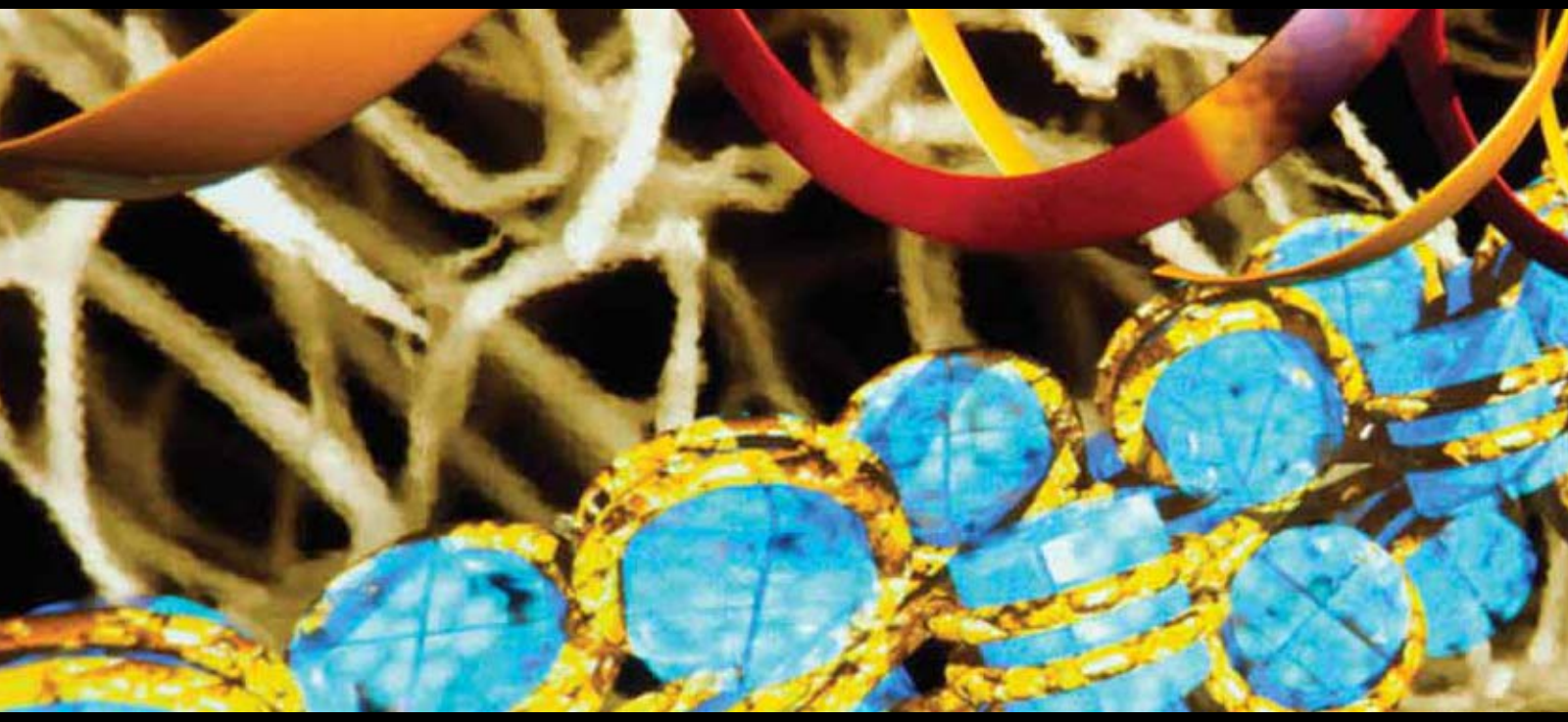
Molecular Biology International

Target Identification and Intervention Strategies against Kinetoplastid Protozoan Parasites

Guest Editors: Hemanta K. Majumder, Wanderley De Souza, and Kwang Poo Chang



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Editorial Board

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Editorial

Target Identification and Intervention Strategies against Kinetoplastid Protozoan Parasites

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The past few decades have been marked by numerous admirable research efforts and promising technological advancements in the field of research on protozoan parasites. The parasites of this genre cause some devastating diseases that pose alarming threat to the mankind. Though several intervention strategies have been developed to get rid of these parasites, they always seem to frustrate the efforts of the scientific community sooner or later. The intervention strategies include identification of novel drug targets, development of target-based therapy, and development of vaccines. that provide significant impetus in the field of research pertaining to these parasites. In this context, several reviews have appeared in the past few years elucidating different drug targets in these parasites. For example, Das et al. [1], Balaña-Fouce et al. [2], and others have described the role of topoisomerases as potential drug targets in these kinetoplastid protozoa. Urbina [3] has described the lipid biosynthetic pathway as a possible chemotherapeutic target whereas McConville [4] has elucidated the potential of parasite surface glycoconjugates as possible drug targets. Other targets include cysteine peptidases [5] and histone deacetylases [6] of the trypanosomatid parasites.

Parasites of the genus *Trypanosoma* and *Leishmania* are kinetoplastid protozoan parasites that cause trypanosomiasis and leishmaniasis, respectively. Parasites belonging to the genus *Plasmodium* mainly cause malaria. These diseases are prevalent in tropical and subtropical countries and cause significant morbidity and mortality. However, these diseases are of the lowest priority because they offer little or no commercial incentives to the pharmaceutical companies.

This special issue is a much needed and timely compilation of selected research and review articles in the concerned field. Though the selected papers are not a comprehensive representation of the field, but they represent a rich mixture of multifaceted knowledge that we have the pleasure of sharing with the readers. We would like to thank all the authors for their excellent contributions and also the reviewers for their efforts in assisting us.

This special issue contains thirteen papers, of which five are research papers, and the rest are review articles. The five research articles mainly focus on development of new drugs and targets and also shed light on novel therapeutic intervention strategies. In the first paper, S. Sengupta et al. have established cryptolepine-induced cell death in the protozoan parasite *L. donovani*. Interestingly, the death process is augmented when the autophagic mechanism is inhibited by specific chemical inhibitors, and this finding may form the skeleton for novel therapeutic intervention strategies.

In the second paper, S. Teixeira de Macedo-Silva et al. have investigated the effect of the antiarrhythmic drug amiodarone on the promastigotes and amastigotes form of *L. amazonensis*. They have shown that this drug has antiproliferative effect on *L. amazonensis* promastigotes and amastigotes and causes depolarization of mitochondrial membrane potential in both forms which ultimately leads to cell death of the parasites. So this compound may serve as a potential starting material for antileishmanial drug development.

In the third paper, L. Major and T. K. Smith. have screened the MayBridge Rule of 3 Fragment Library to identify compounds targeting Inositol-3-phosphate synthase

(INO1) which has previously been genetically validated as a drug target against *Trypanosoma brucei*, the causative agent of African sleeping sickness. By this approach, they have identified 38 compounds that significantly altered the Tm of TbINO1. Four compounds showed trypanocidal activity with ED50s in the tens of micromolar range, with 2 having a selectivity index in excess of 250.

Topoisomerases are key enzymes that play a pivotal role in various cellular processes and also serve as an important drug target. In the paper, A. Roy et al. have described a synthetic peptide, WRWYCRCK, with inhibitory effect on the essential enzyme topoisomerase I from the malaria-causing parasite *Plasmodium falciparum*. Although *Plasmodium falciparum* does not belong to the order kinetoplastida, but still it has several features common with the kinetoplastid protozoan parasite *T. brucei*, for example, antigenic variation. The transition step from noncovalent to covalent DNA binding of *P. falciparum* topoisomerase I is specifically inhibited by this peptide while the ligation step of catalysis remains unaffected. Molecular docking analyses further provide a mechanistic explanation for this inhibition. This work provides evidence that synthetic peptides may represent a new class of potential antiprotozoan drugs.

In the fifth paper, J. Kaur et al. have performed bioinformatic analysis of the *Leishmania donovani* long-chain fatty acid Co-A ligase (LCFA) as a novel drug target. The authors have previously found this enzyme to be differentially expressed by *Leishmania donovani* amastigotes resistant to antimonial treatment. In the present study, the authors have confirmed the presence of long-chain fatty acyl CoA ligase gene in the genome of clinical isolates of *Leishmania donovani* collected from the disease-endemic area in India and propose that this enzyme serves as an important protein and a potential target candidate for development of selective inhibitors against leishmaniasis.

This special issue also features some timely and much needed review articles in the field. In the sixth paper, S. Gupta et al. have validated the role of a key enzyme, glucose-6-phosphate dehydrogenase (G6PDH) in trypanosomatids as an important drug target and discussed the possibility of drug discovery targeting this enzyme. G6PDH is the first enzyme of the pentose phosphate pathway and is essential for the defense of the parasite against oxidative stress. *T. brucei* and *T. cruzi* G6PDHs are inhibited by steroids such as dehydroepiandrosterone and derivatives in an uncompetitive way. The *Trypanosoma* enzymes are more susceptible to inhibition by these compounds than the human G6PDH. These compounds are presently considered as promising leads for the development of new parasite-selective chemotherapeutic agents.

In the seventh paper, A. F. Coley et al. have discussed the possibility of therapeutic development targeting glycolysis in African trypanosomes. The parasite is limited to using glycolysis of host sugar for ATP production while infecting the human host. This dependence on glucose breakdown presents a series of targets for potential therapeutic development, many of which have been explored and validated as therapeutic targets experimentally and has been addressed in this paper in detail.

