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Antileishmanial Effect of 3-Aminooxy-1-Aminopropane Is Due to Polyamine Depletion▽

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The polyamines putrescine, spermidine, and spermine are organic cations that are required for cell growth and differentiation. Ornithine decarboxylase (ODC), the first and rate-limiting enzyme in the polyamine biosynthetic pathway, catalyzes the conversion of ornithine to putrescine. As the polyamine biosynthetic pathway is essential for the growth and survival of *Leishmania donovani*, the causative agent of visceral leishmaniasis, inhibition of the pathway is an important leishmaniacidal strategy. In the present study, we examined for the first time the effects of 3-aminooxy-1-aminopropane (APA), an ODC inhibitor, on the growth of *L. donovani*. APA inhibited the growth of both promastigotes in vitro and amastigotes in the macrophage model, with the 50% inhibitory concentrations being 42 and 5 μM, respectively. However, concentrations of APA up to 200 μM did not affect the viability of macrophages. The effects of APA were completely abolished by the addition of putrescine or spermidine. APA induced a significant decrease in ODC activity and putrescine, spermidine, and trypanothione levels in *L. donovani* promastigotes. Parasites were transfected with an episomal ODC construct, and these ODC overexpressers exhibited significant resistance to APA and were concomitantly resistant to sodium antimony gluconate (Pentostam), indicating a role for ODC overexpression in antimonial drug resistance. Clinical isolates with sodium antimony gluconate resistance were also found to overexpress ODC and to have significant increases in putrescine and spermidine levels. However, no increase in trypanothione levels was observed. The ODC overexpression in these clinical isolates alleviated the antiproliferative effects of APA. Collectively, our results demonstrate that APA is a potent inhibitor of *L. donovani* growth and that its leishmaniacidal effect is due to inhibition of ODC.

Leishmaniasis, caused by a protozoan parasite, constitutes a wide spectrum of diseases ranging from the simple self-limiting cutaneous form to the debilitating visceral form, which is often fatal if it is left untreated. Although pentavalent antimonials (sodium antimony gluconate [SAG]) are the standard first-line treatments for visceral leishmaniasis (VL) (30), in more recent times, increasing resistance to SAG has emerged as a major barrier in the treatment of VL. There has been an epidemic of primary resistance to SAG in parts of India (30), and an urgent need exists to look for newer and more effective drugs.

The polyamine biosynthetic pathway is one such pathway that has been exploited successfully for the development of antiparasitic drugs (18). Polyamines are organic cations required for cell growth and differentiation (31). Ornithine decarboxylase (ODC), the first and rate-limiting enzyme in the polyamine biosynthetic pathway, catalyzes the conversion of ornithine to putrescine (21), and therefore, its inhibition offers a promising approach to antiparasitic therapy (18). α-Difluoromethylornithine (DFMO), the enzyme-activated, irreversible ODC inhibitor, is an effective trypanocidal drug for the treatment of patients with West African sleeping sickness (18), even those refractory to currently available antitrypanosomal drugs and those with central nervous system involvement (22). DFMO is also effective against other parasitic protozoa, e.g., *Plasmodium falciparum* and *Plasmodium berghei* (2). It has been demonstrated earlier that DFMO treatment results in lowering of the intracellular polyamine content and has been shown to be effective against promastigotes of the 1S strain of *Leishmania donovani* (12). In addition to DFMO, several inhibitors of ODC, like 3-aminooxy-1-aminopropane (APA), have been reported to be effective in blocking the proliferation of parasites and tumor cells (9, 13, 14, 16, 25, 29).

In the present study we show for the first time the inhibitory effect of APA, a competitive inhibitor of mammalian ODC, on parasite growth, ODC activity, and cellular polyamine and trypanothione [$T(SH)_2$] concentrations in *L. donovani*. Furthermore, the *L. donovani* ODC gene was used to generate ODC overexpressers, and these ODC overexpressers proved to be resistant to sodium antimony gluconate, pointing toward a role for ODC overexpression in antimonial drug resistance. To understand the role of ODC in antimonial drug resistance, we analyzed the polyamine synthesis pathway in field isolates collected from patients with visceral leishmaniasis who did not respond to SAG treatment. All the SAG-resistant isolates showed an increase in ODC activity concomitant with high levels of putrescine and spermidine. Furthermore, the ODC overproducers raised in the laboratory and clinical isolates

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resistant to sodium antimony gluconate were also resistant to APA, confirming that ODC is the primary intracellular target.

MATERIALS AND METHODS

Chemicals. Growth media and antibiotics were purchased from Sigma (St. Louis, MO), and fetal bovine serum (FBS) was purchased from Gibco/BRL (Life Technologies, Scotland, United Kingdom). All other chemicals were of analytical grade. Sodium antimony gluconate powder was obtained from Glaxo-Wellcome.

