

ACTIVITY OF TWO DIFFERENT TRIAZOLES IN A MURINE MODEL OF PARACOCCIDIOIDOMYCOSIS

Susana RESTREPO, Angela M. TABARES & Angela RESTREPO

SUMMARY

A new orally absorbable triazole (Schering 39304) with a long serum half-life in man (60 hours), was tried in a murine model of progressive paracoccidiodomycosis and compared with itraconazole, another triazole which has proven effective in this mycosis. Only 15% of the infected, untreated mice survived while 53 to 75% of the animals receiving itraconazole survived. Mice treated with Schering 39304 exhibited higher (86 - 100%) survival rates. Statistically, the 5 mg/kg Sch 39304 was superior to the 50 mg/kg itraconazole dose. Lung cultures showed that 20 mg/kg/day of Sch achieved sterilization of the infectious foci. These results indicate that the new triazole will have a place in the treatment of paracoccidiodomycosis.

KEY WORDS: Paracoccidiodomycosis; Experimental; Treatment; Triazole; Itraconazol; Schering 39304.

INTRODUCTION

Therapy with imidazole derivatives have greatly improved the prognosis of paracoccidiodomycosis (PCM), a severe systemic mycosis prevalent in Latin America⁽⁶⁾. Experience to date indicates that ketoconazole is one of the best therapeutic options available^(12, 14). The newer triazole derivative, itraconazole, is also very effective and allows shorter courses of therapy (3-6 months)^(11, 13, 14). Both drugs, however, require daily dosing. Most patients with PCM are agricultural workers who have limited access to regular medical consultation and who, additionally, find it difficult to purchase an expensive drug for prolonged periods of time⁽⁶⁾. Consequently, it would be desirable to find a more potent medication which could permit short-term therapies or less frequent dosing.

Sch 39304 is a new triazole derivative which is potent *in vitro* against a variety of pathogenic fungi. It is well absorbed after oral administration and has a long serum half-life in man (60 hrs) which allows alternate day dosing^(7,8). These data stimulated interest in comparing the efficacy of

this new triazole agent with itraconazole, a drug which has been previously shown to protect mice against a lethal paracoccidiodal challenge⁽⁹⁾.

MATERIAL AND METHODS

Experimental animals:

A total of 91 BALB/c mice, 39 males and 52 females, 3-4 weeks old, obtained from the breeding colony of the Corporación para Investigaciones Biológicas (CIB), Medellín, Colombia, were used in the challenge experiments. Mice were supplied with sterile food and bedding and acidified water (pH 2.5-.30) in sterilized bottles^(1,10). Mice of both sexes were used as previous experiments⁽⁹⁾ had shown that there were no differences in susceptibility to *P. brasiliensis* infection between males and females. Animals were weighed before inoculation and weekly thereafter. Food and water were furnished *ad libitum*.

Fungus:

Paracoccidoides brasiliensis isolate Gar obtained from a Colombian patient was used. The iso-

late was initially passed through mice and re-isolated from their tissues by culturing at room-temperature. The fungus was then converted into the yeast form by subculturing at 36°C in Muller-Hinton agar plus thiamine⁽¹⁵⁾. Five days later when vigorous growth was obtained, subcultures were made to a chemically-defined solid medium⁽¹⁶⁾.

After two more passages, the growth was transferred to the same medium but in liquid form, and incubated for 5 days at the same temperature under constant shaking. Yeast cells were harvested, washed 3 times in phosphate buffered saline (PBS) at pH 7.2 and sonicated for 28 seconds in order to obtain single-cell suspensions. Viability was confirmed by the ethidium bromide fluorescent technique⁽²⁾. A yeast inoculum consisting of 6×10^7 viable units was prepared the day of inoculation.

Antifungal agents:

Two antifungal agents were tested for their efficacy in this murine model. Sch 39304 was obtained from Schering Plough Research, Bloomfield, New Jersey; itraconazole (ITZ) was obtained from Janssen Pharmaceutica, Beerse, Belgium. Sch 39304 was suspended in 0.3%. Noble agar (Difco) in water and ITZ was dissolved in polyethylene glycol 400 (PEG) (Fisher), as described by GRAYBILL & ARHENS⁽⁵⁾. Sch 39304 was prepared in concentrations varying from 0.5. to 0.8 mg/ml according to the weight of the animals and given at doses of 5 mg/kg; for the 20 mg/kg dose and depending on mouse weight, Sch 39304 was prepared in concentrations ranging from 2.2 mg/ml to 4.0 mg/ml. Itraconazole was prepared in concentrations of 1.4 - 3.1 mg/ml and given at a dose of 20 mg/kg; for the 50 mg/kg dose, itraconazole was prepared in concentrations of 5.1 to 7.9 mg/ml.

