Biochemical and Biophysical Research Communications 375 (2008) 168–172

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Novel agmatine analogue, γ-guanidinooxypropylamine (GAPA) efficiently inhibits proliferation of *Leishmania donovani* by depletion of intracellular polyamine levels

Sushma Singh [a], Anupam Jhingran [a], Ankur Sharma [a], Alina R. Simonian [b], Pasi Soininen [c], Jouko Vepsalainen [c], Alex R. Khomutov [b], Rentala Madhubala [a,*]

[a] School of Life Sciences, Jawaharlal Nehru University, New Mehrauli Road, New Delhi 110067, India
[b] Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow 119991, Russia
[c] Department of Chemistry, University of Kuopio, P.O. Box 1627, FI-70211 Kuopio, Finland

ARTICLE INFO

Article history:
Received 29 July 2008
Available online 8 August 2008

Keywords:
γ-Guanidinooxypropylamine (GAPA)
Agmatine analogue
Leishmania donovani
ODC-overexpressors
Ornithine decarboxylase
Polyamines
Putrescine transport

ABSTRACT

The efficacy of γ-guanidinooxypropylamine (GAPA), a novel agmatine analogue against protozoan parasite, *Leishmania donovani* was evaluated. Wild-type and ornithine decarboxylase-overexpressors of *L. donovani* were used to study the effect and mode of action of this inhibitor. GAPA inhibited the growth of both promastigotes and amastigotes. Ornithine decarboxylase (ODC) activity and polyamine levels were markedly lower in cells treated with GAPA and proliferation was rescued by addition of putrescine or spermidine. GAPA inhibited *L. donovani* recombinant ODC with K_i value of ~60 μM. The ODC-overexpressors showed significant resistance to GAPA. GAPA has pK_a 6.71 and at physiological pH the analogue can mimic protonated state of putrescine and can probably use putrescine transport system. Transport of putrescine in wild-type *L. donovani* promastigotes was inhibited by GAPA. We for the first time report that GAPA is a potential antileishmanial lead compound and it possibly inhibits *L. donovani* growth by depletion of intracellular polyamine levels.

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Leishmania are protozoan parasites that result in a spectrum of diseases, ranging from benign cutaneous lesions through metastasizing mucocutaneous forms to the often fatal visceralizing form [1]. Pentavalent antimonials are the standard first line choice against the disease [2]. There has been an epidemic of primary resistance to antimonials in parts of India [2]. Hence, there is an urgent need to look for more effective drugs and also to identify novel molecular targets on which to base future treatment strategies.

Polyamines are ubiquitous organic cations that play a critical role in key cellular processes [3]. The cellular content of polyamines is regulated by biosynthesis, degradation and transport [4]. Since polyamines are especially important to rapidly growing cells, the polyamine pathway has been targeted in a multiplicity of antineoplastic and antiparasitic drug regimens. Most notably, α-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC) has been reported to be effectively curative against the late stage African sleeping sickness caused by the protozoan parasite *Trypanosoma brucei gambiense* [5,6].

We for the first time report the inhibitory effect of γ-guanidinooxypropylamine (GAPA), an agmatine analogue on *Leishmania donovani*. Agmatine [(4-aminobutyl) guanidine], an arginine metabolite has been postulated to suppress cell proliferation by affecting polyamine metabolism [7]. It is actively transported into rat hepatocytes by putrescine transporter and affects polyamine homeostasis [8]. Agmatine is formed by decarboxylation of arginine by arginine decarboxylase [9]. An important distinct feature of *Escherichia coli* and plants is that they can synthesize putrescine from arginine via arginine decarboxylase–agmatine ureohydrolase pathway [10]. A low amount of agmatine has been reported in eukaryotes [11]. There is no report of arginine decarboxylase presence in *L. donovani* and ornithine decarboxylase is reported to be the sole enzyme that initiates polyamine biosynthesis [12].

In this study, we for the first time report that γ-guanidinooxypropylamine (GAPA) inhibits *L. donovani* growth by depletion of polyamines. ODC-overexpressing *L. donovani* were used to study the effect and mode of action of this inhibitor.

