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Oral putrescine restores virulence of ornithine decarboxylase-deficient *Leishmania donovani* in mice

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Abstract

Administration of putrescine as a 1% solution in the drinking water ameliorated the profound loss of virulence exhibited by ornithine decarboxylase (ODC) deficient *Leishmania donovani* in mice. Furthermore, supplying α -difluoromethylornithine, an ODC inhibitor, at 2% in the drinking water reduced but did not eliminate infection with wild type *L. donovani* in the mouse model. Taken collectively, these findings: 1) demonstrate that oral putrescine can access the phagolysosome of macrophages in which the parasite resides in mice; 2) establish that the loss of virulence due to the *Δodc* lesion is a consequence of the inability of the mutant parasite to synthesize sufficient polyamines *de novo*; 3) imply that the *L. donovani* amastigote cannot access host polyamines in sufficient amounts for survival and growth; 4) and validate ODC as a drug target, although oral administration of DFMO is an unlikely therapeutic paradigm for visceral leishmaniasis.

Keywords

Leishmania donovani; polyamines; ornithine decarboxylase; putrescine; α -difluoromethylornithine

Leishmania donovani, the etiologic agent of visceral leishmaniasis, is a digenetic parasite that exists as the extracellular promastigote in its insect vector and as the intracellular amastigote within the phagolysosome of macrophages and other reticuloendothelial cells of the mammalian host. The current arsenal of drugs that is used to treat visceral and other forms of leishmaniasis is far from ideal. The mainstay drugs include pentavalent antimonials, pentamidine, amphotericin B, and miltefosine, and the chemotherapeutic regimens that employ these drugs are complicated by toxicity, often invasive routes of administration, and the emergence of drug refractoriness/resistance. Consequently, the need for new and more efficacious antileishmanial drugs is acute, and identifying and validating new targets for leishmaniasis is a critical component of this process.

One pathway that has stimulated considerable interest among scientists and clinicians alike for the treatment of parasitic diseases is that for the biosynthesis of polyamines, aliphatic cations that play vital roles in key cellular processes such as growth, differentiation, and macromolecular synthesis. The first intimation that inhibitors of polyamine biosynthesis

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could significantly impact the viability of protozoan parasites was the observation by Bacchi and coworkers that α -difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC) [1], could eradicate *Trypanosoma b. brucei* infections in mice [2]. This seminal contribution was followed by the demonstration that DFMO was an extremely effective agent (>95% cure) against early- and late-stage African sleeping sickness caused by *T. b. gambiense*, including many arsenical-refractory cases that would otherwise have proven fatal [3–4]. Subsequent studies by Wang, Coffino, and coworkers revealed that the selectivity of DFMO toward African trypanosomes could be ascribed to the extremely rapid rate of turnover of the mammalian ODC compared to the trypanosome counterpart, a lability that is conferred by a 39 amino acid COOH-terminal extension rich in PEST sequences [5].

DFMO is also effective against *Leishmania* promastigotes propagated axenically *in vitro* [6]. However, DFMO appears to be much less effective against the infectious amastigote form of the parasite. DFMO can reduce parasite loads in short-term experimental *Leishmania* infections in mice and hamsters but is not curative [6–8]. For instance, Keithly and Fairlamb reported that DFMO in the drinking water could partially suppress the parasitemia in affected organs of Balb/c mice infected with either *L. donovani* or *L. braziliensis* and that the antiparasitic effects of DFMO could be synergistic, additive, or have no effect when combined with other antileishmanial agents [6]. A later study by Gradoni *et al.* [8] showed that oral DFMO administration markedly suppressed *L. infantum* infections in Balb/c mice. Additionally, Mukhopadhyay and Madhubala [7] reported that a 2% DFMO solution provided to Syrian golden hamsters 2–6 days post-infection with *L. donovani* suppressed the number of parasites in liver and spleen by 90% and 99%, respectively. In none of these experimental infections did DFMO achieve elimination of the parasite, but it should be noted that the studies were preliminary and the time courses of drug administration limited. As a consequence, it has been generally viewed that ODC and the other enzymes of the polyamine biosynthetic pathway are not realistic drug targets for curing leishmaniasis, primarily due to the notion that the phagolysosome in which the parasite resides is replete with salvageable polyamines that can bypass a pharmacological block of ODC.