Parasite and culture conditions. Promastigotes of Indian *Leishmania donovani* clone GE1 (MHOM/IN/80/GE1F8R) (1), *Leishmania donovani* strain (MHOM/IN/80/AG83) (wild type), and three untyped clonal strains (strains S-1, R-1, and R-2) were isolated from patients with VL and were routinely cultured at 22°C in medium M-199 with Hanks' salts, including 25 mM HEPES buffer (Sigma) supplemented with 10% FBS and 100 μg/ml gentamicin (Sigma).

Clinical isolates sensitive to SAG included S-1 and AG83, whereas the three SAG-resistant isolates were GE1, R-1 and R-2. The SAG-resistant isolates were maintained in the absence of drug pressure in vitro. The isolates have been passaged through hamsters or BALB/c mice to retain their virulence; and importantly, their chemosensitivity profiles have remained unchanged, as measured periodically by the amastigote-macrophage infectivity assay described below.

Effect of drugs on cell growth. To estimate the 50% inhibitory concentrations (IC_{50}s) of the drugs, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) micromethod was used, as described previously (15). Briefly, late-log-phase promastigotes were seeded in a 96-well flat-bottom plate (200 μl per well; Nunc) in the presence or absence of drugs. To examine the effects of exogenous polyamines, the cultures were additionally supplemented with 1 mM putrescine or spermidine. After 72 h of incubation, MTT (10 mg/ml, 10 μl per well) was added to each well and the plates were incubated for an additional 4 h. The enzyme reaction was then stopped by addition of acidic isopropanol (0.4 ml 10 N HCl in 100 ml isopropanol, 100 μl per well), and the absorbances were measured at 570 nm. Two or more independent experiments were performed in triplicate for each drug.

DNA constructs and transfection. Construct pGEM7Zf-αHYGα-ODC, which contains the ODC gene of *L. donovani* cloned into a *Leishmania* expression vector (pGEM7Zf-αHYG-α) (6), and an episomal *Leishmania* expression vector (pGL-αNEOαLUC) containing luciferase-encoding DNA and neomycin phosphotransferase-selectable marker (26) were used in the present study. Clinical isolates were transfected with pGL-αNEOαLUC. Twenty micrograms of the construct was transfected into *L. donovani* promastigotes by electroporation in 2-mm-gap cuvettes at 450 V and 500 μF (BTX Electro Cell Manipulator 600). Transfectants were selected for resistance to either hygromycin B (100 μg/ml) or G418 (50 μg/ml), as described previously (20). *L. donovani* strain AG83 was used for overexpression of the *L. donovani* ODC gene by transfection. Transfectants overexpressing ODC were routinely maintained in α-minimum essential medium supplemented with 50 μg/ml hygromycin B. FBS was excluded to avoid polyamine oxidase-mediated toxicity (12).

Effects of drugs on amastigote-macrophage model. Stationary-phase *Leishmania* promastigotes expressing the luciferase gene (pGL-αNEOαLUC) were used to infect J774A.1 macrophages. Macrophage cell line J774A.1 (American Type Culture Collection) was maintained at 37°C in RPMI 1640 medium (Sigma) containing 10% FBS, as described previously (11). Briefly, J774A.1 murine macrophages (1×10^5 cells/250 μl/per well) were infected with 1×10^6 promastigotes in medium M-199 with 10% FBS (26). After 3 h, the noninternalized parasites were washed off and drug was added at different concentrations. After 5 days of drug exposure, plates containing adherent macrophages were washed and luciferase activity was determined (26). The IC_{50} was determined from a graph representing different concentrations of drug plotted against the number of relative light units produced by luciferase-expressing parasites.

Enzyme assay. *L. donovani* promastigotes (1×10^7) in late log phase were treated with APA, and the cells were harvested 48 h later by centrifugation at $2,000 \times g$ for 15 min at 4°C. The cell pellet was washed with phosphate-buffered saline (pH 7.4) and resuspended in 50 mM Tris-HCl (pH 7.5), 10 μM EDTA, and 2.5 mM dithiothreitol (DTT). The cells were lysed by freezing-thawing in liquid nitrogen. The lysate was centrifuged at $15,000 \times g$ (20 min, 4°C), and the supernatant was assayed for ODC activity and polyamine and protein concentrations. Protein concentrations were determined by the method of Bradford (3), with bovine serum albumin used as the standard. ODC activity was assayed by monitoring the release of $^{14}CO_2$ from [L-^{14}C]ornithine (24, 27). The standard assay mixture, which contained the supernatant, 200 μM pyridoxal phosphate, 12.5 mM DTT, 250 mM Tris (pH 7.5), 2 mM ornithine, and 3 μCi of the radiolabeled ornithine, was incubated at 37°C for 1 h. The reaction was terminated by injecting 5 N H_2SO_4; and the activity was expressed in enzyme units, in which 1 unit is the number of nmol of CO_2/mg protein/h.