Materials and methods

Chemicals. Growth media and antibiotics were purchased from Sigma (St. Louis, MO) and fetal bovine serum (FBS) from Gibco/BRL (Life Technologies Scotland, United Kingdom). GAPA was synthesised following earlier published method [13]. 1,4-[^{14}C]-putres-

* Corresponding author. Fax: +91 11 26742630.
E-mail address: madhubala@mail.jnu.ac.in (R. Madhubala).

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doi:10.1016/j.bbrc.2008.07.143

cine dihydrochloride (specific activity 110 mCi/mmol) was obtained from American Radiolabelled Chemicals Inc. (St. Louis, MO).

Parasite and culture condition. Promastigotes of Indian *L. donovani* strain MHOM/IN/80/AG83 were routinely cultured at 22 °C in M199 medium with Hanks' salts including 25 mM HEPES buffer (Sigma, USA) supplemented with 10% heat inactivated fetal bovine serum and 0.13 mg/mL penicillin and streptomycin [14]. For drug studies and polyamine estimation, cells were grown in α-Minimum Essential Medium (αMEM) minus FBS in order to avoid polyamine oxidase-mediated toxicity.

Drug susceptibility assay. The effect of GAPA on the growth of the promastigotes was determined in microtiter plates, each containing 96 wells. Briefly, 1×10^6 parasites in 0.2 ml of α-Minimum Essential Medium minus FBS were placed in each well and incubated with various concentrations of the drug. After 72 h of incubation, cell densities were determined by the Neubeur heamocytometer. The concentrations of GAPA which inhibited the growth of the cells by 50% were determined. Two or more independent experiments in triplicate were performed for the determination of sensitivity to each drug.

DNA constructs and transfection. Leishmania donovani strain AG83 was used for overexpression of the *L. donovani* ODC gene by transfection with an episomal *Leishmania* expression vector (pGL-αNEOαLUC) containing luciferase encoding DNA and neomycin phosphotransferase selectable marker [15]. The construction of the ODC overexpressing strain (ODC+) has been described previously [16]. Transfectants overexpressing ODC were routinely maintained in α-Minimum Essential Medium (αMEM) supplemented with 50 μg/ml hygromycin B.

Macrophage infection and intracellular amastigote drug susceptibility assay. Stationary phase *Leishmania* promastigotes expressing the luciferase gene (pGL-αNEOαLUC) were used to infect J774A.1 macrophages. Briefly, J774A.1 murine macrophages (1×10^5 cells/250 μl/per well) were infected with 1×10^6 promastigotes [15]. After 3 h, the non-internalized parasites were removed by washing and drug was added at different concentrations. After 3 days of drug exposure, plates containing adherent macrophages were washed and luciferase activity was determined [15]. The 50% inhibitory concentration (IC_{50}) was determined from the graph representing different concentrations of drug plotted against relative light units (RLU) produced by luciferase expressing parasites.

Characterization of L. donovani ODC protein expressed in E. coli. Recombinant expression and purification of the *Leishmania* ODC was carried-out by cloning *L. donovani* ODC gene encoding the enzyme into the bacterial expression vector pET-30 Xa/LIC and expressing in *E. coli*. The presence of the His-tag was used to purify the recombinant protein in large quantities to homogeneity by immobilized metal affinity chromatography. Recombinant *L. donovani* ODC was used for the ODC assay.

ODC activity. Ornithine decarboxylase activity was assayed by following the release of $^{14}CO_2$ from L- [-^{14}C] ornithine [17]. Protein concentrations were determined by the method of Bradford [18] using bovine serum albumin as standard. The activity was expressed in enzyme units in which one unit is nmol of CO_2 /mg protein/h.

ODC inhibition. The inhibition constant for GAPA was determined using recombinant *Leishmania* ODC (rODC) under standard assay conditions by the addition of various concentrations of inhibitor (5–100 μM). The K_i values were calculated by using the programme GraphPad Prism 4.03.

Putrescine uptake. Parasites were harvested during the mid logarithmic phase of growth. Cells were separated by centrifugation at 2100g, for 10 min at 4 °C and washed twice with phosphate buffered saline (PBS) supplemented with 1% D-glucose (PBSG) at pH 7.4. Parasite suspensions (100 μl, containing 2×10^7 cells) were warmed to 25 °C and mixed with 100 μl of assay buffer containing labelled molecule (0.2 μCi) plus or minus GAPA at the concentration indicated in the figure legend. Putrescine uptake was performed as described previously [19].

