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Experimental Evaluation of Second-Line Oral Treatments of Visceral Leishmaniasis Caused by *Leishmania infantum*

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In a murine model of *Leishmania infantum* visceral leishmaniasis, metronidazole, ketoconazole, fluconazole, itraconazole, and terbinafine were less effective than antimonial agents in reducing hepatic parasite load. Ketoconazole potentiated the effect of meglumine antimoniate reference therapy through its marked activity against spleen infection.

Visceral leishmaniasis (VL) caused by *Leishmania infantum* remains difficult to treat in patients with AIDS because of parasite resistance and high rates of relapse (1, 4, 13). There is a need for alternatives to antimonial agents and amphotericin B, especially for drugs that are effective by the oral route. Metronidazole and sterol biosynthesis inhibitors (ketoconazole, fluconazole, itraconazole, and terbinafine) are well-tolerated drugs that are potentially active against *Leishmania* when given by mouth. However, their use in the treatment of cutaneous and visceral leishmaniasis caused by different *Leishmania* species has produced conflicting results (2, 3, 7–9, 21–28). Mbongo et al. (23) recently showed that *Leishmania donovani* experimentally resistant to amphotericin B was highly susceptible to ketoconazole. Although it is now agreed that the therapeutic response varies with the parasite species (6), few data are available on *L. infantum* sensitivity. Before these compounds are used again for the treatment of VL in human immunodeficiency virus (HIV) patients, it seems judicious to evaluate their specific anti-*L. infantum* activity in order to better define the rationale for their prescription.

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In an experimental mouse model previously used to test the efficacy of amphotericin B, lipid formulations of amphotericin B, and aminosidine (10–12), we compared the efficacies of metronidazole and antifungal agents against *L. infantum* VL. Control groups included untreated mice and mice treated with meglumine antimoniate. Adult female BALB/c mice (Iffa Credo, Lyon, France) were infected on day 0 by intravenous injection of 10^7 stationary-phase promastigotes of *L. infantum* MON-1 after zymodeme analysis (MHOM/FR/91/LEM2259V), isolated from a patient with AIDS.

The first experiment consisted of drug screening. To evaluate drug activity, parasite loads in the liver and spleen of mice treated from day 7 to day 17 were determined on day 20, by use of a culture microtitration method (5). Parasite load was expressed as the $\log_{10}$ number of parasites per gram of tissue, and the mean (±standard error) parasite load for four mice was calculated. Drug administration was daily, by mouth, for treatments with metronidazole (70 and 140 mg/kg; Specia Rhône-Poulenc Rorer, Paris, France), ketoconazole (50 and 100 mg/kg; Janssen-Cilag, Boulogne-Billancourt, France), fluconazole (50 and 100 mg/kg; Pfizer, Orsay, France), itraconazole (50 and 100 mg/kg; Janssen-Cilag), and terbinafine (100 mg/kg; Novartis, Rueil-Malmaison, France) and daily, by intraperitoneal injection, for treatment with meglumine antimoniate (200 mg/kg; Specia Rhône-Poulenc Rorer). None of the test compounds administered alone from day 7 to day 17 significantly reduced the parasite load in the liver by day 20, in comparison to that in infected untreated control mice (Table 1). By contrast, the parasite load in the spleen of treated mice fell by 1 to 4 $\log_{10}$ parasites/g relative to the parasite load in the controls. Ketoconazole was the most effective of the drugs tested, since the parasite loads were markedly reduced at a dose of 50 mg/kg/day and were undetectable in mice treated with 100 mg/kg/day ($P < 0.01$, compared to results with untreated mice; Newman Keuls test). Treatments with metronidazole, and treatment with fluconazole at a dose of 50 mg/kg/day, also significantly reduced the parasite load in the spleen, in comparison to that in infected untreated control mice. Surprisingly, treatment with fluconazole at 50 mg/kg/day seemed slightly more effective in the spleen infection than that at 100 mg/kg/day. However, this effect is limited and individual variations between mice may partially explain such a difference.

A second experiment (Table 2) was designed to identify possible synergistic activity between meglumine antimoniate, which is highly effective in the liver but less effective in the spleen (10–12), and drugs with significant activity against spleen infection. We thus examined the efficacies of ketoconazole and metronidazole alone and in combination with meglumine antimoniate and compared the results with those in untreated mice and in mice treated with meglumine antimoniate alone. Each group comprised 12 mice. Meglumine antimoniate was highly effective in the liver, and treatment with ketoconazole resulted in the lowest spleen load. Interestingly, no parasites were detected on day 20 in the liver or spleen of mice treated with meglumine antimoniate plus ketoconazole or metronidazole. However, relapses were observed with both combinations at day 60, with parasite counts in the spleen comparable to those in mice treated with meglumine antimoniate alone.