TABLE 1. Effects of APA and DFMO on promastigotes and amastigotes of *Leishmania donovani* and macrophage cell line J774A.1

Inhibitor	IC_{50} (μM)[a]		
	Promastigote	Amastigote	Macrophage cell line (J774A.1)
APA	42 ± 8	5 ± 2.1	>200
DFMO	>10,000	50 ± 3.2	ND

[a] The results are the means ± SDs of three independent experiments for all data sets. IC_{50}s for promastigotes were determined after 72 h of drug addition. IC_{50}s for intracellular amastigotes were determined after 5 days of drug addition. IC_{50}s for macrophage cell line J774A.1 were determined after 24 h of drug addition. ND, not determined.

Western blot analysis. Late-log-phase promastigotes (1×10^8) were harvested, and the resultant cell pellet was resuspended in lysis buffer (morpholinepropanesulfonic acid [20 mM, pH 7.2], DTT [1 mM], phenylmethylsulfonyl fluoride [2 mM], and leupeptin and aprotinin [0.5 μg ml^{-1} each]). Promastigotes (wild type and ODC overexpressers) were sonicated in the lysis buffer mentioned above, and cell supernatants were prepared by centrifugation at $20,000 \times g$. Aliquots containing 50 μg of protein were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane with an electrophoretic transfer cell (Bio-Rad). Proteins were detected by the enhanced chemiluminescence method (Amersham Pharmacia Biotech). A polyclonal antibody (1:100 dilution) against purified *L. donovani* ODC, which was generated in rabbits (kindly provided by Buddy Ullman, Portland, OR), was used. Autoradiograms of the proteins were analyzed by using a model FLA 5000 imaging densitometer (Fuji, Japan). The results shown are representative of at least three independent experiments.

Polyamine analysis. *L. donovani* promastigotes (1×10^7) were harvested at 48 h of growth by centrifugation at $2,000 \times g$ (15 min, 4°C). Quantitative determination of the polyamines in acid extracts of *L. donovani* was performed by C_{18} reversed-phase high-performance liquid chromatography after precolumn derivatization with dansyl chloride (28).

Thiol analysis. Thiols were derivatized with monobromobimane and were separated by high-performance liquid chromatography, as described elsewhere (17).

Northern blot analysis. Total RNA was isolated from wild-type *L. donovani* promastigotes (strain AG83, 2×10^8) and from ODC overexpressers by using the TRI reagent (Sigma). For Northern blot analysis, 15 μg of total RNA was fractionated by denaturing agarose gel electrophoresis and transferred onto a nylon membrane by standard procedures. Following the transfer of the RNA onto nylon membranes, the nucleic acids were UV cross-linked to the membrane in a Stratagene UV cross-linker. Prehybridization was done at 65°C for 4 h in a buffer containing 0.5 M sodium phosphate, 7% sodium dodecyl sulfate, 1 mM EDTA (pH 8.0), and 100 μg/ml sheared and denatured salmon sperm DNA. The blots were hybridized with a denatured [α-^{32}P]dCTP-labeled DNA probe (the PCR probe comprised the *L. donovani* ODC-coding region) at 10^6 cpm/ml, which was labeled by random priming (NEBlot kit; New England BioLabs). The membranes were washed and air dried, visualized with a Fujifilm FLA-5000 imaging system, and quantified with ImageQuant software.

Statistical analysis. Data were statistically analyzed by the Student's *t* test. The data represent mean ± standard deviations (SDs) of at least three determinations from two independent experiments. A P value of <0.05 was considered significant.

RESULTS

Effect of APA on growth of promastigotes and amastigotes. *L. donovani* promastigotes were cultured in the presence of increasing concentrations of APA. APA inhibited the growth of promastigotes in a dose-dependent manner; the effective concentration that caused 50% inhibition of growth (IC_{50}) after 72 h of drug addition was 42 μM (Table 1). The sensitivities of the amastigotes were tested in an intracellular amas-