Polyamine analysis. Leishmania donovani promastigotes (1×10^7) were harvested at 48 h of growth by centrifugation at 2000g (15 min, 4 °C). Quantitative determination of polyamines in crude lysates of *L. donovani* was performed by C_{18} reversed-phase high performance liquid chromatography (HPLC) after pre-column derivatization with dansyl chloride [20].

Thiol analysis. Thiol content was determined by fluorescence detection following pre-column derivatization with monobromobimane and separated by high performance liquid chromatography (HPLC) as described elsewhere [21]. Analysis was performed in triplicates for two independent experiments.

Statistical analysis. The Student's *t* test, with significance at *P* values of <0.05, <0.005, and <0.001, was used for analysis. The data represent means ± SD of at least three determinations from two independent experiments.

Results

Effect of GAPA on the growth of promastigotes and amastigotes of wild-type and ODC overexpressors

To determine the inhibitory effect of GAPA on the promastigotes of the wild-type and the ODC overexpressors, parasites were cultured in the presence of increasing concentrations of GAPA. GAPA inhibited the growth of wild-type and ODC overexpressing promastigotes in a dose dependent manner. Table 1 summarizes the calculated IC_{50}s. The results indicated that ODC overexpression conferred resistance to GAPA thereby suggesting that the primary cellular toxicity of GAPA could be mediated via the polyamine biosynthetic pathway enzyme.

The sensitivity of amastigotes was then tested in intracellular amastigotes-macrophage model. Table 1 summarizes the IC_{50}s, of *L. donovani* amastigotes and J774A.1 cell line. Both amastigotes of wild-type and ODC overexpressors of *L. donovani* exhibited higher sensitivity to GAPA (4- and ~1.4-, respectively) when compared to promastigotes of the wild-type and the ODC overexpresssors. Concentration of GAPA as high as 200 μM did not affect the viability of J774A.1 macrophage cell line at either 24 or 72 h after drug addition.

In order to find out whether the antiproliferative effect observed with GAPA is due to polyamine depletion, 1 mM of putrescine or spermidine was added to promastigotes after 24 or 48 h of drug treatment. Putrescine and spermidine supplementation reversed the antiproliferative effect of GAPA (40 μM) (Fig. 1A and B).

Table 1
Effect of GAPA on promastigotes, amastigotes of wild-type (WT) and ODC overexpressing *Leishmania donovani* and macrophage cell line J774A.1

	IC_{50} (μM)		
	Promastigotes	Amastigotes	Macrophage cell line (J774A.1) (μM)
WT	36 ± 7.0	9 ± 1.0	
ODC overexpressions	90 ± 2.0	66 ± 8.0	
Macrophage cell line J774A.1	–	—	>200

IC_{50}s were determined after 3 days of drug addition as reported in the Methods section.
The results are means ± standard deviation of three independent experiments for all data sets.