This assumption that ODC and the polyamine pathway are not valid drug targets has been challenged by the recent observation that a *Δodc* lesion, which renders the parasite auxotrophic for polyamines, profoundly impairs the ability of *L. donovani* to sustain an infection in Balb/c mice [9]. Parasite burdens in liver and spleen were reduced by six and two orders of magnitude, respectively, in mice inoculated with *Δodc* knockout lines compared to mice injected with wild type *L. donovani* [9]. Moreover, complementation of the *Δodc* with an ODC-containing plasmid rescued the virulence defect and restored polyamine prototrophy. The finding that an *Δodc* lesion profoundly compromises *L. donovani* virulence in a manner that can be circumvented by episomal complementation provides compelling genetic evidence that ODC is a potential therapeutic target for leishmanial infections. These results do not, however, prove that the damaging effect of an ODC lesion on *L. donovani* virulence is due to a consequence of a deleterious effect on polyamine biosynthesis. It is plausible that ODC may have an additional essential function in the amastigote that is unrelated to its acknowledged metabolic role and that the avirulence of the *Δodc* lines is due to the loss of that second function.

In order to authenticate that the mechanism of avirulence in the *Δodc* parasites is attributable to a loss of polyamine biosynthetic capacity in amastigotes, we evaluated whether dispensing putrescine as a 1% solution (w/v) in the drinking water could rescue the detrimental effects of a *Δodc* null mutation on parasite infection in mice. This approach mirrors that taken by Bacchi and coworkers when they demonstrated that polyamine administration could antagonize DFMO-elicited cures of *T. brucei* infections in mice [10]. Oral putrescine was chosen as a route of administration since the bioavailability of

polyamines in the gut is proficient [11], and 1% putrescine in the drinking water has been provided to mice for periods up to 10 weeks without side effects [12–13]. The four week course of oral 1% putrescine increased the parasite burden of livers of mice infected with *Δodc L. donovani* by almost three orders of magnitude, while the parasite loads in mouse spleens were augmented approximately 10-fold (Fig. 1). Putrescine had no effect on parasite numbers in mice infected with wild type *L. donovani* (data not shown). The substantial reversal of the virulence deficit of *Δodc* parasites by putrescine indicates that the orally administered diamine can access the phagolysosome of infected macrophages in liver and spleen. Furthermore, this partial virulence reversal provides a proof-of-concept that the loss of virulence in the null parasites is due to the inability of the mutant parasite to synthesize sufficient polyamines *de novo* and not to an alternative *ODC* function in the *L. donovani* amastigote. Furthermore, these data imply that *Δodc L. donovani* amastigotes fail to thrive because they cannot retrieve host polyamines in adequate quantities for survival and growth under physiological conditions. It should be noted, however, that infectivity rates of *Δodc* parasites were not restored to wild type levels by oral administration of putrescine (Fig. 1). The explanation for this, although uncertain, could be ascribed to limitations in putrescine absorption or pharmacokinetics that prevent unhindered access of the amastigote sequestered in the phagolysosome of macrophages in liver and spleen to the orally administered diamine.

To determine whether *L. donovani* *ODC* could be pharmacologically targeted by DFMO in an experimental murine infection using a regimen that more closely imitates the experimental paradigm used for the *Δodc* parasites ([9] and Fig. 1) than the previous short-term rodent studies with DFMO [6–8], Balb/c mice infected with wild type *L. donovani* were treated with a 2% DFMO solution in the drinking water for three weeks. DFMO is known to be well-absorbed (50% oral bioavailability) after oral administration, although the half-life in serum is short [14], and this DFMO concentration and route of administration is curative for *T. brucei* infections after three days [2,10] and dramatically reduces *L. donovani* parasitemias in hamster spleen and liver after four days [7]. Furthermore, it is known that DFMO is a particularly well-tolerated agent in experimental animals, and provision of DFMO as a 2% solution in the drinking water for up to 28 days shows no visible signs of toxicity in mice [15–16]. The effects of oral administration of DFMO on parasite burdens in mice infected with wild type parasites are shown in Fig. 2. Whereas the negative *Δodc* control parasites exhibited the expected dramatically reduced parasite loads in both liver and spleen, the three week exposure of mice inoculated with wild type *L. donovani* to DFMO only reduced parasite levels by an order of magnitude compared to untreated controls (Fig. 2) and had no effect on the splenic amastigote numbers. These data are similar to those reported previously for experimental DFMO treatments of *Leishmania* infections [6–8]. The discrepancies in the extents by which genetic ablation and pharmacologic inhibition of *ODC* affect *L. donovani* virulence can be ascribed to limited DFMO bioavailability or to the lack of DFMO access to the intracellular parasite and indicate that there is little rationale for exploiting DFMO as an antileishmanial drug. The data do not, however, negate *ODC* as a drug target for leishmaniasis and encourage the discovery of potent inhibitors with better therapeutic properties as a valid therapeutic paradigm.