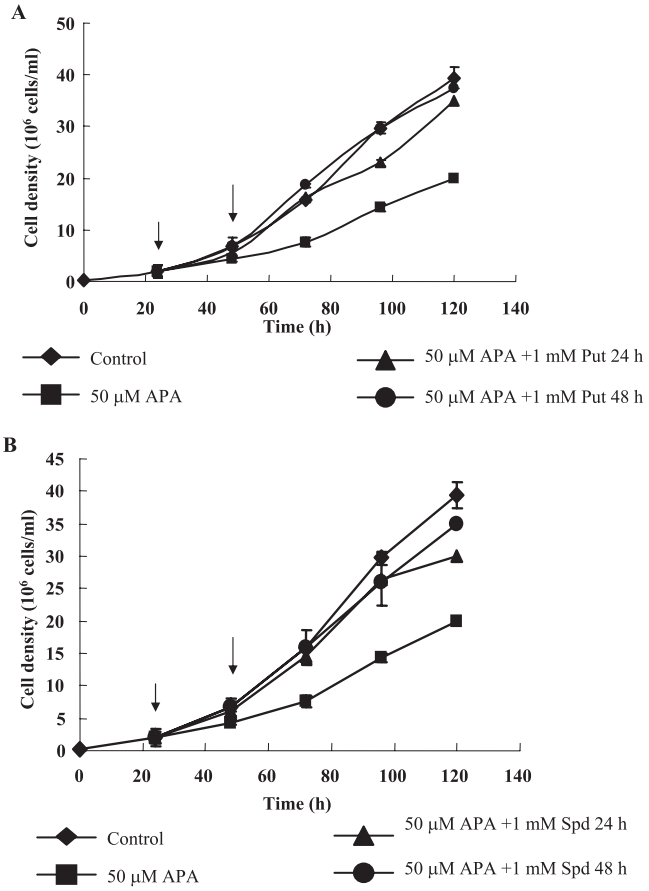

FIG. 1. Reversal of APA-mediated promastigote growth inhibition by exogenous polyamines. The *L. donovani* culture medium containing an inhibitory concentration of APA was supplemented with 1 mM putrescine (A) or 1 mM spermidine (B). Control cells were cultured in the absence of APA. The growth of *L. donovani* was monitored over 120 h in the presence of 50 μM APA. Putrescine at 1 mM (A) or spermidine at 1 mM (B) was added after APA treatment for 24 h or 48 h. Parasites were enumerated every 24 h by counting with a hemocytometer. The arrows indicate the times of addition of putrescine (A) and spermidine (B). Each datum point represents the mean ± SD of three determinations. If the SD was <5%, the means are not shown.

tigote-macrophage model. The *Leishmania* expression vector (pGL-αNEOαLUC) containing the luciferase gene was transfected into the parasite, and the resulting transfectants were used for susceptibility assays, as described in Materials and Methods. The IC_{50} of APA for amastigotes after 5 days of drug treatment was 5 μM (Table 1). At these concentrations, APA did not affect the viability of the macrophage cell line J774A.1, with the IC_{50} being >200 μM after 24 h of drug addition. The *L. donovani* promastigotes and amastigotes exhibited higher sensitivities to APA (at least 4.7- and 40-fold, respectively) than the macrophage cell line J774A.1 did (Table 1). DFMO inhibited amastigote growth in the macrophage model with an IC_{50} of 50 μM, but concentrations of DFMO as high as 10 mM did not have any inhibitory effect on the promastigotes (Table 1). It is evident from Fig. 1A and B that addition of putrescine (1 mM) or spermidine (1 mM) after 24 h or 48 h of drug treatment resulted in reversal of the growth inhibition caused by APA (50 μM) in promastigotes.

Effects of APA on ODC activity and polyamine and trypanothione levels in promastigotes. APA inhibited the ODC activities of the *L. donovani* promastigotes (Fig. 2A). Treatment with 50 μM APA for 48 h inhibited ODC activity by ~76%. Treatment with APA (50 μM) for 48 h also resulted in the concomitant reduction of putrescine and spermidine levels, with 50 μM APA resulting in an ~70% reduction of putrescine levels (Fig. 2B) and a 44% reduction of spermidine levels compared to those for the untreated cells (Fig. 2B). APA (50 μM) inhibited trypanothione levels by ~79% (Fig. 2C).

Characterization of *L. donovani* transfectants overexpressing ODC. Promastigotes from the *L. donovani* AG83 strain overexpressing ODC were isolated after transfection of an episomal ODC gene construct, as described in Materials and Methods. The growth patterns of the wild-type and ODC-overexpressing cells were very similar, with population doubling times of ~32 h and ~29 h, respectively (results not shown). Northern blot analysis of total *L. donovani* RNA prepared from promastigotes of the wild type and ODC overexpressers revealed the presence of the 4.8-kb and 6.5-kb ODC transcripts in both cell lines (Fig. 3A). The presence of two ODC transcripts in *L. donovani* species has been reported earlier (8).

However, the 4.8-kb transcript was more abundant in the ODC overexpressers than in the wild-type cells (~6.2-fold). Interestingly, no major difference in the expression of the 6.5-kb transcript between the ODC-overexpressing and wild-type cells was observed (Fig. 3A). Western blot analysis showed that in the ODC-overexpressing cells there was an increase in the level of ODC protein (~4.4-fold) compared to that in wild-type promastigotes, which corresponded to a similar difference in ODC activity (~3.8-fold) (Fig. 3B and C). Although overexpression of ODC in the transfectants was observed both by Northern analysis and by Western blot analysis, the increase in the level of ODC mRNA expression was higher than the increase in the level of the ODC protein. This discrepancy may be due to posttranscriptional regulation, which seems to be the mechanism of choice for gene expression in *Leishmania* and other lower organisms (4).