In the eighth paper, S. L. de Castro et al. have given a good overview of experimental chemotherapy in the Chagas disease which is caused by *Trypanosoma cruzi*, and it affects approximately eight million individuals in Latin America. The authors have presented a nice biochemical and proteomic overview of potential *T. cruzi* targets with reference to amidine derivatives and naphthoquinones that have showed the most promising efficacy against *T. cruzi*.

In the ninth paper, A. K. Haldar et al. have classically demonstrated the current status and future directions for the use of antimony in the treatment of leishmaniasis. The standard treatment of Kala-azar in the recent past has been the use of pentavalent antimonials (SbV) but there has been progressive rise in treatment failure to Sb(V) due to the problem of chemoresistance that has limited its use in the treatment program in the Indian subcontinent. However, it has been shown recently that some of the peroxovanadium compounds have Sb(V) resistance modifying ability in experimental infection with Sb(V) resistant *Leishmania donovani* isolates in murine model. Thus vanadium compounds may be used in combination with Sb(V) in the treatment of Sb(V) resistance cases of kala-azar.

In the tenth paper, R. Duncan et al. have presented a comprehensive overview of the genes involved in *Leishmania* pathogenesis with reference to the potential for drug target selection. Proteins that are differentially expressed or required in the amastigote life cycle stage found in the patient are likely to be effective drug targets. Several examples and their potential for chemotherapeutic disruption have been presented in this paper. The programmed cell death pathway that is now recognized among protozoan parasites is reviewed for some of its components and evidence that suggests that they could be targeted for anti-parasitic drug therapy has been presented.

In the next paper, A. Biswas et al. have discussed the role of cAMP signaling in the survival and infectivity of the protozoan parasite *Leishmania donovani*. While invading macrophages, *L. donovani* encounters striking shift in temperature and pH that act as the key environmental trigger for differentiation and increase cAMP level and cAMP-mediated responses. A differentially expressed soluble cytosolic cAMP phosphodiesterase (LdPDEA) might be related to infection establishment by shifting trypanothione pool utilization bias toward antioxidant defense. This paper explains the significance of cAMP signaling in parasite survival and infectivity.

In the twelfth paper, Md. Shadab and N. Ali have elegantly discussed the evasion of host defense mechanism by *L. donovani*. They have presented a detailed account of the subversion and signaling pathways that allow the parasites to get rid of the host defense mechanism.

In the last paper, A. Ghoshal and C. Mandal have presented a detailed perspective of sialic acids that serve as important determinants influencing the parasite biology. Despite the steady progress in the field of parasite glycobiology, sialobiology has been a less traversed domain of research in leishmaniasis. This paper focuses on identification, characterization, and differential distribution of sialoglycotope having the linkage-specific 9-*O*-acetylated sialic acid in

promastigotes of different *Leishmania sp.* causing different clinical ramifications.

There are other areas of relevance not covered in the volume, that is, prophylactic and therapeutic vaccination, targeted drug delivery, and antigenic variation. However, the present issue covers a significant area of the subject and will be of immense interest to the readers.

Hemanta K. Majumder
Wanderley de Souza
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Research Article

Cryptolepine-Induced Cell Death of *Leishmania donovani* Promastigotes Is Augmented by Inhibition of Autophagy

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Leishmania donovani are the causative agents of visceral leishmaniasis worldwide. Lack of vaccines and emergence of drug resistance warrants the need for improved drug therapy and newer therapeutic intervention strategies against leishmaniasis. In the present study, we have investigated the effect of the natural indoloquinoline alkaloid cryptolepine on *L. donovani* AG83 promastigotes. Our results show that cryptolepine induces cellular dysfunction in *L. donovani* promastigotes, which leads to the death of this unicellular parasite. Interestingly, our study suggest that cryptolepine-induced cell death of *L. donovani* is counteracted by initial autophagic features elicited by the cells. For the first time, we show that autophagy serves as a survival mechanism in response to cryptolepine treatment in *L. donovani* promastigotes and inhibition of autophagy causes an early increase in the amount of cell death. This study can be exploited for designing better drugs and better therapeutic strategies against leishmaniasis in future.

1. Introduction

Species of the genus *Leishmania* are the causative agents of various parasitic infections which manifest itself in a variety of clinical forms depending upon the species of *Leishmania* and the immunological status of the host. *Leishmania donovani* is the causative agent of visceral leishmaniasis (VL) or "Kala-azar", which is fatal if patients are left untreated and is more common in less developed countries [1]. The organism has a digenic life cycle residing as flagellated extracellular promastigotes in the gut of insect vector and as nonflagellated amastigotes in mammalian host macrophages [2]. The drugs recommended for treatment of VL, namely, pentavalent antimonials, amphotericin B, and lipid formulations of amphotericin B, have many limitations like long course of treatment, toxic side effects and high costs [1]. Moreover, the occurrence of chemoresistance against classical drugs has worsened the situation further [3, 4]. Thus, search for new drugs, new molecular targets, and novel therapeutic strategies are justified.

In search of better leishmanicidal compounds, plant derived compounds have long been evaluated [5]. There has been considerable public and scientific interest in the use of plant derived compounds to combat human diseases. Cryptolepine is an indoloquinoline alkaloid which was first isolated from the roots of *Cryptolepis triangularis* collected in Belgian Congo and afterward from the roots of *Cryptolepis sanguinolenta* from Nigeria [6]. This species has been used traditionally to treat malaria, hypertension, hyperglycemia, inflammation and cancer [7, 8]. Although there are reports of antiparasitic activity of CLP [9], its effect on *Leishmania donovani* is yet to be evaluated.

Programmed cell death (PCD) appears to be the most preferred mechanism for mediating parasiticidal activity, as has been observed in kinetoplastids in response to diverse stimuli, for example, heat shock, chemotherapeutic agents

such as pentostam, amphotericin B [10], camptothecin [4], oxidants such as H_2O_2 [11] or even serum deprivation [12]. Apoptosis involves a series of morphological and biological changes including ROS production, decrease in cellular GSH levels, and so forth, which ultimately results in DNA fragmentation [3, 4, 13]. This is considered as the hallmark of apoptosis. However, it has also been suggested that autophagy provides the front line of defense against oxidative stress [14] and can actually protect cells by preventing them from undergoing apoptosis [15]. Autophagy is an evolutionarily conserved mechanism for the degradation of cellular components in the cytoplasm [16] resulting in eventual breakdown and recycling of macromolecules [17]. Though autophagic cell death has been suggested to be involved in various systems [18], the precise role of this catabolic process in dying cells is not clear [16]. In fact, autophagy may have originally arisen as a mechanism to protect unicellular organisms against any form of environmental stress [19]. Autophagy plays a role in lifespan extension and Sir2 has been suggested to be involved in the process [20]. However, role of autophagy as a survival mechanism in response to drug in *Leishmania* remains to be elucidated.

In the present study, we have evaluated the effect of naturally occurring indoloquinoline alkaloid cryptolepine (CLP) on *L. donovani* AG83 promastigotes. We show that CLP induces ROS in the cells, ultimately resulting in DNA fragmentation which is a hallmark of apoptosis. For the first time, we identified that the parasites try to combat against initial CLP-induced stress response by initiating an autophagic response as a survival mechanism and activation of silent information regulator protein Sir2 plays a role in the process. This study has a great potential in understanding the role of autophagy in the cell death mechanism of *Leishmania* and will be helpful in identifying new drugs and newer therapeutic strategies to combat leishmaniasis in future.

2. Materials and Methods

2.1. Chemicals. Cryptolepine (Figure 1) hydrochloride was synthesized using isatin and O, N-acetylindoxyl as described previously [21] and was dissolved at 20 mM concentration in 100% DMSO and stored at −20°C. *N*-Acetyl-L-cysteine (NAC) was purchased from Sigma-Aldrich, was dissolved in 100% DMSO at 50 mM and stored at −20°C. FM4-64 and monodansylcadaverine (MDC) and monochlorobimane were purchased from Molecular Probes and stored at −20°C and room temperature, respectively.

2.2. Parasite Culture and Maintenance. The *L. donovani* strain AG83 promastigotes were grown at 22°C in Ray's modified media [22] and in M199 liquid media supplemented with 10% fetal calf serum as described previously [3].

2.3. Cell Viability Test by MTT Assay. The effect of drug on the viability of *L. donovani* AG83 promastigote cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) assay [2, 23]. The cells at the exponential phase were collected and transferred into

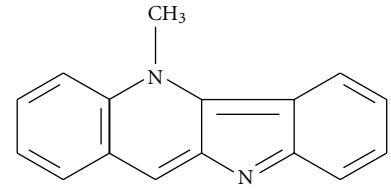


Figure 1: Structure of Cryptolepine.

24-well plate (approximately 4×10^6 cells/well). The cells were then incubated for various time periods in the presence of different concentrations of cryptolepine hydrochloride (CLP). After incubation, the cells were centrifuged and the supernatant was aspirated. The cell pellet was washed with PBS (1X) twice and then finally suspended in 100 μL of PBS (1X) in 96-well plates. Ten microliters of MTT solution (10 μg/mL) were added in each sample of 96-well plates and samples were incubated for 4 h. After incubation, 100 μL of stop solution (stock: 4963 μL of isopropanol and 17 μL of concentrated HCl) was added and kept for 20 min at room temperature. The optical density was taken at A_{570} on an ELISA reader (Multiskan EX; Thermo Fisher Scientific, Waltham, MA).