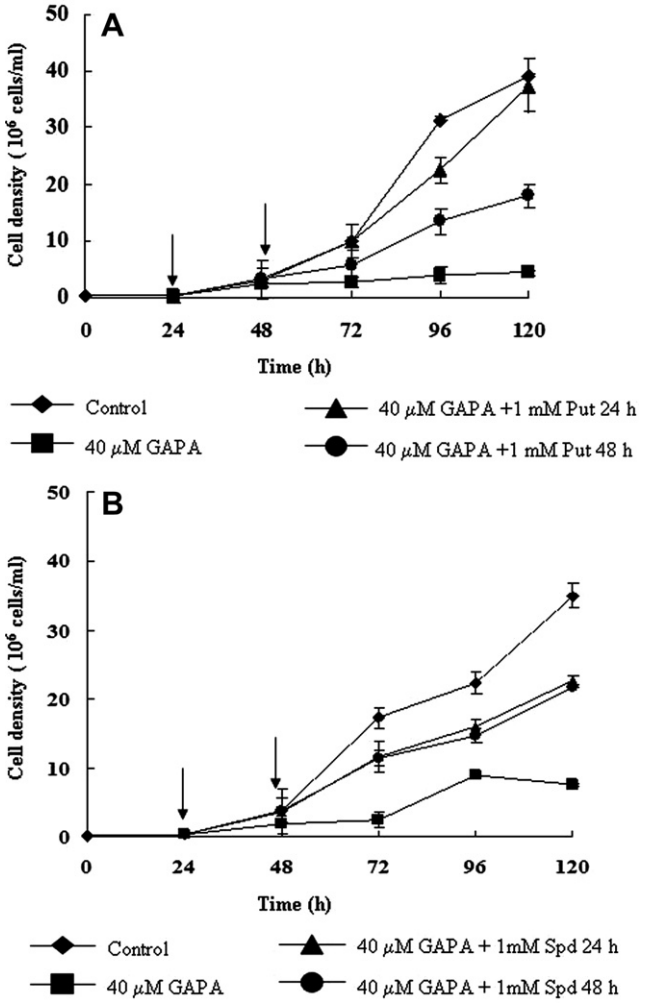

Fig. 1. Reversal of growth inhibition by GAPA in the presence of exogenous polyamines. The *L. donovani* culture medium containing inhibitory concentration of GAPA was supplemented by 1 mM of putrescine (A) and 1 mM of spermidine (B). Control cells were cultured solely in the presence of the drug. The growth of *L. donovani* was monitored over a period of 120 h in the presence of 40 μM GAPA. One millimolar of putrescine (A) and 1 mM spermidine (B) was added after treatment for 24 or 48 h. Parasites were enumerated every 24 h by counting using hemocytometer. Arrow indicates the time of the addition of putrescine (A) and spermidine (B).

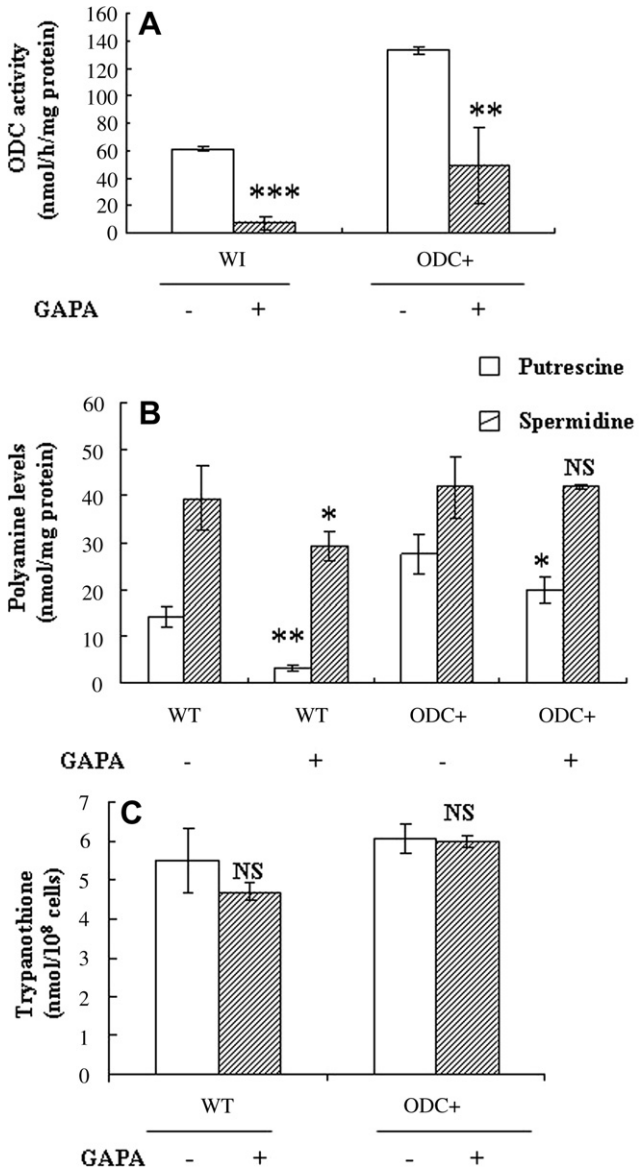

Fig. 2. Effect of GAPA on ODC activity, polyamines and trypanothione levels of *L. donovani* promastigotes. (A) ODC activity in WT and ODC overexpressors. (B) Putrescine and spermidine levels in WT and ODC overexpressors. (C) Trypanothione levels in WT and ODC overexpressors. GAPA (40 μM) was added to the log phase promastigotes and cells were harvested 48 h later as described under Materials and methods. These pools were measured in αMEM medium which is a complete medium and does not contain FBS. FBS was excluded to avoid polyamine oxidase-mediated toxicity [26]. Results are means ± SD. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to the corresponding control values. NS, not significant.