The increased activity of ODC in the promastigotes of the overexpressers resulted in a twofold increase in the putrescine content compared to that in wild-type cells (Table 2), but no corresponding difference in the spermidine content was observed between the two cell lines. The absence of spermine in both the wild type and the ODC overexpressers is consistent with the previous observation that *L. donovani* lacks spermine synthase (12). We also quantified the $T(SH)_2$ levels in the wild type and the ODC overexpressers (Table 2). Surprisingly, there were no significant differences in the $T(SH)_2$ levels between the wild type and the ODC overexpressers.

A link between polyamine metabolism and antimony drug resistance in leishmaniasis has been suggested (17, 32). To evaluate whether the level of expression of ODC in *L. donovani* affected sensitivity to the antimonial drug sodium antimony gluconate, the effect of this drug was determined in the wild-type and the ODC-overexpressing cell lines. As shown in Table 3, the ODC overexpressers exhibited significant resistance to sodium antimony gluconate compared to the resistance of wild-type cells for both promastigotes and amastigotes. The concentrations of sodium antimony gluconate that

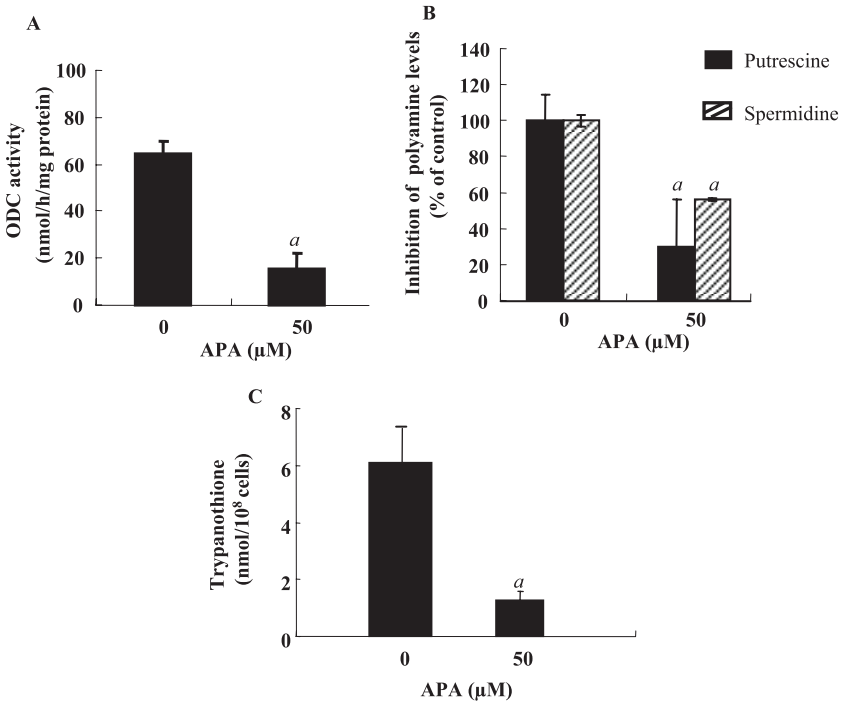

FIG. 2. Effects of APA on ODC activity, polyamine pattern, and trypanothione levels of *L. donovani* promastigotes. (A) ODC activity, (B) polyamine levels, and (C) trypanothione levels were determined as described in Materials and Methods. APA (50 μM) was added to the log-phase promastigotes, and cells were harvested 48 h later, as described in the Materials and Methods. Results are mean ± SDs ($n = 3$). *a*, the values are significantly different from those for the controls, to which APA was not added ($P < 0.05$).

inhibited growth of the wild-type promastiogtes and amastigotes by 50% were 57 μM and 15 μM, respectively, whereas the IC_{50}s for promastiogtes and amastigotes of the ODC overexpressers were 190 μM and >76 μM, respectively, indicating that overexpression of ODC can confer resistance to antimonials in both forms of the parasite. Similarly, the competitive ODC inhibitor APA effectively inhibited growth, with the IC_{50}s being 42 μM and 5 μM for wild-type promastigotes and amastiogotes, respectively (Table 3), and 155 μM and >100 μM for ODC-overexpressing promastigotes and amastigotes, respectively (Table 3). In comparison, no difference in growth inhibition was observed between the two cell lines when the antitrypanosomal diamidine compound berenil was added to the growth medium (IC_{50}s, ~40 μM for both cell lines [results not shown]).