2.4. Study of Parasite Ultrastructure by Transmission Electron Microscopy. Transmission electron microscopy (TEM) was carried out with both CLP treated and untreated cells as described previously [4, 13]. Sections were cut with a Du-point diamond knife in an LKB Ultramicrotome, stained on copper grids with uranyl acetate and lead acetate for 10–15 min, respectively, and examined under JEOL 100CX TEM.

2.5. Double Staining and Confocal Microscopy. L. donovani AG83 promastigotes (approximately 10^6 cells/mL) were cultured in 24-well plates with different treatments. FM4-64 (40 μM) (Excitation wavelength = 505 nm, emission wavelength = 725 nm) was added directly in the culture medium and kept for 90 min at room temperature. The cells were then washed twice with 1X PBS and stained with 50 μM MDC (Excitation wavelength = 335 nm, emission wavelength = 518 nm) for 10 min at room temperature. The cells were further washed twice with 1X PBS and live promastigotes were immobilized by mounting under poly-L-lysine coated coverslips as described previously [24]. Samples were viewed with a Nikon A1 R laser confocal microscope.

2.6. Measurement of Intracellular ROS Levels. Intracellular ROS level was measured in CLP-treated and untreated leishmanial cells as described previously [3]. In brief, after treatment with CLP and NAC for different time periods, cells (approximately 10^6) were washed and resuspended in 500 μL of medium 199 and were then loaded with a cell-permeate probe CM-H_2DCFDA for 1 h. This is a nonpolar compound that is hydrolyzed within the cell to form a nonfluorescent derivative, which in presence of a proper oxidant converted to a fluorescent product. Fluorescence was measured through spectrofluorometer using 507 nm as excitation and 530 nm as emission wavelengths.

Table 1: List of gene-specific primers used for real-time PCR analysis.

Target	Primer sequence
ATG 8	Forward: 5′-ATG TCT TCC AGA GTA GCT GGG-3′
	Reverse: 5′-ATT GAA GAG GTC GCT CAT GAG-3′
Sir2	Forward: 5′-TTT CGC TCA TCT GAC ACC GGG-3′
	Reverse: 5′-CCG CTG CCT TCT CCA GAC CAT-3′
GAPDH	Forward: 5′-AGA AGA CGG TGG ATA GTC ACT-3′
	Reverse: 5′-GCC ACA CCG TTG AAG TCT GAA-3′

2.7. Measurement of GSH Level. GSH level was measured by monochlorobimane dye that gives a blue fluorescence when bound to glutathione [2, 3]. *L. donovani* promastigotes (approximately 10^6 cells) were treated with or without CLP at different times. The cells were then pelleted down and lysed by cell lysis buffer according to the manufacturer's protocol (ApoAlert glutathione assay kit; Clontech, Mountain View, CA). Cell lysates were incubated with monochlorobimane (2 mM) for 3 h at 37°C. The decrease in glutathione levels in the extracts of nonapoptotic and apoptotic cells were detected by spectrofluorometer with 395-nm excitation and 480-nm emission wavelengths.

2.8. Measurement of Total Fluorescent Lipid Peroxidation Product. CLP-treated and -untreated *L. donovani* cells were pelleted down and washed twice with 1X PBS. The pellet was dissolved in 2 mL of 15% SDS-PBS solution. The fluorescence intensities of the total fluorescent lipid peroxidation products were measured with excitation at 360 nm and emission at 430 nm as described previously [3, 4].

2.9. Flow Cytometric Analysis. The *L. donovani* promastigotes were treated with CLP at 20 μM and 3-methyladenine (3-MA) at 10 mM for different times and washed twice with PBS. The cells were then resuspended in 100 μL of binding buffer provided with the FLUOS-annexinV staining kit (Roche Diagnostics). The cells were stained with annexin V-FITC and PI as per instructions given by the manufacturer, and then they were scanned for fluorescence intensity of cell population in different quadrants. The fraction of cell population in different quadrants was analyzed using quadrant statistics [3, 13]. Cells treated with 3-MA alone served as the control for the experiment.

2.10. Real-Time PCR Analysis. Total RNA was prepared from *L. donovani* AG83 promastigotes after different treatments for different times using the Total RNA isolation kit (Roche Biochemicals). cDNA was synthesized from 60 ng of total RNA using Superscript II RNaseH$^-$ Reverse Transcriptase (Invitrogen) and oligo $(dT)_{12-18}$ primers (Invitrogen) following manufacturers instructions. For amplification of the desired cDNA, gene-specific primers were designed from sequencing data bank website (Table 1). Real-Time PCR was performed for ATG 8, Sir2, and GAPDH genes. Three separate reactions were carried out using three different RNA preparations in 25 μL volume using SYBR-Green Super mix (Applied Biosystem) and same primer sets in a 7300 Real-Time PCR system (Applied Biosystem). Reactions were carried out using the following profile: initial denaturation at 95°C for 5 min followed by 35 cycles with denaturation at 95°C for 45 s, annealing at 52°C for 45 s and extension at 68°C for 45 s. The PCR was followed by a melt curve analysis to ascertain that the expected products were amplified. Values for each gene were normalized to expression levels of GAPDH using the $2^{-\Delta\Delta Ct}$ method [25, 26]. The fold expression was calculated as described previously [25, 26] using the following equation:

$$\text{Fold expression} = 2^{-\Delta\Delta Ct}. \quad (1)$$

2.11. DNA Fragmentation Assay. The assay was performed as described previously [4, 13]. Briefly, genomic DNA was isolated from the parasites (approximately 10^6 cells/mL) after different treatments using an apoptotic DNA ladder kit (Roche Diagnostics). The DNA was quantified and equivalent amount of DNA was electrophoresed in a 1.5% agarose gel at 75 V for 2 h and thereafter stained with EtBr and photographed under UV illumination.

3. Results

3.1. Cryptolepine (CLP) Causes Loss of Cell Viability of L. donovani Promastigotes. *L. donovani* AG83 promastigotes (4×10^6 cells/mL) were incubated with five different concentrations of CLP (2, 5, 10, 15, and 20 μM) for 6, 12, and 24 h after which the cell viability was determined by MTT assay (Figure 2(a)). At 12 h, 80% growth was inhibited by 20 μM CLP which was comparable with the inhibition achieved by 10 μM CLP at 24 h and 92% growth was inhibited by 20 μM CLP at 24 h. The effect of CLP was to cause both time- and concentration-dependent decrease in cell viability of *L. donovani* promastigotes. The IC_{50} value of CLP was calculated to be 8.2 μM at 12 h in *L. donovani* AG83 promastigotes. As a positive control, cells were treated with different concentrations of camptothecin (CPT) (2, 5, and 10 μM) for 6, 12, and 24 h and cell viability was determined by MTT assay (Figure 2(b)).

3.2. Parasite Ultrastructural Studies Using Transmission Electron Microscopy. To understand the effect of CLP on *L. donovani* promastigotes in detail, we carried out transmission electron microscopy (TEM) with CLP-treated and -untreated cells for different time points. DMSO treated parasites (control cells) retain the normal nuclear architecture with a prominent central or slightly eccentrically localized nucleolus, while chromatin was usually distributed peripherally beneath the nuclear membrane (Figure 3(a)). Treatment with CLP for 2 h revealed the appearance of multiple cytoplasmic vacuoles, but the nucleus appeared normal with minimum evidence of chromatin condensation. There is also one mitochondrion profile which is swollen, and the matrix appears to be lost (Figure 3(b)). However, treatment with CLP for 6 h causes extensive damage to the cells. The cells exhibited condensed and marginated chromatin and fragmented nucleus. The integrity of the plasma membrane

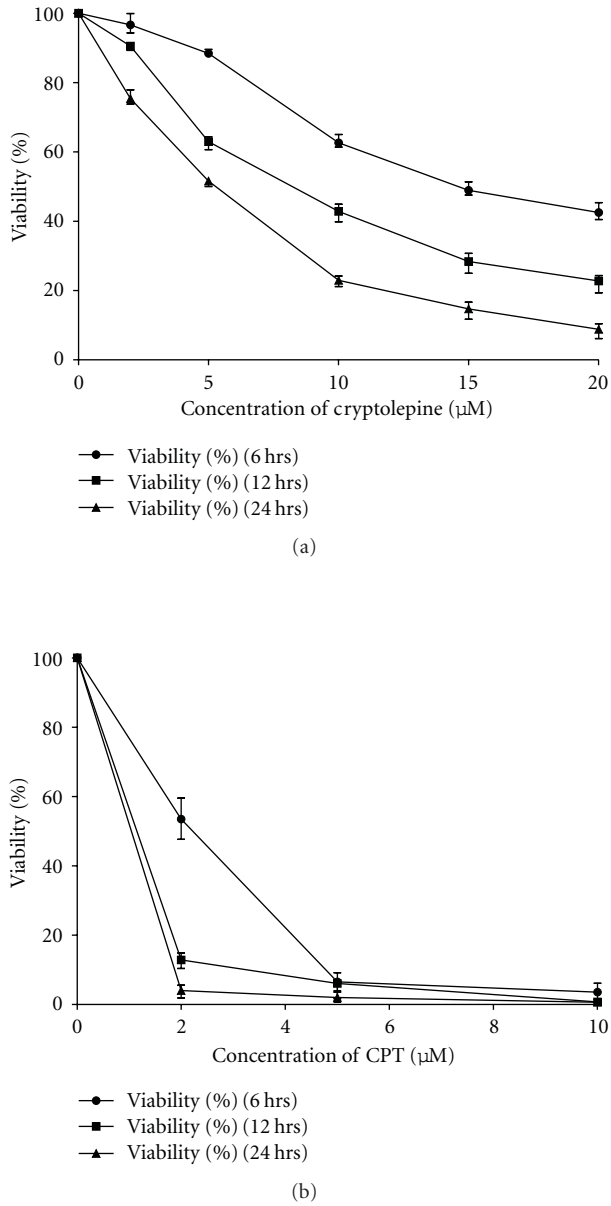


Figure 2: Measurement of cell viability by MTT assay. Log phase *L. donovani* AG83 promastigote cells (4×10^6 cells/mL) were treated with different concentrations of CLP (2, 5, 10, 15, and 20 μM) for different time periods (6, 12, and 24 h) (a) and CPT (2, 5, and 10 μM) for different time periods (6, 12, and 24 h) (b) and percentage of cell viability was measured by MTT assay. Data are represented as Mean ± SEM ($n = 3$).

was apparently maintained and membrane blebbing was also observed (Figure 3(c)). Taken together, these results suggest the involvement of initial autophagic response on treatment of *L. donovani* promastigotes with CLP. However, at a later time period, cells exhibit features of apoptotic like cell death.