With our model, we can assess drug efficacy in several organs, particularly in the liver and spleen. The threshold of detection of our microtitration method is 50- to 100-fold lower than that of microscopic examination of stained organs and is estimated to be 10^3 parasites per g (6). In the present study, we

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TABLE 1. Parasite load on day 20 in the liver and spleen of *L. infantum*-infected mice treated from days 7 to 17

Expt 1 treatment	Dose (mg/kg/day)	Day	Log_{10} parasites/g of tissue[a]	
			Liver	Spleen
None (control)		7	4.66 ± 0.18	1.83 ± 2.14
		20	4.99 ± 0.31	3.82 ± 0.30
Ketoconazole	50	20	4.66 ± 0.21	0.86 ± 1.76
	100	20	4.90 ± 0.13	0*
Fluconazole	50	20	4.94 ± 0.28	0.85 ± 1.70*
	100	20	5.04 ± 0.16	1.74 ± 2.01
Itraconazole	50	20	4.72 ± 0.30	2.51 ± 2.19
	100	20	4.48 ± 0.60	1.14 ± 1.98
Terbinafine	100	20	4.57 ± 0.49	2.29 ± 1.10
Metronidazole	70	20	4.55 ± 0.23	1.13 ± 2.26*
	140	20	4.67 ± 0.53	1.03 ± 2.06*

[a] Values are means ± standard deviations for four mice. *, significantly different from value for untreated mice at $P < 0.01$ (by Newman Keuls test).

confirm that pentavalent derivatives of antimony are highly effective, since parasite burdens were at an undetectable level in the liver. However, parasite foci persisted in the spleen, and this probably explains the occurrences of relapses. Our results with itraconazole and terbinafine alone argue against their therapeutic use in cases of VL due to *L. infantum*. Although fluconazole showed good activity in the spleen, its current use for the treatment of oral candidiasis in HIV-infected patients and the risk of selecting for resistant *Candida* strains limit its use in AIDS patients with VL. Despite being less effective than meglumine antimoniate in monotherapy, ketoconazole and metronidazole were the most effective second-line treatments in this study. Furthermore, their combination with meglumine antimoniate resulted in marked decreases in parasite loads in both the liver and spleen. However, the relapses that were observed in susceptible BALB/c mice treated with these combinations indicate the inefficacy of the host immune functions in clearing parasites when anti-*Leishmania* drug concentrations decreased and/or when the drug did not reach the parasitophorous vacuole. This was previously described for treatment with free antimony in monotherapy and for amphotericin B deoxycholate treatment, whereas drug delivery systems allowed sustained higher levels of drug and/or drug targeting to the infected site, potentiating the parasite suppression (3, 7, 8, 14). Although drug levels in tissues were not determined in this work, the pharmacokinetic characteristics of ketoconazole and metronidazole are also probably responsible for such relapses. With regard to the use of marketed drug carrier systems with meglumine antimoniate, ketoconazole, or metronidazole in order to reach sustained drug levels and to target the drugs toward infected tissues, our results stress the need for multidrug therapy and maintenance therapy in the treatment of *L. infantum* VL with these drugs.

TABLE 2. Parasite loads on days 20 and 60 in the liver and spleen of *L. infantum*-infected mice treated from days 7 to 17

Expt 2 treatment	Day	Log_{10} parasites/g of tissue[a]	
		Liver	Spleen
None (control)	7	3.87 ± 0.07	1.45 ± 1.74
	20	4.77 ± 0.35	3.56 ± 0.29
	60	4.57 ± 0.27	4.79 ± 0.51
Glucantime (200 mg/kg/day)	20	0*	1.25 ± 1.95*
	60	0*	3.87 ± 0.29
Ketoconazole (50 mg/kg/day)	20	4.69 ± 0.26	0.68 ± 1.66*
	60	3.94 ± 0.35	4.72 ± 0.14
Metronidazole (70 mg/kg/day)	20	4.89 ± 0.32	2.13 ± 1.56
	60	4.50 ± 0.45	4.96 ± 0.35
Meglumine antimoniate (200 mg/kg/day) + ketoconazole (50 mg/kg/day)	20	0*	0*
	60	0*	4.25 ± 0.54
Meglumine antimoniate (200 mg/kg/day) + metronidazole (70 mg/kg/day)	20	0*	0*
	60	0*	3.17 ± 1.62

[a] Values are means ± standard deviations for six mice. *, significantly different from value for untreated mice at $P < 0.01$ (by Newman Keuls test).

In conclusion, this study confirms the marked variability in the susceptibility of *Leishmania* species to drugs. In this experimental model of VL due to *L. infantum*, none of the oral treatments administered alone was more effective than therapy with meglumine antimoniate. Meglumine antimoniate combined with ketoconazole may warrant clinical trials in immunosuppressed hosts, since it clears the parasite from multiple target organs.

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