Challenge and therapy:

Mice were anesthetized with intramuscular ketamine hydrochloride. When breathing deeply, they were inoculated intranasally with the fungus. The inoculum was given in a step-wise fashion: an initial dose of 0.025 ml and 10 minutes later, a second dose of 0.025 ml. (Total dose was .50 ml = 3×10^6 cells/mouse). Deaths within 24 hours post-challenge were probably due to the procedure and were not recorded with the results. Animals were randomized into 3 subgroups of 14 to 16. Subgroup 1 was subdivided into two, one receiving Noble agar and the other, PEG (controls). Subgroup 2 was treated with ITZ and subgroup 3 with Sch, both by gavage.

Therapy was initiated 24 hours after challenge and continued for 30 consecutive days. Animals were observed for 30 days after termination of treatment; however, groups of 4-6 survivors were sacrificed by means of prolonged ether anesthesia at 7, 14 and 21 days during this time. Spontaneous deaths were also recorded. All animals were autopsied, their weights recorded and colony forming units (CFU) of the paracoccidioidal burdens from lungs were determined. Lungs were processed in tissue grinders and 1.0 ml dilutions in PBS were plated in duplicate on modified Sabouraud's agar (Mycosel, BBL).

The plates were incubated at 25-28°C for 30 days and the number of *P. brasiliensis* colonies was recorded.

Statistics: Comparisons between groups were analyzed by the Student's tests, with significance at $P < 0.05$.

RESULTS

There were no significant differences in the survival curves of animals given Noble agar or PEG alone; consequently, the data were pooled. Deaths (shown by culture to be due to paracoccidioidomycosis) occurred between 7 and 21 days and mostly between days 9-11. As shown in Table 1, both ITZ-SCH markedly prolonged survival, but Sch offered greater (88-100%) protection to challenged animals. ITZ protected 64.7-75.0% of the animals, depending on the dose. Untreated control animals exhibited a lower survival rate (14.86%). Statistically, mice treated with either dose of Sch 39304 had better survival than control infected mice ($P < 0.001$). Mice treated with ITZ at 20 and 50 mg/kg had a lower, yet significant survival time, $P < 0.01$ and $P < 0.001$, respectively. When the low doses (20 mg/kg ITZ, 5 mg/kg Sch) were compared, no statistically significant differences were seen; however at higher doses (50 mg/kg ITZ, 20 mg/kg Sch), the latter was more effective. Figure 1 shows the survival curve; however at higher doses (50 mg/kg ITZ; 20 mg/kg Sch) no statistically significant differences were seen. Figure 1 shows the survival curve.

Lung cultures taken from mice that died during the experiment revealed high CFU counts to 1.1×10 to 8.3×10 in all cases, irrespective of therapy (Fig. 2). However, when the counts were done only on the lungs of those animals that sur-

Table 1

Effect of Itraconazol and of Schering 39304 on the mortality of mice challenged with intranasal *P. brasiliensis* yeast cells

	Therapy and dose (mg/kg/day)		Occurrence of death at indicated time time in days (range)		No. Survivors	
	None	(1)*			Total infected (% survival)	
ITZ	20	(2)	9.6	(5-21)	11/17	(64.7)
	50	(3)	10.8	(9-13)	12/16	(75.0)
	None	(1)*	7.6	(5-11)	4/27	(14.8)
SCH	5	(4)	7.0	(6-8)	15-17	(88.2)
	20	(5)	0	(-)	12/12	(100)

* Significances

(P < 0.05)