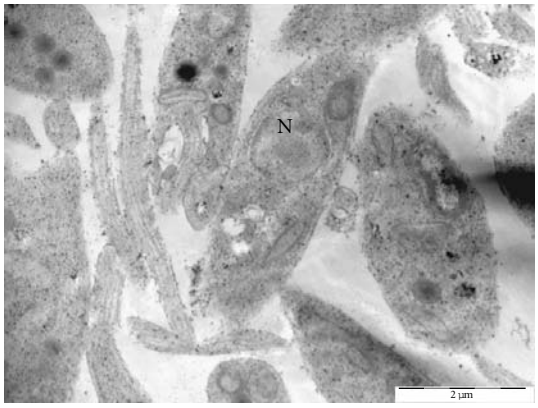
3.3. Double Staining with MDC and FM4-64. To confirm the formation of autophagic vacuoles, we next carried out staining with monodansylcadaverine (MDC). MDC is an autofluorescent, autophagolysosome marker that specifically labels autophagic vacuoles *in vivo* and *in vitro* conditions [27–30]. The autophagic machinery involves the fusion of the autophagic vacuoles with the lysosomal compartment for degradation [31]. The lipophilic dye FM4-64 is a fluorescent endocytic marker which has been used in *Leishmania* as a marker for the MVT-lysosome [24, 32]. FM4-64 was found to localize in a tubular compartment in control cells and no fluorescence of MDC was observed under these conditions. However, upon treatment with 20 μM CLP for 2 h, MDC labeled vesicles were observed which colocalized with FM4-64 labelled compartment (Figure 4). Moreover, pretreatment of cells with 3-methyladenine (3-MA), a specific inhibitor of autophagy [33, 34], caused disappearance of MDC labelled vesicles with no change in FM4-64 labelling pattern. Altogether, these observations suggest the involvement of autophagy in response to CLP treatment.

3.4. CLP Induces the Formation of ROS inside the Cells Resulting in Cellular Oxidative Stress. The results of the EM study suggested that apoptotic like cell death might be occurring in CLP treated parasites at a later time point. A key regulator for induction of apoptosis is intracellular ROS [3, 4]. So, next we wanted to see if CLP causes generation of ROS inside the cells. To measure the status of ROS inside cells, we used a spectrofluorometric assay using CM-H_2DCFDA as described in Section 2. DMSO treated cells (control cells) contained a basal level of ROS whereas treatment with 20 μM CLP caused a 4-fold increase in the ROS levels in parasites at 3 h time period (Figure 5). When cells were pretreated with NAC (20 mM), the level of ROS generation decreased and was nearly same as that of control cells. Thus, it is conceivable from the above result that CLP causes oxidative stress in *Leishmania* parasites.

3.5. CLP-Induced Oxidative Stress Causes Depletion of Cellular GSH Level and Increases the Level of Lipid Peroxidation. One of the most important cellular defenses against intracellular oxidative stress is GSH, which plays a critical role in mediating apoptosis in eukaryotes, including leishmanial cells. GSH is an important molecule for protecting kinetoplastids from ROS or toxic compounds [4]. As shown in Figure 6(a), CLP causes a 49% decrease in GSH level after 3 h and the effect was more pronounced after 6 hrs treatment with CLP. When cells were preincubated with NAC (20 mM) for 1 h, followed by treatment with CLP, GSH level was protected significantly and tends to become normal.

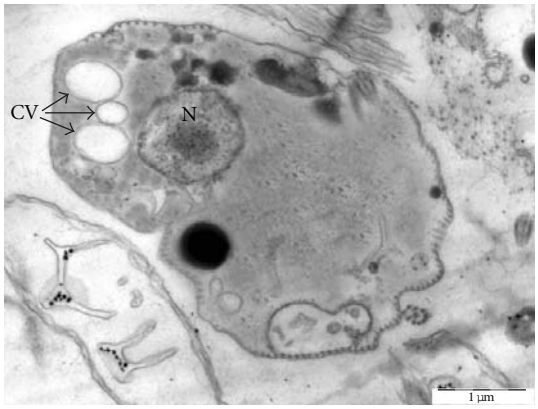
Lipid peroxidation was assessed by measuring the total fluorescent lipid peroxidation products in leishmanial cells after treatment with CLP as described in Section 2. CLP treatment leads to an increase in lipid peroxides after 3 h of drug treatment and reached saturating level after 6 h. In the presence of 20 mM NAC, the level of fluorescent products decreased significantly (Figure 6(b)).

3.6. Inhibition of Autophagy Causes Upregulation of CLP-Induced Cell Death. Although treatment of *Leishmania* parasites with CLP shows initial features of autophagy, apoptosis-like cell death does occur at the later stage. To understand



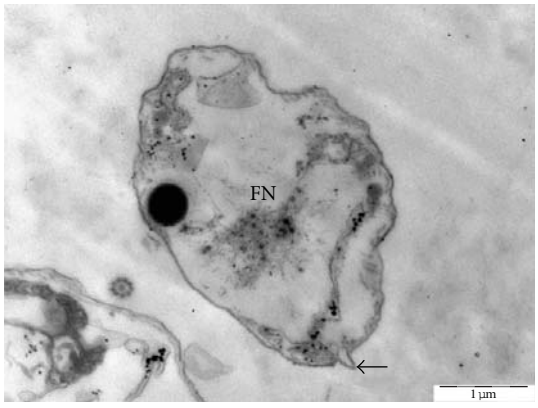
Control

(a)



2 hrs

(b)



6 hrs

(c)

Figure 3: Electron microscopic analysis of *L. donovani* AG83 cells treated with 0.2% DMSO alone and 20 μM CLP for different time periods. Spur blocks were prepared as described in Section 2. (a) Control cells treated with 0.2% DMSO alone, (b) cells treated with 20 μM CLP for 2 h, and (c) cells treated with 20 μM CLP for 6 h. Scale bars are indicated in the figure. N: nucleus, CV: cytoplasmic vacuoles, FN: fragmented nucleus, and closed arrow represents membrane blebbing.

the relationship, if any, of the autophagic features with the cell death mechanism, we first determined the cell viability after CLP treatment when autophagy was inhibited by 3-MA. As evident from Figure 7(a), treatment with 20 μM CLP for 2 h causes a 35% decrease in cell viability compared to control. However, when cells were pretreated with 3-MA, and then treated with 20 μM CLP for 2 h, there was a 58% decrease in cell viability compared to control. Treatment with 3-MA only had no detectable effect on cell viability. These results suggest that pretreatment of *L. donovani* AG83 cells with 3-MA makes them more sensitive to CLP-induced cell death. This was further supported by the flow cytometric analysis. Cells were treated with 20 μM CLP for 2 and 6 h with or without pretreatment with 3-MA and the percentage of apoptotic cells was determined by flow cytometric analysis after staining with annexin V-FITC and PI (Figure 7(b)). Externalization of phosphatidyl serine (stained by annexin V) and presence of impermeant cell membrane (negative PI staining) are hallmarks of PCD [13]. Flow cytometric analysis with annexin V/PI staining showed that when cells were exposed to CLP for 2 h, about 30.5% cells were annexin V positive (Figure 7(b)) but when cells were pretreated with 3-MA and then treated with CLP, about 46.2% cells were annexin V positive (Figure 7(b)). This suggests that inhibition of autophagy by 3-MA causes an increase the number of apoptotic cells. After 6 h of CLP treatment, 49% cells were annexin V positive and when pretreated with 3-MA before treatment with CLP for 6 h, about 50.1% cells were annexin V positive (Figure 7(b)). Interestingly, pretreatment with 3-MA and then adding CLP did not cause any formidable increase in the percentage of annexin V positive cells at 6 h time period. As inhibition of autophagy did not cause any significant increase in the cell death at 6 h time period, we surmise that the autophagic response may not influence the CLP-induced cell death mechanism at a later time period probably due to the prolonged intracellular stress which commits the cells to die.

