Highly Effective Oral Amphotericin B Formulation against Murine Visceral Leishmaniasis

Kishor M. Wasan,[1] Ellen K. Wasan,[1,3] Pavel Gershkovich,[1] Xiaohua Zhu,[4] Richard R. Tidwell,[5] Karl A. Werbovetz,[4] John G. Clement,[2] and Sheila J. Thornton[1]

[1]Faculty of Pharmaceutical Sciences, University of British Columbia, and [2]iCo Therapeutics, Vancouver, and [3]School of Health Sciences, British Columbia Institute of Technology, Burnaby, British Columbia, Canada; [4]Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus; and [5]Department of Pathology and Lab Medicine, Consortium for Parasitic Drug Development, University of North Carolina at Chapel Hill, Chapel Hill

Visceral leishmaniasis is a deadly parasitic disease caused by obligate intramacrophage protozoans of the *Leishmania* genus. The World Health Organization estimates the annual death toll to be 50,000, with 500,000 new cases each year. Without treatment, visceral leishmaniasis is inevitably fatal. For the last 70 years, the firs line of defense has been pentavalent antimonials; however, increased resistance has brought amphotericin B to the forefront of treatment options. Unfortunately, the difficul route of drug administration, toxicity issues, and cost prevent amphotericin B from reaching the infected population, and mortality continues to rise. Our reformulation of amphotericin B for oral administration has resulted in a highly efficaciou antileishmanial treatment that significantl reduces or eradicates liver parasitemia in a murine model of visceral leishmaniasis. This formulation has overcome amphotericin B's signific nt physicochemical barriers to absorption and holds promise for the development of a self-administered oral therapy for the treatment of visceral leishmaniasis.

In developing nations, the need for oral therapies is not a matter of convenience or cost, but instead it is one of survival. The benefi of developing oral therapies is illustrated by the protozoan *Leishmania donovani,* an insidious parasite that is transmitted by the bite of an infected sand fl and places over 350 million people from 88 countries at risk of infection [1]. On the Indian subcontinent alone, >500,000 individuals are infected with the most severe form of this macrophagic infection, which rapidly infiltrate the vital organs and ultimately leads to severe infection of the visceral reticuloendothelial system [2]. Visceral leishmaniasis, also known as Kala-azar, is most prevalent among weak and young persons within a population [3]. Left untreated, almost all infected individuals will die [4]. The therapeutic arsenal against *Leishmania* is limited to a small number of parenterally administered agents, with daily injections of pentavalent antimony compound for 28 days being the usual course of treatment [2]. Increasing resistance to antimonial drugs has severely limited their use in many areas, including northeastern India, where the incidence of Kala-azar is highest [5].

At present, only one oral visceral leishmaniasis therapy exists. Miltefosine (hexadecylphosphocholine), initially developed as an oral anticancer drug, was found to exhibit effi acy against visceral leishmaniasis. In 2002, miltefosine (Impavido; Zentaris GmBH) was registered and licensed in India for oral treatment of visceral leishmaniasis, but teratogenicity restricts its use in women of childbearing age [3, 6–8]. In addition, parasite resistance to miltefosine is easily induced in vitro [3, 6–8]. To prevent widespread reduction in efficac , the administration of miltefosine should occur under direct medical observation.

Amphotericin B is the current secondary treatment of choice against leishmaniasis and has a 97% cure rate with no reported resistance. However, therapy with the first-generatio micellar formulation (Fungizone; Bristol Myers Squibb) involves intravenous administration over a period of 30–40 days and is associated with infusion and drug-related adverse effects (infection of the indwelling catheter, dose-dependent renal toxicity, red blood cell hemolysis, thrombophlebitis, shaking, chills, fever, and bone pain). Although lipid-based formulations exist (ie, Abelcet [lipid complex; Enzon Pharmaceuticals] and AmBisome [liposomal form; Astellas Pharma]), which require a shorter course of therapy (3–5 days), are highly effective, and exhibit lower toxicity when compared with Fungizone, the cost of these formulations is a barrier to widespread use [4, 9]. The amphiphilic nature of amphotericin B prevents gastrointestinal absorption; therefore, current formulations are all administered parenterally. The complications related to intravenous drug administration, toxicity issues, and cost prevent amphotericin B from reaching the infected population, and mortality continues

Received 2 January 2009; accepted 7 March 2009; electronically published 22 June 2009.
Potential conflicts of interest: none reported.
Financial support: Canadian Institutes of Health Research, iCo Therapeutics, and the Consortium for Parasitic Drug Development.
Reprints or correspondence: Dr. Kishor M. Wasan, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, BC, Canada V6T 1Z3 (kwasan@interchange.ubc.ca).