Effect of GAPA on ODC and polyamine metabolism of wild-type and ODC overexpressors

To determine whether the antileishmanial effect of GAPA corresponds to an alteration of the intracellular polyamine levels, the wild-type and the ODC overexpressing *L. donovani* strains were treated with 40 μM of GAPA for 48 h. Treatment of wild-type and ODC overexpressing *L. donovani* promastigotes with 40 μM of GAPA for 48 h inhibited ODC activity by over ~ 88% and 63%, respectively (Fig. 2A).

In order to further confirm the direct inhibitory effect of GAPA on ODC, effect of GAPA on *L. donovani* recombinant ODC (rODC) was studied. The inhibition constant for GAPA was determined to be 60 μM (data not shown).

To show whether GAPA promotes its effect by altering the polyamine concentrations, putrescine and spermidine contents were measured. GAPA resulted in inhibition of putrescine levels by ~77% and ~29% in the wild-type and ODC overexpressing *L. donovani* strains respectively when compared to the respective untreated controls. There was ~2.6-fold more reduction in putrescine levels in wild-type when compared to the ODC overexpressing cell line (Fig. 2B). Spermidine content was significantly inhibited in the wild-type *L. donovani*. However, no significant decrease in spermidine levels was observed in the ODC overexpressing cell line when compared to the corresponding untreated cells. We did not observe any significant change in $T(SH)_2$ levels in wild-type and ODC overexpressors treated with GAPA (Fig. 2C).

Effect of GAPA on putrescine transport

GAPA is an isosteric analogue of agmatine, the latter is known to use putrescine channel to penetrate inside mammalian cells [8]. The pKa value of GAPA was found to be 6.71 and according to the protonation criteria GAPA is closer to putrescine then to

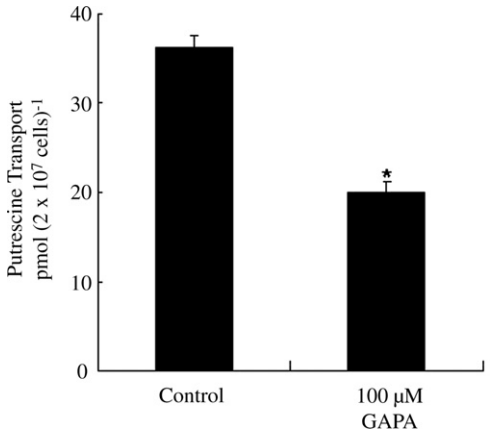

Fig. 3. Effect of GAPA on putrescine uptake in *L. donovani* promastigotes. Transport experiments monitoring uptake of 10 μM of 1,4-[^{14}C]-putrescine dihydrochloride by promastigotes for a 10 min incubation period in the absence, control or presence of 10-fold excess of GAPA were performed as described in the Methods section. Results are means ± SD. ($n = 3$). (*) is statistically different at $P < 0.001$ when compared to the values obtained for controls.

agmatine. Hence, GAPA appears to have semblance to both putrescine and agmatine. We therefore went ahead to check if GAPA is taken up by *L. donovani* through putrescine transporter.

In order to assess the effect of GAPA on the uptake of 1,4-[^{14}C]-putrescine dihydrochloride, promastigotes of the wild-type *L. donovani* in the exponential phase of growth were treated with 10-fold excess of GAPA. As indicated in Fig. 3, GAPA at −10-fold excess was an effective inhibitor of [^{14}C]-putrescine transport and inhibited the putrescine uptake by ∼47%. This data is indicative of a possible role of putrescine transport system in the uptake of GAPA.

Discussion

The critical role that polyamines play in cell proliferation, differentiation, and development [3] coupled with the success of DFMO in the treatment of African trypanosomiasis [5,6] has stimulated considerable interest in the polyamine pathway as a target for potential antiparasitic chemotherapies.