3.7. RT-PCR Analysis. To understand more clearly the role of the autophagic response in response to CLP treatment, we performed RT-PCR analysis with the autophagic gene ATG 8 [34] and Sir2 [35]. Sir2 is a member of silent information regulator family of genes [36] and has been implicated in lifespan extension along with autophagy [20]. Cytoplasmic Sir2 overexpression has been reported to promote survival of *Leishmania* parasites by preventing programmed cell death [36]. Thus, we investigated the effect on *Leishmania* Sir2 in the autophagic response induced by CLP. Treatment with CLP for 2 h causes marked increase in the mRNA level of ATG 8 (Figure 8(a)). CLP caused about 3-fold increase in the level of ATG 8 compared to untreated control at 2 h (Table 2). This confirms the involvement of autophagy in response to CLP treatment. However, cells pretreated with 3-MA before CLP treatment showed no significant change in ATG 8 mRNA levels confirming the inhibition of autophagy by 3-MA. Treatment with 3-MA only had no effect. Interestingly, treatment with CLP for 6 h did not show any significant change in the ATG 8 mRNA levels. This

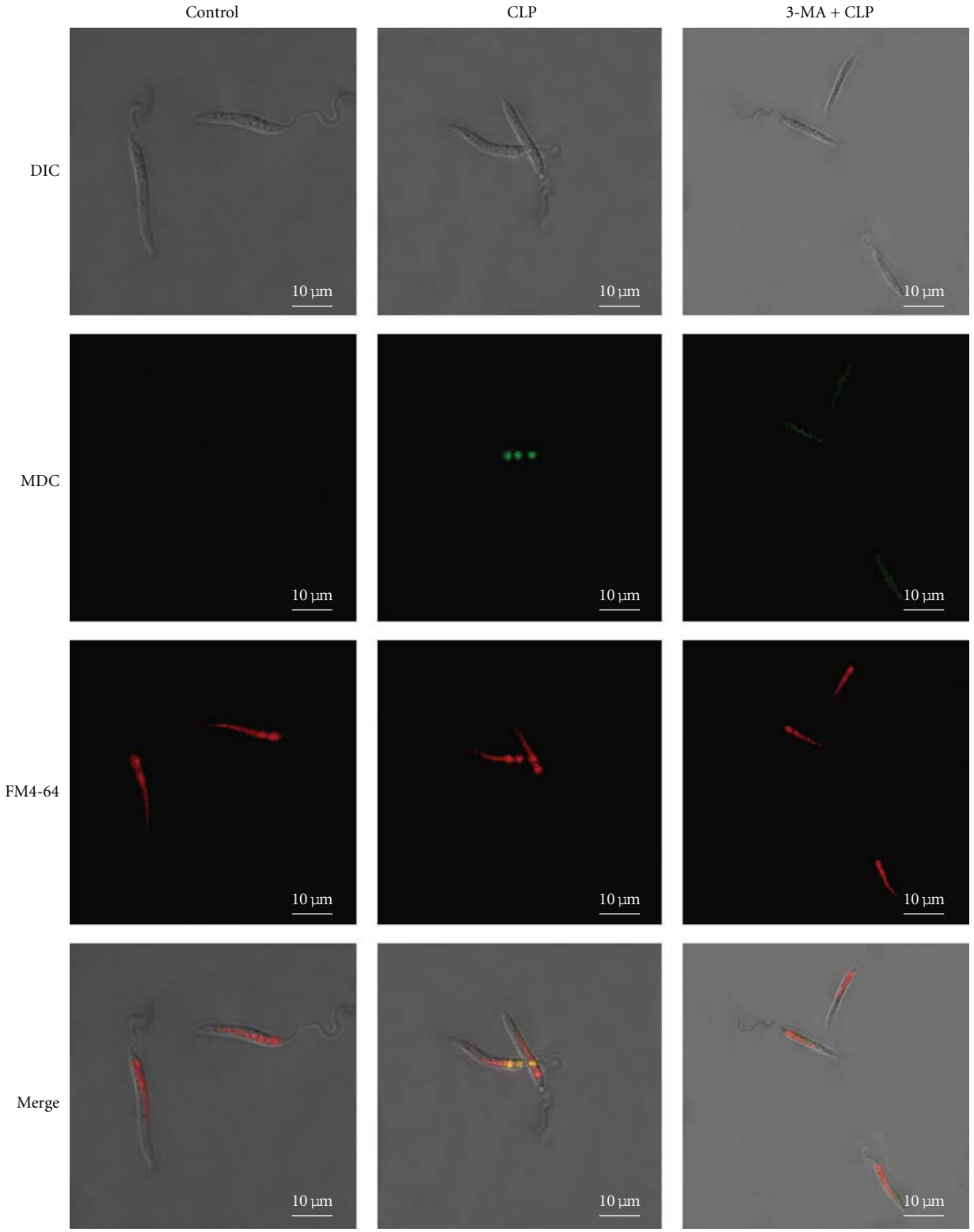


Figure 4: Double staining with FM4-64 and MDC. Slides were prepared as described in Section 2. Confocal microscopic photographs (100X) of control cells, cells treated with CLP (20 μM) for 2 h and cells pretreated with 3-MA (10 mM) and then treated with CLP (20 μM) are shown. DIC denotes differential interference contrast image. FM4-64 signal is shown in red and MDC signal is shown in green. Colocalization of these two markers is shown in yellow. Scale bar is as indicated in the figure.

Table 2: Fold of expression and corresponding fold change of ATG 8 and Sir2 genes relative to internal GAPDH control in treated samples compared with the untreated control.

Condition	ATG 8		Sir2	
	Mean fold expression	Fold change	Mean fold expression	Fold change
Control + 3-MA (6 hrs)	0.867571	~ −1.2	1.132042	~1.1
Control + CLP (2 hrs)	2.936239	~3	2.697552	~2.7
Control + 3-MA + CLP (2 hrs)	1.126242	~1.1	0.873598	~ −1.1
Control + CLP (6 hrs)	1.310307	~1.3	1.460229	~1.5
Control + 3-MA + CLP (6 hrs)	0.774112	~ −1.3	0.659147	~ −1.5

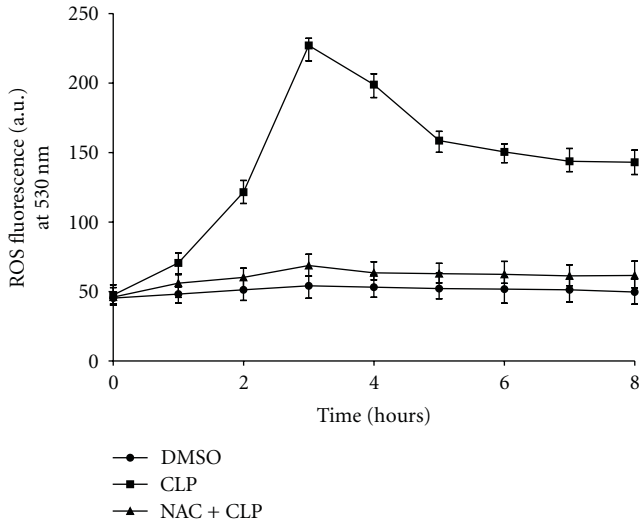


Figure 5: Measurement of CLP-induced generation of ROS. Cells were treated with 20 μM of CLP for different time periods as described in Section 2. Generation of ROS inside the cells was measured after treatment with 0.2% DMSO alone (closed circles), CLP (closed squares) and with NAC prior to treatment with CLP (closed triangles). Data are represented as Mean ± SEM ($n = 3$).

confirms the results of the flow cytometric analysis. Level of Sir2 was elevated after 2 h in response to CLP treatment (Figure 8(a)). CLP caused about 2.7-fold increase in the mRNA level of Sir2 compared to untreated control at 2 h (Table 2). This suggests that the autophagic response serves as a survival mechanism for the cells. However, pretreatment with 3-MA before addition of CLP caused a slight decrease in the mRNA level of Sir2. When cells were treated with CLP for 6 hrs, there was only 1.5-fold increase in the Sir2 mRNA level compared to untreated control suggesting the cells to be committed to death. Pretreatment with 3-MA caused a decrease in the Sir2 level compared to the untreated control cells. Taken together, the above results suggest that treatment of *L. donovani* AG83 promastigotes with CLP causes initial autophagic features as a survival mechanism which can be bypassed by employing specific inhibitor of autophagy (i.e., 3-MA). Moreover, the results also suggest that the survival mechanism cannot cope with the cellular stress at a later time period.

3.8. CLP Induces DNA Fragmentation in L. donovani AG83 Promastigotes. The internucleosomal DNA fragmentation by an endogenous nuclease (genomic DNA fragmentation) is considered as a hallmark of apoptotic cell death [3, 4, 37]. We observed internucleosomal DNA fragmentation in *L. donovani* AG83 cells in response to 20 μM CLP treatment (Figure 8(b)). DNA fragmentation was significantly enhanced by combined treatment of 3-MA and CLP at 2 h compared to CLP alone (Figure 8(b), compare lanes 3 and 4). However, there was no significant difference at 6 h (Figure 8(b), compare lanes 7 and 8). This confirms the involvement of apoptosis-like cell death in *L. donovani* AG83 cells in response to CLP treatment which is augmented by inhibition of autophagy.

4. Discussion

Leishmania donovani is a unicellular protozoan parasite which causes visceral leishmaniasis worldwide. Treatment of leishmaniasis is unsatisfactory due to unavailability of effective vaccines and chemotherapy is still the mainstay for treating this dreaded disease. Moreover, emergence of resistance to traditional drugs has worsened the situation. Thus, there is an urgent need for new drug development and newer therapeutic strategies.