1 vs 2 S < 0.01 . 2 vs 3 NS > 0.5 . 3 vs 4 NS > 0.3

1 vs 3 S < 0.001 . 2 vs 4 NS > 0.1 . 3 vs 5 S < 0.05

1 vs 4 S < 0.001 . 2 vs 5 NS > 0.1 . 4 vs 5 NS > 0.1

1 vs 5 S < 0.001

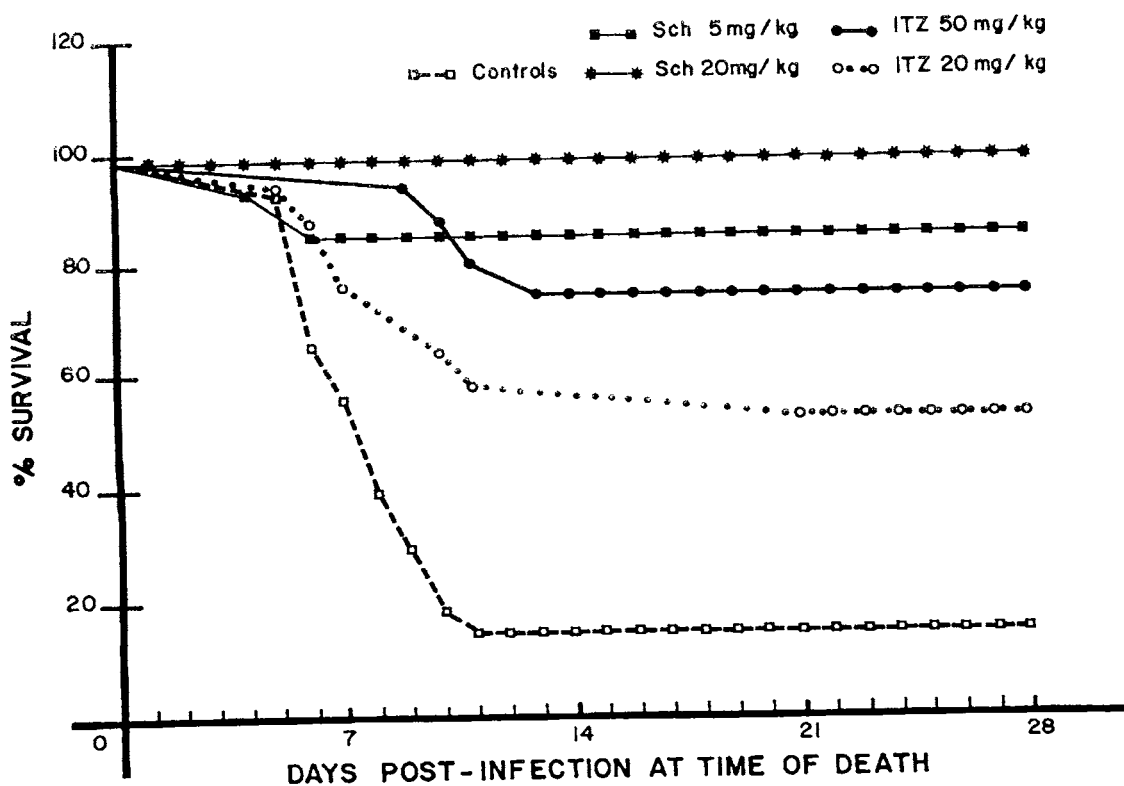


Fig.1 - Survival following the Pulmonary Challenge of mice with *P. brasiliensis* and Treatment with Azoles.

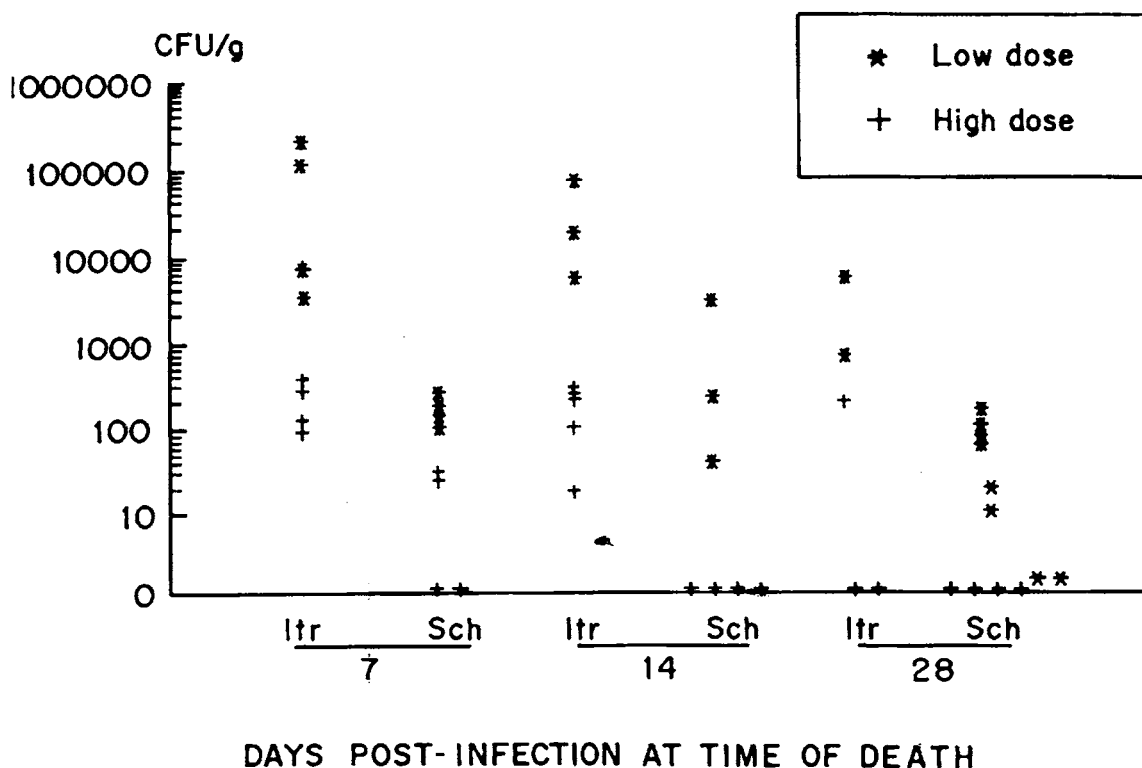


Fig.2 - Lung Cultures in Mice infected with *P.brasiliensis* and treated with Azoles

vived and were sacrificed after the end of therapy, the fungal burden was lower in animals receiving the high dose of ITZ or either of the two Sch doses (Table 2). CFU counts diminished with time and ended (day 28) very low with the 50 mg/kg ITZ dose as well as with the 5 or 20 mg/kg Sch doses. Sch at 20 mg/kg completely sterilized the animals by 14 days after end of therapy.