The Journal of Infectious Diseases 2009; 200:357–60

0022-1899/2009/20003-0006$15.00
DOI: 10.1086/600105

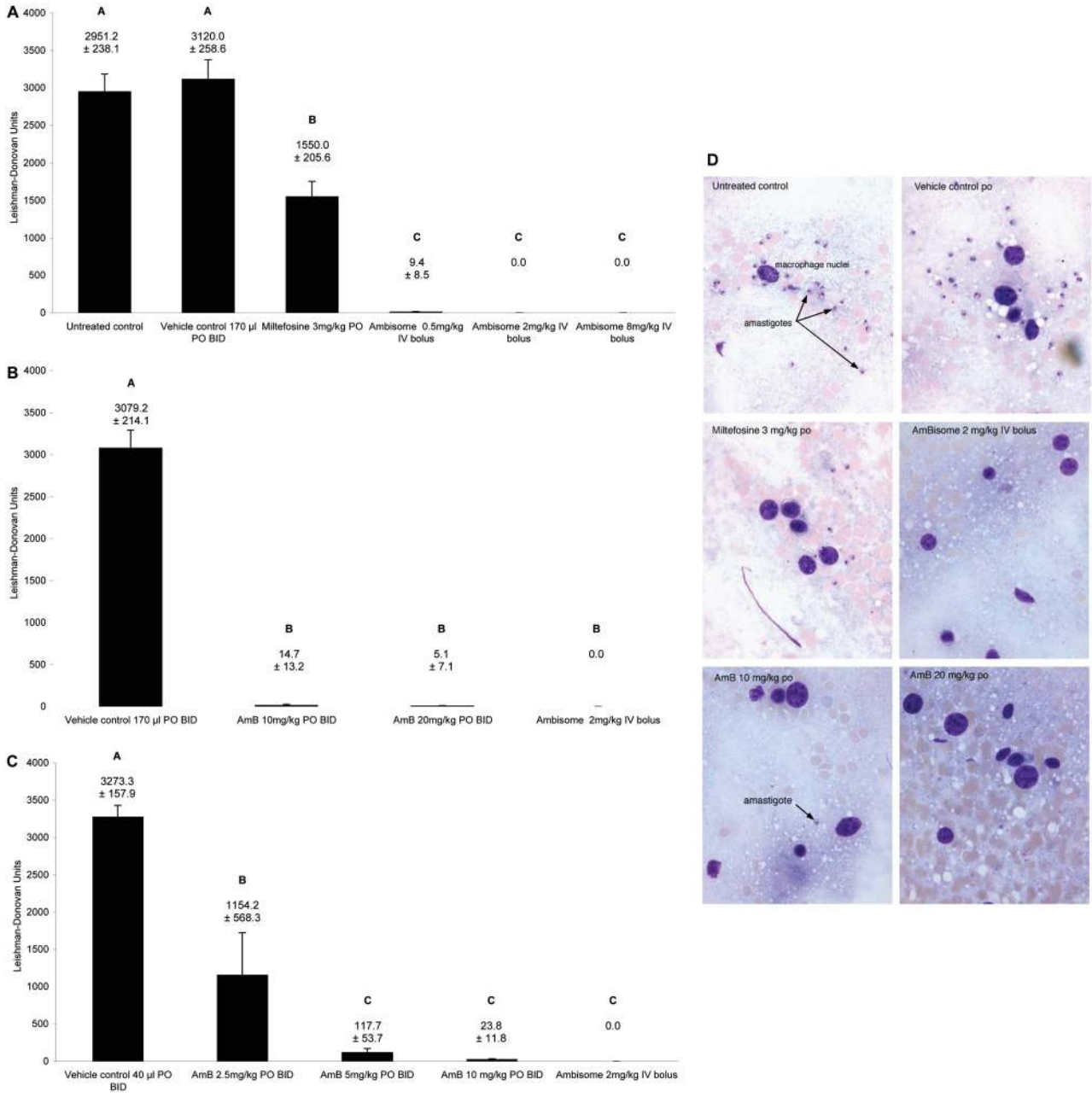

Figure 1. Activity of an oral amphotericin B (AmB) formulation in *Leishmania donovani*–infected BALB/c mice. Animals were infected and treated as described, and Leishman-Donovan units were assessed by microscopic counting of liver smears. All treatments began 7 days after infection. Bars of differing letters indicate statistically significant differences within each figure (1-way analysis of variance with post-hoc Tukey Multiple Comparisons Test); data are expressed as mean values ± standard deviation. *A,* Groups of animals (4 mice in each group) were treated with single 8, 2, or 0.5 mg/kg intravenous (IV) doses of AmBisome; miltefosine at 3 mg/kg orally (PO) daily for 5 days; a lipid-based vehicle control twice daily (BID) PO for 5 days; or a single IV saline injection. *B,* Treatment was performed with oral AmB formulations at 10 and 20 mg/kg BID for 5 days, a single 2 mg/kg IV dose of AmBisome, or a lipid-based vehicle control BID PO for 5 days (5 mice in each group). *C,* BALB/c mice were treated with 2.5, 5, and 10 mg/kg oral AmB BID for 5 days; a single 2 mg/kg IV dose of AmBisome; or a lipid-based vehicle control BID PO for 5 days (4 mice in each group). *D,* Giemsa-stained liver smears were obtained from mice postmortem after no treatment or exposure to vehicle, miltefosine, AmBisome, or the oral AmB formulation at the doses indicated. Different letters above bars indicate statistically significant differences between treatment groups; matching letters indicate groups with no statistically significant difference.

to rise. Over the past 50 years, many attempts have been made to formulate amphotericin B for oral administration, with limited success. A nanosuspension of amphotericin B was developed a by high-pressure homogenization technique in an attempt to facilitate drug uptake across the gastrointestinal tract. Nanosuspensions have been shown to adhere to the gastrointestinal mucosa, increasing drug contact time and potentially enhancing uptake [10]. Oral treatment of *L. donovani*–infected Balb/c mice indicated that the nanosuspension of amphotericin B showed superior drug uptake and some reduction in parasite numbers, compared with oral administration of micronized amphotericin B, Ambisome, or Fungizone, but no curative effect was observed [11]. An oral cochleate formulation of amphotericin B (Bioral Amphotericin B; BioDelivery Sciences International) has been tested in mouse models of candidiasis and aspergillosis and has recently completed Phase I trials [12]. Encapsulation of amphotericin B results in stable, nontoxic lipid particles that facilitate systemic delivery and encourage interaction with biological membranes [13]. Although preliminary studies indicate 100% survivorship of treated animals, the treatment did not eliminate fungal load from the organs. To the best of our knowledge, testing of this formulation in a visceral leishmaniasis model has not been explored.