Cryptolepine is a naturally occurring indoloquinoline alkaloid which has been used as an antimalarial drug in Central and Western Africa. Cryptolepine has a broad spectrum of biological activity and has been reported to have anticancer activity [7]. In the present study, we have investigated the effect of cryptolepine on *L. donovani* AG83 promastigotes *in vitro*.

Our results show that CLP causes a decrease in the cell viability of *L. donovani* AG83 promastigotes in both time- and concentration-dependent manner. CLP causes an increase in cellular ROS production with concominant decrease in cellular GSH levels and increase in the level of lipid peroxidation. Also, CLP causes DNA fragmentation which is a hallmark of apoptosis. Altogether, these observations suggest the involvement of apoptosis-like cell

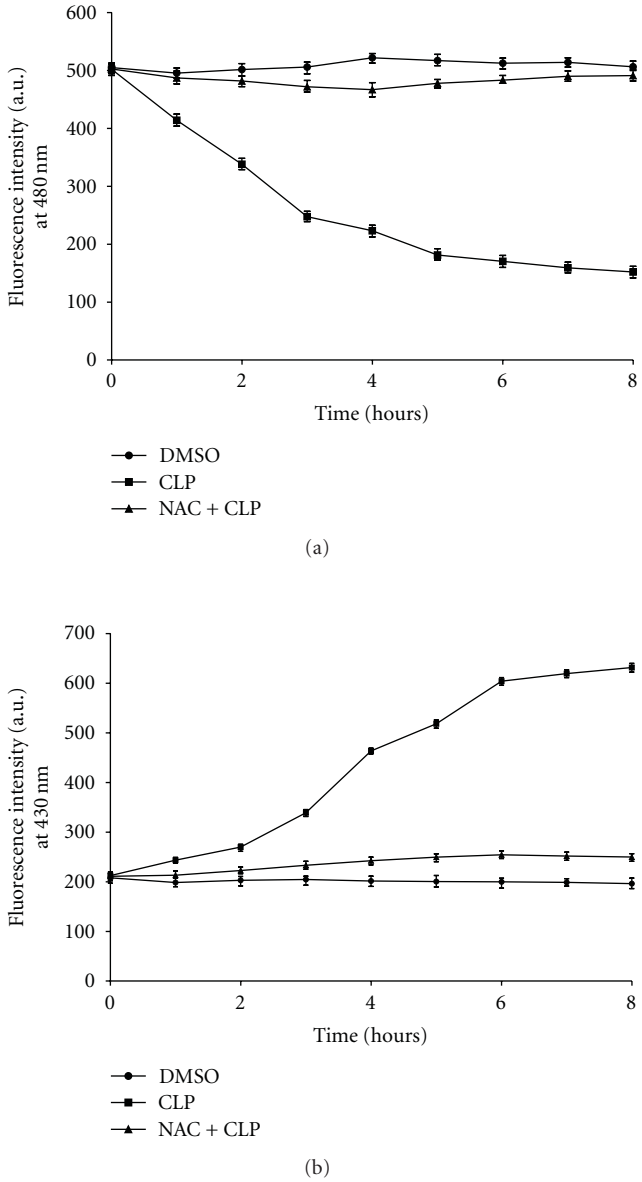


Figure 6: Determination of intracellular GSH level and level of lipid peroxidation in response to CLP treatment. (a) Level of intracellular GSH in treated and untreated *L. donovani* promastigotes. The intracellular GSH level was measured after treatment with 0.2% DMSO (closed circles), 20 μM CLP (closed squares) and with NAC (20 mM) before treatment with CLP (closed triangles). (b) The level of fluorescent products of lipid peroxidation was measured after treatment of leishmanial cells with 0.2% DMSO (closed circles), 20 μM CLP (closed squares) and with NAC (20 mM) before treatment with CLP (closed triangles). Data are represented as Mean $\pm$ SEM ($n = 3$).

death in response to CLP treatment. However, parasite ultrastructural studies by transmission electron microscopy led to some interesting observations. We observed multiple cytoplasmic vacuoles with normal nuclear architecture at an early stage after CLP treatment. This type of vacuolization was suggestive of autophagy [38, 39]. To understand the mechanism in more detail, we carried out staining with MDC which specifically labels autophagic vacuoles. It has been suggested previously that *Leishmania* contain a multivesicular tubule which is lysosomal in nature [31] and constitutes the endocytic compartment which is intimately involved with the autophagic pathway [32]. We observed clear MDC-positive vacuoles which colocalized with the multivesicular tubular compartment (FM4-64 positive) after treatment with CLP. These results confirm the involvement of autophagy in *L. donovani* AG83 promastigotes in response to CLP treatment.

To understand the relationship between autophagy induction and apoptosis-like cell death in more detail, we next carried out our study with 3-MA which is a specific inhibitor of autophagy [34]. MTT assay revealed that 3-MA and CLP cotreatment causes further decrease in the number of viable cells compared to CLP alone. This was further confirmed by flow cytometric analysis suggesting that autophagy serves as a survival mechanism and inhibition of autophagy can amplify the effect of CLP on *L. donovani* AG83 promastigotes. However, this effect is true only at an initial time period (2 h) as we observed no significant changes by inhibiting autophagy at a later time period (6 h). We surmise that at 6 hrs, CLP causes extensive damage to the cells which commits them to die rendering them unable to elicit the survival response.

Real-time PCR analysis revealed that there is a significant upregulation of ATG 8 transcript level in response to CLP treatment for 2 h though there was no significant change in the ATG 8 transcript level at 6 h compared to control untreated cells. This again confirmed the involvement of autophagy in the initial phase of CLP treatment. During past few years, the silent information regulator SIR2 protein family has attracted great interest due to its implication in an organism's life span extension [40]. It has been reported previously that Sir2 over expression promote survival of *Leishmania* parasites by preventing programmed cell death [36]. Also, transient overexpression of Sir2 has been clearly shown to stimulate the basal level of autophagy [20, 41]. Thus, we anticipated a role of Sir2 in CLP-induced cell death of *L. donovani* promastigotes. Rightfully, real-time PCR analysis revealed a significant upregulation in the Sir2 transcript level at 2 h after CLP treatment. This suggests that Sir2 may signal the onset of autophagy in response to CLP treatment. The fact that Sir2 can form molecular complex with several ATG genes and can deacetylate these proteins [41] explains the importance of Sir2 in the process.

From an evolutionary perspective, autophagy has been suggested to have originally evolved as a protective mechanism for unicellular eukaryotes against starvation and other environmental stresses [19]. Though the connection between autophagy and apoptotic cell death is not clear, autophagy has been reported to promote [42] or inhibit [33] apoptosis in cancer cells. There are also reports of autophagic cell death (type II cell death) in response to antimicrobial peptides in *L. donovani* [27] and in response to naphthoimidazoles in *T. cruzi* [34]. In the present study, we provide experimental evidence to show for the first time that autophagy represents a defense mechanism against

