–480.
11. **Longman, L. P., and M. V. Martin.** 1987. A comparison of the efficacy of itraconazole, amphotericin B and 5-fluorocytosine in the treatment of *Aspergillus fumigatus* endocarditis in the rabbit. J. Antimicrob. Chemother. **20:**719–724.
12. **Patterson, T. F., D. George, P. Miniter, and V. T. Andriole.** 1991. The role of fluconazole in the early treatment and prophylaxis of experimental invasive aspergillosis. J. Infect. Dis. **164:**575–580.
13. **Patterson, T. F., D. George, P. Miniter, and V. T. Andriole.** 1992. Saperconazole therapy in a rabbit model of invasive aspergillosis. Antimicrob. Agents Chemother. **36:**2681–2685.
14. **Patterson, T. F., P. Miniter, J. Dijkstra, F. C. Szoka, Jr., J. L. Ryan, and V. T. Andriole.** 1989. Treatment of experimental invasive aspergillosis with novel amphotericin B/cholesterol-sulfate complexes. J. Infect. Dis. **159:**717–724.
15. **Patterson, T. F., P. Miniter, J. L. Ryan, and V. T. Andriole.** 1988. Effect of immunosuppression and amphotericin B on aspergillus antigenemia in an experimental model. J. Infect. Dis. **158:**415–422.
16. **Polak, A.** 1987. Combination therapy of experimental candidiasis, cryptococcosis, aspergillosis and wangiellosis in mice. Chemotherapy. **33:**381–395.
17. **Sabetta, J. R., P. Miniter, and V. T. Andriole.** 1985. The diagnosis of invasive aspergillosis by an enzyme-linked immunosorbent assay for circulating antigen. J. Infect. Dis. **152:**946–953.
18. **Schmitt, H. J., F. Edwards, J. Andrade, Y. Niki, and D. Armstrong.** 1992. Comparison of azoles against aspergilli in vitro and in an experimental model of pulmonary aspergillosis. Chemotherapy **38:**118–126.
19. **Van Cutsem, J., F. Van Gerven, M. A. Van de Ven, M. Borgers, and P. A. Janssen.** Itraconazole, a new triazole that is orally active in aspergillosis. Antimicrob. Agents Chemother. **26:**527–534.