Collectively, these data reveal that the avirulent phenotype of the *Δodc L. donovani* [9] can be ascribed to a loss of metabolic function rather than to an unknown complementary function for the *ODC* gene and *ODC* protein. The partial restoration of *Δodc* virulence by putrescine suggests that the orally supplied diamine can reach the phagolysosome of macrophages in liver and spleen, the milieu in which *L. donovani* resides. This further suggests that *L. donovani* amastigotes in the normal physiological setting cannot salvage adequate amounts of host polyamines to bypass a genetic or pharmacologic deficiency in *ODC* and that the *Δodc* avirulent phenotype cannot be ascribed to polyamine transport down

regulation in the amastigote as has been reported for the *L. major* polyamine transporter [17]. These results further validate ODC as a potential therapeutic target, although DFMO is clearly not a leading candidate drug, and reinforce efforts to discover additional ODC inhibitors. Finally, these findings provide a foundation for subsequent investigations to determine whether genetic lesions in other components of the polyamine pathway, e.g., ARG, ADO METDC, and SPDSYN, impact virulence of *L. donovani* and are, therefore, putative therapeutic candidates [18–19].

Abbreviations

DFMO	α -difluoromethylornithine
ODC	ornithine decarboxylase

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References

1. Metcalf BW, Bey P, Danzin C, Jung MJ, Casara P, Vevert JP. Catalytic irreversible inhibition of mammalian ornithine decarboxylase (E.C.4.1.1.17) by substrate and product analogues. *J Am Chem Soc* 1978;100:2551–2553.
2. Bacchi CJ, Nathan HC, Hutner SH, McCann PP, Sjoerdsma A. Polyamine metabolism: a potential therapeutic target in trypanosomes. *Science* 1980;210:332–334. [PubMed: 6775372]
3. Burri C, Brun R. Eflornithine for the treatment of human African trypanosomiasis. *Parasitol Res* 2003;90 Supp 1:S49–S52. [PubMed: 12811548]
4. Van Nieuwenhove S, Schechter PJ, Declercq J, Bone G, Burke J, Sjoerdsma A. Treatment of gambiense sleeping sickness in the Sudan with oral DFMO (DL- α -difluoromethylornithine), an inhibitor of ornithine decarboxylase; first field trial. *Trans R Soc Trop Med Hyg* 1985;79:692–698. [PubMed: 3938090]
5. Ghoda L, Phillips MA, Bass KE, Wang CC, Coffino P. Trypanosome ornithine decarboxylase is stable because it lacks sequences found in the carboxyl terminus of the mouse enzyme which target the latter for intracellular degradation. *J Biol Chem* 1990;265:11823–11826. [PubMed: 2365702]
6. Keithly, JS.; Fairlamb, AH. Inhibition of *Leishmania* species by α -difluoromethylornithine. In: Hart, DT., editor. *Leishmaniasis: The Current Status and New Strategies for Control*. New York: Plenum Press; 1987. p. 749–756.
7. Mukhopadhyay R, Madhubala R. Effect of a bis(benzyl)polyamine analogue, and DL- α -difluoromethylornithine on parasite suppression and cellular polyamine levels in golden hamster during *Leishmania donovani* infection. *Pharmacol Res* 1993;28:359–365. [PubMed: 8140036]
8. Gradoni L, Iorio MA, Gramiccia M, Orsini S. In vivo effect of eflornithine (DFMO) and some related compounds on *Leishmania infantum* preliminary communication. *Farmaco* 1989;44:1157–1166. [PubMed: 2517472]
9. Boitz JM, Yates PA, Kline C, Gaur U, Wilson ME, Ullman B, et al. *Leishmania donovani* ornithine decarboxylase is indispensable for parasite survival in the mammalian host. *Infect Immun* 2009;77:756–763. [PubMed: 19064633]
10. Nathan HC, Bacchi CJ, Hutner SH, Rescigno D, McCann PP, Sjoerdsma A. Antagonism by polyamines of the curative effects of α -difluoromethylornithine in *Trypanosoma brucei* infections. *Biochem Pharmacol* 1981;30:3010–3013. [PubMed: 6797437]
11. Milovic V. Polyamines in the gut lumen: bioavailability and biodistribution. *Eur J Gastroenterol Hepatol* 2001;13:1021–1025. [PubMed: 11564949]