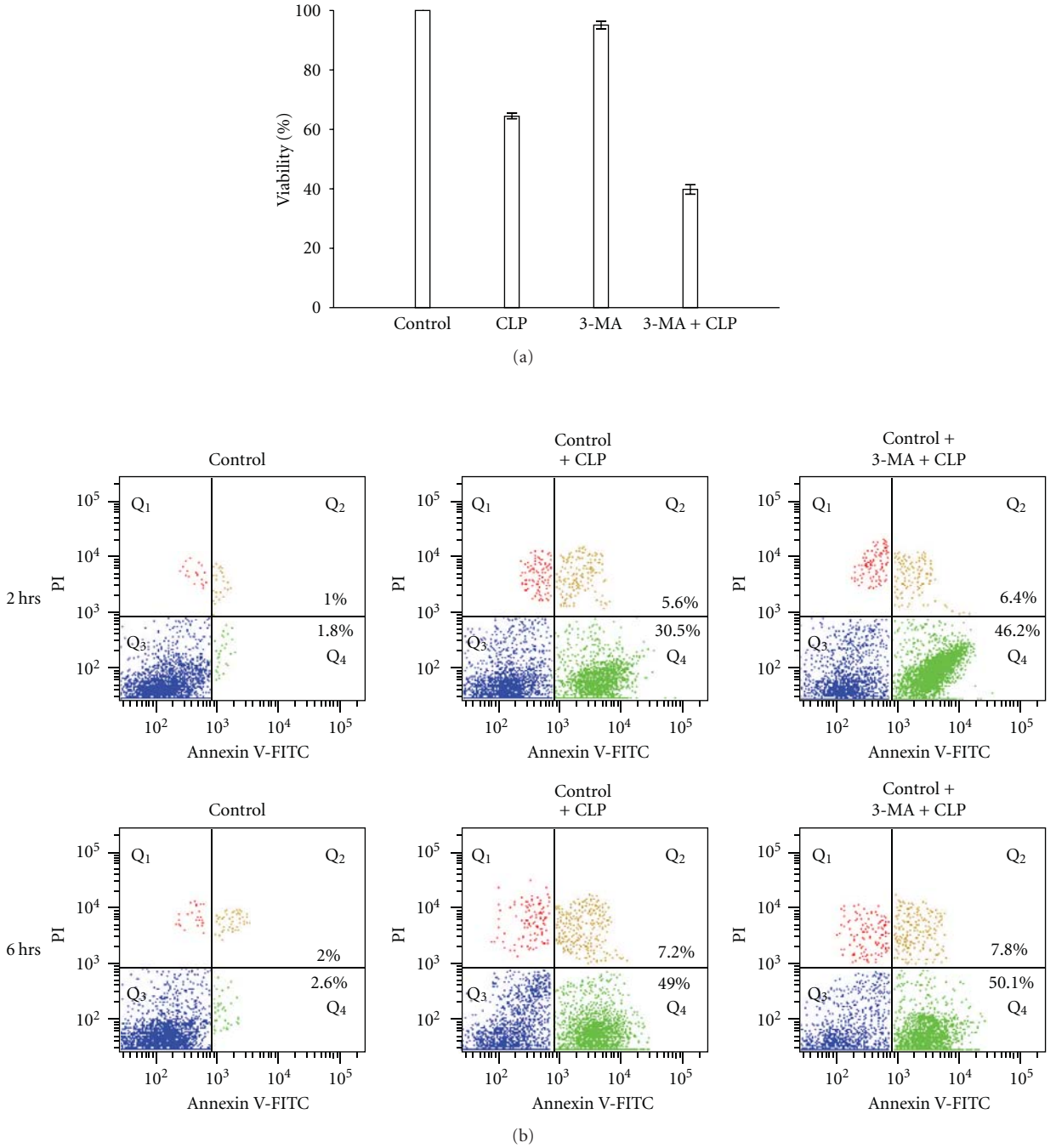
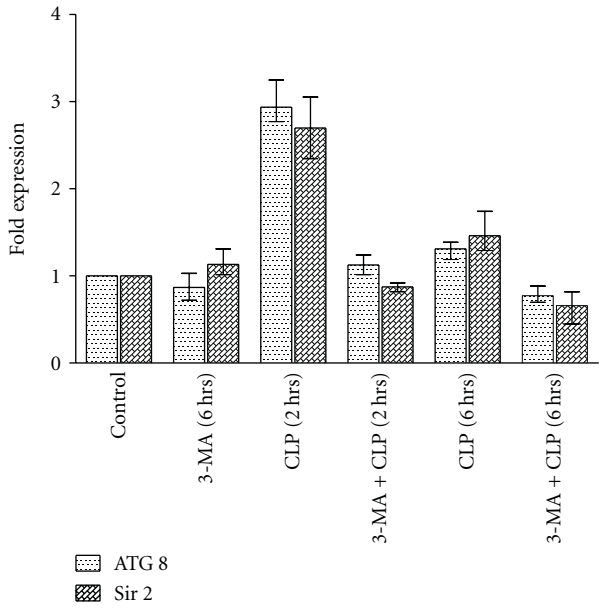
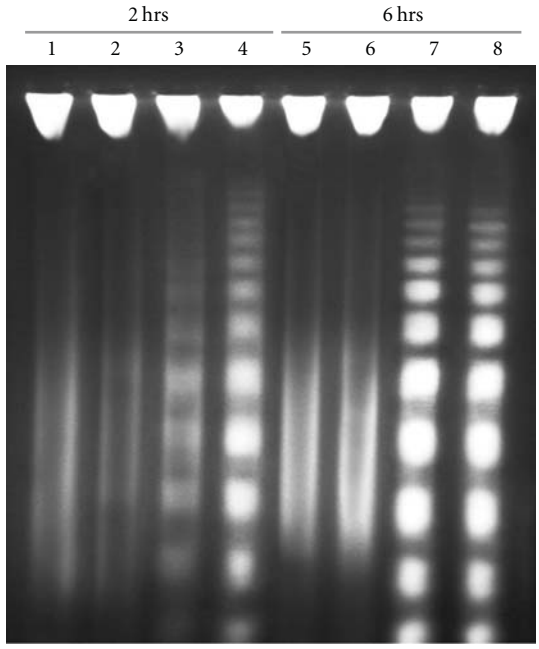


Figure 7: Effect of inhibition of autophagy by 3-MA, on CLP-induced cell death. (a) *L. donovani* promastigotes were treated with 0.2% DMSO, 20 μM CLP, 10 mM 3-MA and 10 mM 3-MA prior to treatment with CLP for 2 h and percentage of cell viability was measured by MTT assay. Data are represented as Mean ± SEM ($n = 3$). (b) Flow cytometric analysis using annexin V and PI in FL-1 versus FL-2 channels. The cells were subjected to different treatments as shown in the figure for 2 h and 6 h, respectively, as described in Section 2. The annexin V positive cells (bottom right quadrant) denote apoptotic population.

CLP-induced cell death in *L. donovani* AG83 promastigotes. We have also shown that the morphological and biochemical changes associated with autophagy precede the onset of apoptosis-like cell death in these unicellular kinetoplastid protozoan parasites. Though dissection of the underlying molecular events is beyond the scope of this study, we surmise that Sir2 is an important candidate in the regulation of the autophagic response. Moreover, our findings also suggest that inhibition of autophagy by 3-MA can actually increase the effectivity of CLP-mediated cell killing. This finding can lead to development of new therapeutic strategies to combat leishmaniasis in future.



(a)



(b)

Figure 8: (a) Real-time PCR analysis for the expression level of ATG 8 and Sir2 genes from *L. donovani* promastigotes. The expression of ATG 8 and Sir2 were estimated relative to GAPDH in the treated samples compared with the untreated control. The fold expression was calculated as described in Section 2. The mean fold expression values are given in Table 2. Data are represented as Mean ± SEM ($n = 3$). (b) Fragmentation of genomic DNA in the presence and in the absence of 3-MA and CLP for different time periods. Genomic DNAs were isolated from *L. donovani* promastigotes after treatment with 0.2% DMSO alone for 2 h (lane 1), 10 mM 3-MA for 2 h (lane 2), 20 μM CLP for 2 h (lane 3), and 20 μM CLP for 2 h after pretreatment with 10 mM 3-MA (lane 4). Lanes 5–8, the same as lanes 1–4, respectively, but for 6 h.

Abbreviations

CLP:	Cryptolepine
CPT:	Camptothecin
Sir2:	Silent information regulator 2
ATG 8:	Autophagic gene 8
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PI:	Propidium iodide
ROS:	Reactive oxygen species
DMSO:	Dimethyl sulfoxide
NAC:	*N*-Acetyl-L-cysteine
CM-H_2DCFDA:	5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester
MDC:	Monodansylcadaverine
PBS:	Phosphate buffered saline
3-MA:	3-methyladenine
FM4:	64-*N*-(3-triethylammoniumpropyl)-4-(6-(4(diethylamino)phenyl) hexatrienyl)pyridinium dibromide
MVT:	Multivesicular tubule.

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Research Article

Antiproliferative, Ultrastructural, and Physiological Effects of Amiodarone on Promastigote and Amastigote Forms of *Leishmania amazonensis*

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Amiodarone (AMIO), the most frequently antiarrhythmic drug used for the symptomatic treatment of chronic Chagas' disease patients with cardiac compromise, has recently been shown to have also specific activity against fungi, *Trypanosoma cruzi* and *Leishmania*. In this work, we characterized the effects of AMIO on proliferation, mitochondrial physiology, and ultrastructure of *Leishmania amazonensis* promastigotes and intracellular amastigotes. The IC_{50} values were 4.21 and 0.46 μM against promastigotes and intracellular amastigotes, respectively, indicating high selectivity for the clinically relevant stage. We also found that treatment with AMIO leads to a collapse of the mitochondrial membrane potential ($\Delta\Psi m$) and to an increase in the production of reactive oxygen species, in a dose-dependent manner. Fluorescence microscopy of cells labeled with JC-1, a marker for mitochondrial energization, and transmission electron microscopy confirmed severe alterations of the mitochondrion, including intense swelling and modification of its membranes. Other ultrastructural alterations included (1) presence of numerous lipid-storage bodies, (2) presence of large autophagosomes containing part of the cytoplasm and membrane profiles, sometimes in close association with the mitochondrion and endoplasmic reticulum, and (3) alterations in the chromatin condensation and plasma membrane integrity. Taken together, our results indicate that AMIO is a potent inhibitor of *L. amazonensis* growth, acting through irreversible alterations in the mitochondrial structure and function, which lead to cell death by necrosis, apoptosis and/or autophagy.

1. Introduction

Leishmaniasis is a parasitosis caused by different species of the *Leishmania* genus that affects about 12 million people around the world, with 90% of the cases reported in Afghanistan, Pakistan, Iran, Iraq, Syria, Saudi Arabia, India, Bangladesh, Nepal, Sudan, Algeria, Ethiopia, Brazil, Bolivia, Colombia, Ecuador, Peru, and Venezuela [1]. Approximately 21 species have been described to cause three different clinical manifestations: (1) cutaneous (CL), where the lesions are confined to the site of the inoculation by the sandfly; (2) mucocutaneous (MCL), which affects the mucosal tissues; (3) visceral (VL), where the parasites have a tropism for phagocytes mainly localized in the spleen and the liver. Visceral leishmaniasis is fatal if not treated, while some forms of cutaneous manifestations can cure spontaneously. According to WHO, around 70,000 deaths per year occur throughout the world [1]. In Brazil, *Leishmania amazonensis* is one of the species responsible for the cutaneous disease; however, in some individuals the immune system fails to

mount an appropriate response against the parasite, leading to clinical manifestations of diffuse cutaneous leishmaniasis [2].