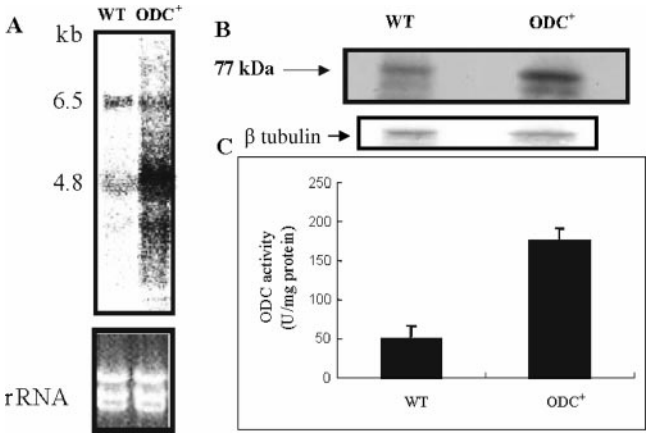

FIG. 3. Analysis of ODC overexpression in promastiogtes of AG83 *L. donovani* derived by transfection with an episomal ODC gene construct. (A) Northern blot analysis of the ODC transcripts in wild-type (WT) and ODC-overexpressing (ODC^+) cells in log-phase growth (48 h) (top). Ethidium bromide-stained rRNA served as a loading control (bottom). (B) The ODC protein contents of wild-type and ODC-overexpressing cells in log-phase growth were determined by Western blot analysis. The blot was probed with antibody against β-tubulin to normalize the loading onto each lane. (C) ODC activity was measured radiometrically in wild-type and ODC-overexpressing cells in logarithmic growth. Data are means ± SDs ($n = 3$).

Characterization of ODC and polyamine and trypanothione levels in promastigotes of clinical isolates. The sensitivities of various clinical isolates to sodium antimony gluconate was tested both in promastigotes and in intracellular amastigotes. The IC_{50}s of promastigotes of the wild-type strain and strain

TABLE 2. Polyamine and trypanothione levels in wild-type and ODC-overexpressing *L. donovani* promastigotes

Strain	Putrescine level[a] (nmol/mg protein)	Spermidine level[a] (nmol/mg protein)	Trypanothione level[b] (nmol/10^8 cells)
Wild type	14.3 ± 2.3	39.6 ± 17.0	6.1 ± 1.2
ODC overexpresser	27.5 ± 4.3	42.0 ± 6.5[c]	5.9 ± 0.5[c]

[a] Polyamines were measured in acid extracts of promastigotes (strain AG83) harvested at 48 h of growth. Data are means ± SDs ($n = 3$).

[b] Intracellular levels of trypanothione were measured by derivatization with monobromobimane and separation by high-performance liquid chromatography. Data are means ± SDs ($n = 3$).

[c] Not significantly different from the results for the wild type.

TABLE 3. IC_{50}s for wild-type parasites and ODC-overproducing parasites with sodium antimony gluconate and APA

Parasite form	IC_{50} (μM)[a]			
	Wild type		ODC overexpressers	
	Pentostam	APA	Pentostam	APA
Promastigotes	57 ± 0	42 ± 8	190 ± 0	155 ± 16
Amastigotes	15 ± 6	5 ± 2	>76	>100

[a] The effects of sodium antimony gluconate and APA on the growth of promastigotes and amastigotes of wild-type and ODC-overexpressing *L. donovani* strains are indicated as IC_{50}s, which were determined as described in Materials and Methods. The results are means ± SDs (n = 3).

S-1, which is a sensitive strain from a SAG-responsive patient, were 57 ± 0 and 47 ± 5.6 μM, respectively (Table 4), whereas the IC_{50}s of promastigotes from SAG-unresponsive patients were ~1.4- to 3.4-fold higher than that for sensitive strain S-1 and ranged from 68 to 164 μM (Table 4). The IC_{50}s of sodium antimony gluconate for amastigotes of the wild type and strain S-1 were 15 ± 6.0 and 13 ± 1.5 μM, respectively (Table 4), whereas field isolates R-1, R-2, and GE1, which came from SAG-unresponsive patients, had IC_{50}s of 62 ± 3.4, 23 ± 1.4, and >95 μM, respectively (Table 4). ODC activity in the SAG-resistant promastigotes (strains R-1 and GE1) was ~2.0-fold higher than that in SAG-sensitive promastigotes of strain S-1 (Table 5). However, strain R-2 had fivefold higher ODC activities than SAG-sensitive strain S-1 (Table 5). The levels of spermidine and its precursor, putrescine, were also measured in the promastigotes of clinical isolates (Table 5). In SAG-resistant strains R-1, R-2, and GE1, putrescine levels were 1.8, 3.4-, and 2.9-fold higher, respectively, than the level in SAG-sensitive strain S-1. The levels of spermidine were ~1.6- to 1.9-fold higher in all the SAG-resistant isolates than in the SAG-sensitive strain. However, there were no significant differences in the $T(SH)_2$ levels between the SAG-sensitive and SAG-resistant isolates (Table 5).

The competitive ODC inhibitor APA was shown to effectively inhibit the growth of amastigotes, with an IC_{50} of about 10 μM for the SAG-sensitive amastigotes of strain S-1, whereas concentrations of APA as high as 200 μM did not inhibit SAG-resistant amastigotes of strains R-1, R-2, and GE1 (Table 4). Thus, ODC overexpression in these clinically resistant isolates alleviated the antiproliferative effects of APA.