In this study, we report on the antileishmanial activity of a novel lipid-based oral amphotericin B formulation that overcomes the barriers preventing oral absorption. Amphotericin B exhibits low solubility, instability at gastric pH, and, due to its physicochemical properties, is unable to penetrate the brush border membrane of the small intestine. In addition to the physicochemical challenges, we were also faced with the task of reducing or eliminating nephrotoxicity.

Methods. To determine the antileishmanial activity of our oral amphotericin B formulation, the following studies were completed. BALB/c mice were intravenously infected with 5×10^7 *Leishmania donovani* LV82 promastigotes (obtained by culturing amastigotes taken directly from the spleen of an infected hamster) 7 days prior to treatment. After the 7 days, mice were administered either a single intravenous dose of AmBisome at 8, 2, or 0.5 mg/kg; 5 daily doses of miltefosine at 3 mg/kg orally; or amphotericin B formulations at 2.5, 5, 10, and 20 mg/kg orally twice daily for 5 consecutive days. Appropriate vehicle controls were also assessed. Animals were sacrifice 14 days after infection, and Leishman-Donovan units were assessed in livers of mice postmortem via microscopic enumeration of Giemsa-stained liver smears.

Results. Treatment with a single 2 mg/kg intravenous dose of AmBisome administered 1 week after infection completely eradicated liver parasites. AmBisome is thus more active in our model, compared with its activity in earlier studies [14]. When given in 5 daily doses at 3 mg/kg orally, miltefosine resulted in a 47.5% ± 7.0% inhibition of liver parasitemia, consistent with other reports [15]. Leishman-Donovan unit values were not significantl different between groups of animals receiving oral doses of a lipid-based vehicle control twice daily for 5 days versus those receiving a single intravenous saline injection (fi - ure 1*A*). Oral amphotericin B formulations at 10 and 20 mg/kg twice daily for 5 days resulted in 99.5% ± 0.4% and 99.8% ± 0.2% inhibition of parasitemia, respectively, compared with control mice that received formulation only (f gure 1*B*). Dose response data from treatment of *L. donovani*–infected BALB/c mice with 2.5, 5, and 10 mg/kg oral amphotericin B twice daily for 5 days is reported in figu e 1*C*. The 10 mg/kg twice daily dose again resulted in almost complete eradication of liver parasitemia, whereas a dose-dependent effect on infection was observed in the other groups. Identical treatment groups between the 3 studies resulted in consistent and repeatable results (data from vehicle control, AmBisome 2 mg/kg IV bolus [1-way analysis of variance], and oral 10 mg/kg amphotericin B [*t* test] studies were not signif cantly different) (figu e 1*C*). Figure 1*D* presents representative liver smears from select groups. On the basis of data from our antifungal studies that indicated no renal toxicity (data not shown) and the level of antileishmanial activity observed (figu e 1), we are now confiden that a self-administered oral formulation of amphotericin B is attainable.