DISCUSSION

Paracoccidioidomycosis remains a difficult infection to treat despite the use of azole derivatives, because it still requires prolonged therapy⁽¹⁶⁾. Consequently, drugs that appear to be more potent *in vitro* or that have prolonged half-lives may play an important role in the control of this mycotic disorder. Using a previously reported murine model⁽¹⁾, it is possible to produce a chronic, progressive form of paracoccidioidomycosis which mimics to some extent, human disease. Therapeutical interaction is then feasible in such a model.

In survival studies, only 14.8% of infected, untreated, mice survived. Survival of mice treated

with Sch ranged from 88 to 100% when given 5 or 20 mg/kg/day respectively. ITZ treated mice also survived although the survival rates were lower (20 mg/kg, 64.7%; 50 mg/kg, 75%).

The protection seen with ITZ in this study was not as high as that reported previously (100%) by McEWEN et al.,⁽⁹⁾. We used the same isolate but passed it through mice just before attempting the experimental infection. We probably had a more virulent isolate for our experiments. CASTAÑEDA has shown that animal passage is required to obtain reproducible results in a murine model of paracoccidioidomycosis⁽³⁾.

Statistically the 20 mg/kg Sch dose was superior to the corresponding high dose (50 mg) of ITZ; both triazoles, however, proved equally effective when used at lower concentrations.

No deaths were observed in those mice receiving 20 mg/kg Sch. Furthermore, organ cultures showed that 14 days after the end of therapy, this dose of Sch sterilized the lung. With ITZ, the mean CFU counts diminished both with dose and time of observation

Table 2

Recovery of *P. brasiliensis* from lungs of Triazole-treated Mice

Time (days) of sampling after end therapy in treatment groups	Mean CFU per gr. of lung* (No. mice)
Itraconazole 20 mg	
7.....	8.3 x 10 ⁴ (4)
14.....	3.1 x 10 ⁴ (3)
28.....	3.0 x 10 ³ (2)
Itraconazole 50 mg	
7.....	2.1 x 10 ² (4)
14.....	2.0 x 10 ² (5)
28.....	6.7 x 10 ¹ (3)
Schering 5 mg	
7.....	1 x 10 ² (5)
14.....	7.6 x 10 ² (4)
28.....	4.8x10 ¹ (8)
Schering 20 mg	
7.....	1.4 x 10 ¹ (4)
14.....	0 (4)
28.....	0 (4)

* Limit of detection of the plates: 8 CFU/g.

post-therapy. However, no cultures became negative during the observation period suggesting that ITZ inhibits but does not kill the fungus.

The azoles are typically fungistatic agents. Nevertheless, Sch prolonged survival of all mice given the higher dose as well as of 88% of those receiving the 5 mg/kg/day dose.

We may conclude that Sch 39304 is an effective therapy for pulmonary-induced murine paracoccidioidomycosis. Furthermore, this triazole appears capable of eradicating the fungus from the infected mice. Although ITZ also protected 75% of the animals treated with the 50 mg/kg/day dose, no eradication of the fungal lung foci was obtained.

RESUMEN

Actividad de dos triazoles diferentes en un modelo murino de paracoccidioidomycosis

Un nuevo derivado triazólico Schering 39304 que tiene una vida promedio prolongada (60 horas), así como otro triazol (itraconazole) de eficacia comprobada en la paracoccidioidomycosis, fueron ensayados en un modelo murino. Se encontró que solo sobrevivían el 15% de los animales infectados no tratados; del grupo tratado con Itraconazol sobrevivieron el 53% de los que recibieron 20 mg/kg/día y el 86% de aquellos con 50 mg/kg/día. De los ratones que recibieron Sch 39304 sobrevivieron el 86% de los tratados con la dosis baja (5 mg/kg) y el 100% con la dosis mayor (20 mg/kg). Se encontraron diferencias estadísticamente significativas entre esta última droga y el itraconazol. Además, los cultivos de pulmón mostraron que solo Sch 39304 a la dosis alta, era capaz de erradicar el hongo de los tejidos. Estos resultados indican que tal triazol tendrá importancia en el tratamiento de la paracoccidioidomycosis humana.

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