DISCUSSION

The polyamine biosynthetic pathway is a potential target for the development of new drugs with activities against parasitic protozoa (18). The polyamines are essential for cell proliferation and differentiation, and interference with their biosynthesis is an established strategy for the treatment of West African trypanosomiasis caused by *Trypanosoma brucei gambiense* (18, 22). Recent results obtained with null mutants of *L. donovani* in which genes in the polyamine biosynthetic pathway have been silenced have clearly demonstrated that this pathway is also a promising target for drugs with activities against the protozoa that cause leishmaniasis (10, 23).

In the present study we examined the effect of APA, an ODC inhibitor, on *L. donovani* growth. APA is a structural analog of putrescine, and the aminooxy group in this compound forms an oxime with the pyridoxal phosphate cofactor in the active site of ODC. APA inhibited the growth of both promastigotes in vitro and amastigotes in the macrophage model, with the 50% inhibitory concentrations being 42 and 5 μM, respectively. All drugs with activities against parasitic organisms that cause disease should exhibit selective activity against the pathogen and not the host; and in this regard, APA at concentrations up to 200 μM had no effect on macrophages, thus establishing the usefulness of *L. donovani* ODC as a target for the treatment of leishmaniasis. Earlier data on the effect of APA on human T24 bladder carcinoma cells (IC_{50}, ~24.4 μM) (29) further confirm that *Leishmania* amastigotes exhibit higher sensitivities to APA than the mammalian cells. The antileishmanial effect of APA is mediated by the decrease in ODC activity and putrescine and spermidine levels, as corroborated by the abolition of antileishmanial activity following the addition of putrescine or spermidine. It is likely that the effect of putrescine is due to its conversion to spermidine, as has been reported previously (25). In those studies, APA (50 μM) was found to significantly inhibit trypanothione levels in the wild-type cells.

Surprisingly, in this study, addition of an irreversible inhibitor of ODC, DFMO, to promastigotes even up to a concentration of 10 mM showed no effect on growth. In an earlier study by Kaur et al. (12), DFMO was reported to cause polyamine depletion and growth inhibition of *L. donovani* promastigotes. Interestingly, in the present study, DFMO inhibited

TABLE 4. Comparative analysis of the susceptibilities of the sodium antimony gluconate-sensitive and -resistant clinical isolates to sodium antimony gluconate and APA[a]

Isolate	IC_{50} (μM)			
	Promastigotes		Intracellular amastigotes	
	Sodium antimony gluconate	APA	Sodium antimony gluconate	APA
Wild type	57 ± 0	42 ± 8	15.0 ± 6.0	5 ± 2.0
S-1	47 ± 5.6 (1)	19 ± 0.7	13.0 ± 1.5 (1)	10 ± 2.5
R-1	128 ± 3.3 (2.7)*	>400	62.0 ± 3.4 (5)*	>200
R-2	68 ± 4.2 (1.4)*	46 ± 2.8	23.0 ± 1.4 (1.8)*	>200
GE1	164 ± 5.1 (3.4)*	>400	>95.0 (>7.6)*	>200

[a] Stationary-phase *L. donovani* isolates transfected with pGL-αNEOαLUC were used to infect J774A.1 macrophages, as described in Materials and Methods. Luciferase activity was determined after 5 days of drug exposure. IC_{50}s were determined from the graph representing the concentration of drug plotted against the number of relative light units produced by luciferase-expressing parasites. IC_{50}s are given as the means ± SDs of at least three independent determinations. The fold increase in the IC_{50} compared to that of strain S-1 is given in parentheses. *, The resistance levels observed in the SAG-resistant isolates are statistically different from that in SAG-sensitive strain S-1 ($P < 0.002$).

TABLE 5. Quantification of intracellular polyamine and trypanothione levels in promastigotes of sodium antimony gluconate-sensitive and -resistant isolates[a]

Strain	Putrescine level (nmol/h/mg protein)	Spermidine level (nmol/h/mg protein)	Trypanothione level (nmol/10^8 cells)	ODC activity (nmol/mg of protein)
S-1	17.7 ± 1.8 (1)	12.39 ± 4.2 (1)	7.8 ± 1.1	28.2 ± 3.4 (1)
R-1	33.1 ± 5.8* (1.8)	19.97 ± 3.5* (1.6)	6.6 ± 0.4[b]	58.7 ± 4.5** (2.0)
R-2	59.7 ± 3.0** (3.4)	23.85 ± 6.5*(1.9)	4.7 ± 0.5[b]	144.3 ± 2.8*** (5.1)
GE1	52.67 ± 4.78*** (2.9)	22.75 ± 2.1*(1.8)	5.7 ± 0.6[b]	62.6 ± 5.5** (2.2)