Discussion. The demonstrated efficac of this formulation is likely attributable to a combination of increased solubility, improved gastrointestinal stability, and enhanced membrane permeability. In addition, oral administration of a lipid-based formulation favors lymphatic transport. As visceral leishmaniasis parasites disseminate through the lymphatic and vascular system, infecting macrophages and infiltratin the bone marrow, liver, and spleen, the lipid carrier may assist in delivering the drug to the site of greatest infection. It is important to note that the 20 mg/kg oral amphotericin B therapy completely eradicated *L. donovani* from the liver in 3 of 5 animals. Incomplete suppression may resolve symptoms but may also be accompanied by a significan incidence of recurrence. Taking into consideration the short treatment course in this study, it is conceivable that longer treatment would completely eradicate visceral leishmaniasis in all treated animals. Taken together, these data, to the best of our knowledge, represent the fi st oral amphotericin B formulation with significan efficac against *L. donovani* (the parasite responsible for visceral leishmaniasis) in an infected mouse model.

Acknowledgments

The authors acknowledge the assistance of Dr. Abhay Satoskar (The Ohio State University) for helpful discussions.

References

1. Singh RK, Pandey HP, Sundar S. Visceral leishmaniasis (kala-azar): challenges ahead. Indian J Med Res **2006**; 123:331–44.
2. Sundar S, Chatterjee M. Visceral leishmaniasis—current therapeutic modalities. Indian J Med Res **2006**; 123:345–52.
3. Chappuis F, Sundar S, Hailu A, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol **2007**; 5:873–82.
4. Thakur CP, Pandey AK, Sinha GP, Roy S, Behbehani K, Olliaro P. Comparison of three treatment regimens with liposomal amphotericin B (AmBisome) for visceral leishmaniasis in India: a randomized dose-find ng study. Trans R Soc Trop Med Hyg **1996**; 90:319–22.
5. Olliaro PL, Guerin PJ, Gerstl S, Haaskjold AA, Rottingen JA, Sundar S. Treatment options for visceral leishmaniasis: a systematic review of clinical studies done in India, 1980–2004. Lancet Infect Dis **2005**; 5: 763–74.
6. Croft SL, Snowdon D, Yardley V. The activities of four anticancer alkyllysophospholipids against *Leishmania donovani*, *Trypanosoma cruzi* and *Trypanosoma brucei*. J Antimicrob Chemother **1996**; 38:1041–7.
7. Le Fichoux Y, Rousseau D, Ferrua B, et al. Short- and long-term eff cacy of hexadecylphosphocholine against established *Leishmania infantum* infection in BALB/c mice. Antimicrob Agents Chemother **1998**; 42:654–8.
8. Berman JJ. Treatment of leishmaniasis with miltefosine: 2008 status. Expert Opin Drug Metab Toxicol **2008**; 4:1209–16.
9. Thakur CP, Narayan S. A comparative evaluation of amphotericin B and sodium antimony gluconate, as first-lin drugs in the treatment of Indian visceral leishmaniasis. Ann Trop Med Parasitol **2004**; 98: 129–38.
10. Golenser J, Domb A. New formulations and derivatives of amphotericin B for treatment of leishmaniasis. Mini Rev Med Chem **2006**; 6: 153–62.
11. Kayser O, Olbrich C, Yardley V, Kiderlen AF, Croft SL. Formulation of amphotericin B as nanosuspension for oral administration. Int J Pharm **2003**; 254:73–5.
12. Delmas G, Park S, Chen ZW, et al. Efficac of orally delivered cochleates containing amphotericin B in a murine model of aspergillosis. Antimicrob Agents Chemother **2002**; 46:2704–7.
13. Zarif L, Graybill JR, Perlin D, Najvar L, Bocanegra R, Mannino RJ. Antifungal activity of amphotericin B cochleates against *Candida albicans* infection in a mouse model. Antimicrob Agents Chemother **2000**; 44:1463–9.
14. Escobar P, Yardley V, Croft S. Activities of hexadecylphosphocholine (miltefosine), AmBisome, and sodium stibogluconate (Pentostam) against *Leishmania donovani* in immunodeficien scid mice. Antimicrob Agents Chemother **2001**; 45: 1872–1875.
15. Sundar S, Jha TK, Thakur CP, et al. Oral miltefosine for Indian visceral leishmaniasis. N Engl J Med **2002**; 347:1739–46.