12. Ignatenko NA, Besselsen DG, Roy UK, Stringer DE, Blohm-Mangone KA, Padilla-Torres JL, et al. Dietary putrescine reduces the intestinal anticarcinogenic activity of sulindac in a murine model of familial adenomatous polyposis. *Nutr Cancer* 2006;56:172–181. [PubMed: 17474863]
13. Waters WR, Reinhardt TA, Harp JA. Oral administration of putrescine inhibits *Cryptosporidium parvum* infection of neonatal C57BL-6 mice and is independent of nitric oxide synthesis. *J Parasitol* 1997;83:746–750. [PubMed: 9267420]
14. Haeghele KD, Alken RG, Grove J, Schechter PJ, Koch-Weser J. Kinetics of alpha-difluoromethylornithine: an irreversible inhibitor of ornithine decarboxylase. *Clin Pharmacol Ther* 1981;30:210–217. [PubMed: 6788425]
15. Herr HW, Kleinert EL, Conti PS, Burchenal JH, Whitmore WF Jr. Effects of alpha-difluoromethylornithine and methylglyoxal bis(guanyldrazone) on the growth of experimental renal adenocarcinoma in mice. *Cancer Res* 1984;44:4382–4385. [PubMed: 6432312]
16. Miyaji S, Katakura K, Matsufuji S, Murakami Y, Hayashi S, Oku Y, et al. Failure of treatment with alpha-difluoromethylornithine against secondary multilocular echinococcosis in mice. *Parasitol Res* 1993;79:75–76. [PubMed: 8469673]
17. Hasne MP, Ullman B. Identification and characterization of a polyamine permease from the protozoan parasite *Leishmania major*. *J Biol Chem* 2005;280:15188–15194. [PubMed: 15632173]
18. Roberts SC, Jiang Y, Jardim A, Carter NS, Heby O, Ullman B. Genetic analysis of spermidine synthase from *Leishmania donovani*. *Mol Biochem Parasitol* 2001;115:217–226. [PubMed: 11420108]
19. Roberts SC, Scott J, Gasteier JE, Jiang Y, Brooks B, Jardim A, et al. S-adenosylmethionine decarboxylase from *Leishmania donovani*. Molecular, genetic, and biochemical characterization of null mutants and overproducers. *J Biol Chem* 2002;277:5902–2909. [PubMed: 11734561]
20. Buffet PA, Sulahian A, Garin YJ, Nassar N, Derouin F. Culture microtitration: a sensitive method for quantifying *Leishmania infantum* in tissues of infected mice. *Antimicrob Agents Chemother* 1995;39:2167–2168. [PubMed: 8540741]

Fig. 1.

Putrescine partially reverses avirulent phenotype of Δodc *L. donovani*. Three cohorts of five 6–7 week old female Balb/c mice were inoculated with 5×10^6 stationary phase promastigotes by tail vein injection. One group was injected with wild type parasites and the other two with Δodc *L. donovani* knockout. One of the groups of mice injected with Δodc parasites was given a 1% putrescine solution to drink for 28 days while the remaining groups were provided with unadulterated drinking water. Mice were weighed and fluid intake measured at weekly intervals. No differences in body weights or fluid consumption were observed among the three cohorts, reflective of negligible toxicity triggered by the putrescine solution. Mice were sacrificed at four weeks and livers and spleens harvested and processed by standard surgical techniques. Single cell suspensions of mouse organs were prepared by passage through a 70 μ m cell strainer (BD Falcon), and parasitemias were then determined in 96-well microtiter plates using the standardized limiting dilution assay of Buffet *et al.* [20]. For this infectivity study, parasite strains were cycled back and forth between promastigotes and axenic amastigotes just prior to animal inoculation in order to ensure optimal infectivity. This experiment has been repeated one time with similar findings. Data presented are the average results and standard error from five animals.

Fig. 2.

Oral administration of DFMO reduces parasite burden in livers but not spleens of Balb/c mice infected with *L. donovani*. Two groups of five 6–7 week old female Balb/c mice were infected with 5×10^6 wild type promastigotes and one set of five mice was inoculated with an equivalent number of the *Δodc* mutant via tail-vein injection. Seven days post-infection, one of the two groups of mice infected with the wild type line was provided with a 2% (w/v) solution of DFMO in the drinking water for three additional weeks. The other group of mice injected with wild type parasites, as well as the group inoculated with the *Δodc* mutant were given water, for 28 days. Mice in each cohort were monitored weekly for weight and fluid intake with no significant differences observed between groups. At 28 days post-infection, mice were harvested as described in Fig. 1, the livers and spleens excised, and parasitemias determined by the limiting dilution method [20]. Data presented are the average results and standard error from five animals.