[a] Each value is the mean ± SD of at least four determinations for putrescine and spermidine levels and three determinations for ODC activity from two independent experiments. For trypanothione levels, each value is the mean ± SD of triplicate experiments. Concentrations were determined by using the values from known amounts of standards. The fold increase compared to the level in strain S-1 is given in parentheses. *, $P < 0.05$ compared to the corresponding values obtained for strain S-1; **, $P < 0.05$ compared to the corresponding values obtained for strain S-1; ***, $P < 0.001$ compared to the corresponding values obtained for strain S-1.
[b] Not significantly different from the value for strain S-1.

amastigote growth in the macrophage model, with an IC_{50} of 50 μM. Differences in sensitivities to DFMO and also to APA between promastigotes and amastigotes is interesting and may be due to differences in the uptake of the inhibitors. Amastigotes of the *L. donovani* strain used in this study were shown to be highly sensitive to the ODC inhibitors DFMO and APA, which inhibited growth in the micromolar range. However, APA was 10-fold more effective than DFMO against the amastigote stage.

Previous results have shown that resistance to antimonial compounds in *Leishmania* is multifactorial, with contributions made by several independent mechanisms (6). A link between polyamines and antimonial resistance has been suggested (19). In trypanosomatids, like *Leishmania*, glutathione is replaced by trypanothione, which is a conjugate between the polyamine spermidine and glutathione. A correlation between resistance to antimonial drugs and increased trypanothione levels appears to exist in *Leishmania* (17, 32). Thus, increased resistance to antimonial drugs was found in cells that overproduce trypanothione as a result of the increased expression of ODC (7) or γ-glutamylcysteine synthetase (6), the rate-limiting enzyme of glutathione biosynthesis. A role of ODC in the development of antimonial resistance is supported by our findings that parasites overexpressing ODC after transfection of the gene became more resistant to sodium antimony gluconate than the wild-type cells. The ODC overexpressers expressed high levels of ODC mRNA and an approximately fourfold increase in ODC activity over that of the wild type. However, there was only a twofold increase in putrescine levels and no significant change in spermidine levels in the ODC overexpressers. No difference in trypanothione levels was observed between the wild type and the ODC overexpressers. It is possible that this discrepancy could be due to the need to maintain polyamines and trypanothione at constant intracellular levels. These ODC overexpressers proved to be resistant to sodium antimony gluconate, indicating a role of ODC overexpression in drug resistance to an antimonial compound. ODC overexpressers exhibited significant resistance to APA. The wild-type cells were more sensitive to APA than the ODC overexpressers, indicating that the target was indeed ODC.

The resistance of clinical isolates of *Leishmania donovani* to sodium antimony gluconate, the mainstay for the treatment of Indian visceral leishmaniasis, has become a critical issue in India. To understand the role of ODC and elevated levels of polyamines in antimonial resistance, we looked into the polyamine synthesis pathway in the clinical isolates. ODC activities were high in all three resistant isolates (strains R-1, R-2, and GE1); and concomitantly, high levels of spermidine and putrescine were also present, indicating that the resistance to sodium antimony gluconate in the clinical isolates was associated with an increase in ODC activity and significant increases in putrescine and spermidine levels. However, no strict correlation between the degree of sodium antimony gluconate resistance, as observed from the IC_{50}s (Table 4), and the corresponding levels of ODC activity in the clinical isolates was observed (Table 5). Thus, it is possible that ODC activity may explain the resistance to sodium antimony gluconate only in part, and several other factors may be responsible for the mechanism of resistance to sodium antimony gluconate. Surprisingly, no difference in the trypanothione levels was observed between the sensitive and the resistant clinical isolates. This was also the case in the ODC overexpressers reported in this study. Earlier reports also showed no change in trypanothione levels in *Leishmania infantum* isolates resistant to antimony [Sb(III)] (5). This is possibly because the levels of $T(SH)_2$ in the *Leishmania tarentolae* strain studied are much lower than those in other strains. It is also possible that the discrepancy observed in the present study could be due to the need to maintain trypanothione at a constant intracellular level. It has been reported earlier that Sb(V) depletes $T(SH)_2$ by efflux of the Sb-trypanothione conjugate (5, 6, 17). This efflux system is possibly enhanced in the resistant field strain, hence leading to increased trypanothione efflux and thus explaining the relatively constant level of trypanothione, despite the increase in ODC activity and polyamine levels.

Our results demonstrate a link between ODC overexpression and antimonial resistance. We have also demonstrated that APA is a potent inhibitor of *L. donovani* and that its leishmaniacidal effect is due to inhibition of ODC. The inhibitory effect of APA on ODC activity correlates well with its effect on parasite growth and depletion of putrescine and spermidine levels. Furthermore, the sodium antimony gluconate-resistant clinical isolates exhibited significant resistance to APA. However, APA exhibited selective activity against the pathogen and not against the host, establishing the usefulness of *L. donovani* ODC as a target for the treatment of leishmaniasis.

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