The compound γ-guanidinooxypropylamine (GAPA), an agmatine analog, was first reported to be naturally present in *Wistaria floribunda* seeds and seedlings of the sword bean, *Canavalia gladiata* [22]. Agmatine is proposed to deplete cell polyamine content by inducing antizyme [23], which in turn inactivates the initial enzyme in polyamine biosynthesis, ODC and then promotes its 26S proteosome dependent degradation [23,24]. One of the several causes of anti-proliferative nature of agmatine is by decreasing ODC activity [25]. Agmatine also results in marked decline of putrescine and comparatively insignificant decline of spermidine in rat liver hepatoma cell line [26]. The polyamine transport system is reported to mediate agmatine uptake in mammalian cells [27]. Agmatine has also been reported to have effect on hepatocytes in an antizyme independent mechanism [28]. Since there is no report on the presence of antizyme in *L. donovani* we explored the possibility whether GAPA affects promasigotes by an antizyme independent mechanism. GAPA is known to suppress mammalian cell proliferation by affecting polyamine metabolism [7].

In the present study we for the first time report the effect and mode of action of γ-guanidinooxypropylamine (GAPA), an agmatine analog on *L. donovani* growth. GAPA inhibited the proliferation of both promatigotes *in vitro* and amastigotes in the macrophage model. *L. donovani* cultures exhibited higher sensitivity to GAPA than the mammalian cells (J774A.1) which showed no response to even up to 200 μM of the inhibitor after 24 h. Its specificity against the parasite and not against the host is essential for future *in vivo* trials.

The finding that addition of 1 mM of putrescine or spermidine reversed the growth arrest is consistent with the hypothesis that the polyamine pool is the main target of GAPA's metabolic effect. In order to further establish whether GAPA actually targets the polyamine pathway, we had created transgenic *L. donovani* strains that have been transfected with an episomal construct of ODC [16]. The promastigotes of ODC overexpressors were ∼3-fold more resistant and intracellular amastigotes were ∼6.5-fold more resistant to GAPA than the wild-type. This suggests that GAPA possibly suppresses ODC enzyme and overexpression of enzyme alleviated the antiproliferative potential of GAPA

Inhibitory effect of GAPA on ODC activity of *L. donovani* and direct inhibitory effect of GAPA on purified *Leishmania* recombinant ODC provides evidence on the involvement of the polyamine biosynthetic pathway. In the present study GAPA decreased putrescine and spermidine levels in the wild-type *L. donovani*. GAPA did not result in any change in the trypanothione content in both the WT and ODC overexpressors.

We further confirmed the effect of GAPA on polyamine biosynthesis by checking the possibility of active transportation of the drug by putrescine transporter. Determination of the pK_a of propoxyguanidine group of GAPA showed it to be 6.71. Therefore at physiological pH the analogue is sufficiently protonated to mimic charge distribution of putrescine and probably uses the same transporter. Our present data indicates that cellular uptake of GAPA is possibly via the putrescine transport system in *L. donovani*. Utilization of the polyamine transport system by GAPA could effectively contribute to the depletion of intracellular polyamine levels and thereby leading to the antiproliferative potential of GAPA.

Our results show that GAPA probably has multi-site action. It not only inhibits ODC activity and intracellular polyamine but also inhibits putrescine transport. Multiple target inhibitors have a better rate of success than the single enzyme inhibitors. For instance, polyamine analogues, possibly due to their multiple targets have met with greater success than the single enzyme inhibitors [29]. These include down regulation of polyamine biosynthesis through inhibition of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase and decreased polyamine uptake.

Our results demonstrate that GAPA is a potential antileishmanial lead compound and it possibly inhibits *L. donovani* growth by depletion of intracellular polyamine levels by acting on the activity of the ornithine decarboxylase enzyme. Inhibition of *L. donovani* recombinant ODC by GAPA and reduction of growth of *L. donovani* upon the addition of putrescine or spermidine further supports the possible involvement of polyamine biosynthetic pathway. Our *in vitro* studies demonstrate that GAPA could possibly be used as an alternative for treating leishmaniasis. However, this needs to be further confirmed using an *in vivo* model.

Acknowledgments

This work is supported by a grant from the Council of Scientific and Industrial Research (CSIR), Government of India. This work is also supported by joint Indo-Russian project proposal (DST-RFBR) to RM (RUSP-883) and ARK (RFBR 08-04-91317). S.S., A.J., and A.S. were supported by the CSIR. We thank Marc Ouellette, Canada for providing the constructs used in this study.

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