Pentavalent antimonials such as meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostan) have been employed as first-line treatment for many decades [3]. In the case of resistance to pentavalent antimonials, second-line treatments using amphotericin B or pentamidine have been successfully used [4]. For visceral leishmaniasis, miltefosine (Impavido) has been recently employed by oral route in India as a first-line treatment [5]. However, the drug is teratogenic and has a narrow chemotherapeutic window [6]. More recently, combination treatments are emerging as first-line treatments for visceral leishmaniasis [7]. Toxic side effects and increasing resistance limit most of the current specific treatments for leishmaniasis, indicating that there is an urgent need to develop new drugs that are efficacious, safe, and more accessible for the patient populations.

Amiodarone (AMIO) is the antiarrhythmic class III drug most frequently used to treat arrhythmias in general as well as in patients with chronic Chagas' disease and cardiac compromise. The antiarrhythmic action in mammals has been well characterized and results from Ca^{2+} and K^{+}channel inactivation, but it has recently been shown that the drug also has selective activity against parasitic protozoa such as *Trypanosoma cruzi* and *Leishmania mexicana* [8–10] as well as a broad-spectrum antifungal action [11, 12]. The mechanisms of action of AMIO reported in these different microorganisms involve the inhibition of sterol biosynthesis, disruption of mitochondrial membrane potential ($\Delta\Psi m$), and Ca^{2+} homeostasis, as well as production of reactive oxygen species [8, 9, 11–13]. Apparently, these alterations in the mitochondrial metabolism trigger a sequence of cellular events leading to apoptosis-like cell death [13]. However, the action of AMIO on the mitochondrion of target cells is controversial, as some groups have implicated inhibition of mitochondrial respiration, mainly by the direct action of AMIO against complex I, II, and F0F1-ATP synthase [13] or by a rapid release of Ca^{2+} from the mitochondrial compartment due to a collapse of mitochondrial membrane potential ($\Delta\Psi m$) (see [8, 9]), while others suggest that AMIO is able to protect mitochondrial function [14–17].

In this study, we investigated the antiproliferative, ultrastructural, and physiological effects of amiodarone on promastigote and intracellular amastigote forms of *Leishmania amazonensis*. The results indicated that AMIO acts mainly by altering the mitochondrial ultrastructure and physiology but other deleterious effects were also observed, including lipid accumulation, loss of the plasma membrane integrity, and presence of autophagic-like structures, suggesting different types of cell death involved in the mechanism of action of AMIO.

2. Material and Methods

2.1. Parasites. MHOM/BR/75/Josefa strain of *Leishmania amazonensis* isolated from a patient with diffuse cutaneous leishmaniasis by C. A. Cuba-Cuba (Universidade de Brasilia, Brazil) was used in the present study. It has been maintained by hamster footpad inoculation and, in the case of promastigotes, axenically cultured in Warren's medium (brain heart infusion plus hemin and folic acid) [18] supplemented with 10% fetal bovine serum at 25°C. Infective promastigotes of the Josefa strain were used to obtain intracellular amastigotes in macrophage cultures.

2.2. Drug. Amiodarone (AMIO), {(2-butyl-3-benzofuranyl) -[4-[2-(diethylamino)ethoxi]-3,5-diiodophenyl]methanone hydrochloride}, was purchased from Sigma, dissolved in dimethyl sulfoxide as a 100 mM stock, and stored at −20°C.

2.3. In Vitro Antiproliferative Activities of Amiodarone. Growth experiments with promastigotes were initiated with 2.0×10^6 parasites/mL, and AMIO was added at different concentrations from concentrated stock solutions in DMSO after 24 h of growth. Cell densities were evaluated daily in a Neubauer chamber during 72 h of growth. To evaluate the effects of the AMIO on the *L. amazonensis* intracellular amastigotes, peritoneal macrophages from CF1 mice were harvested by washing them with RPMI medium (Gibco) and plated in 24-well tissue culture chamber slides, allowing them to adhere to the slides for 24 h at 37°C in 5% CO_2. Adherent macrophages were infected with metacyclic promastigotes at a macrophage-to-parasite ratio of 1 : 10 at 35°C for 2 h. After this time, noningested parasites were removed by washing and infected cultures were incubated for 24 h in RPMI (containing 10% of fetal bovine serum) without AMIO. Different concentrations of AMIO were added after 24 h of interaction, when the number of amastigotes per macrophage was in the range of two to four, and fresh medium with AMIO was added daily for 2 days. The cultures were fixed with 4% freshly prepared formaldehyde in phosphate buffer saline (PBS, pH 7.2) and stained with Giemsa for 15 min. The percentage of infected cells was determined by light microscopy. Association indexes (mean number of parasites internalized per cell, multiplied by the percentage of infected macrophages, and divided by the total number of macrophages) were determined and used as a parameter to calculate the intensity of infection in each condition used in this study. The 50% inhibitory concentrations (IC_{50}s) were calculated with the SigmaPlot (version 10) program. The results are expressed as the means of three independent experiments.

2.4. Tests of Viability in Macrophages. To evaluate the cytotoxicity effects of AMIO against the host cell cultures, macrophages were incubated with different concentrations of AMIO for 48 h, and exclusion tests with 0.1% trypan blue were carried out for 5 min. The percentages of dead and alive cells were determined after counting of 400 macrophages in randomly selected fields by light microscopy.

2.5. Estimation of Mitochondrial Transmembrane Electric Potential ($\Delta\Psi m$). $\Delta\Psi m$ of the control and AMIO-treated (6,10, and 15 μM) promastigotes was investigated using

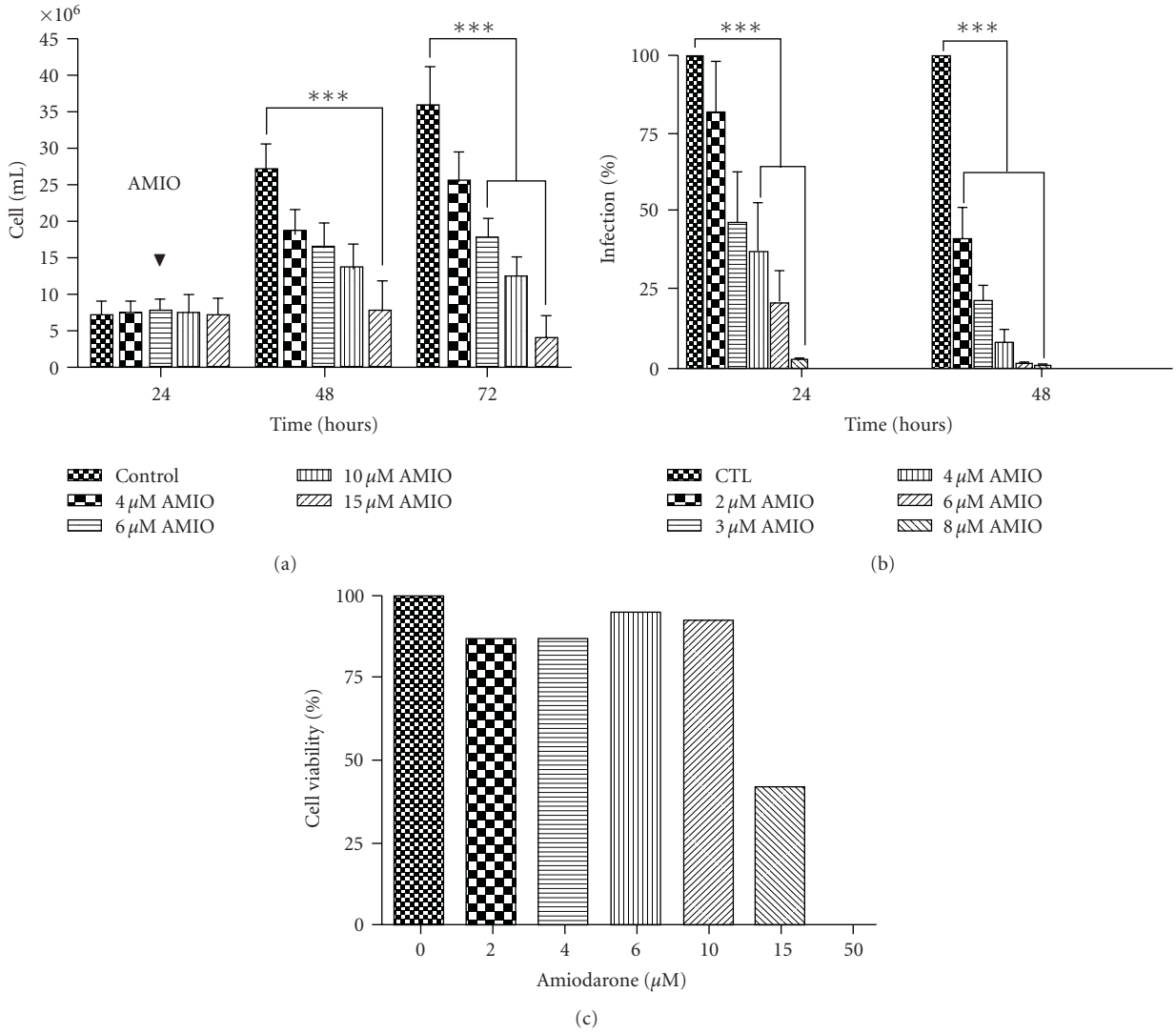

Figure 1: Antiproliferative and cytotoxic effects of amiodarone (AMIO) on *Leishmania amazonensis* promastigotes and intracellular amastigotes. (a) Treatment of promastigotes with different concentrations of AMIO for 48 h. (b) Effects of amiodarone on intracellular amastigote forms cultivated in macrophages. Murine host cells were cultivated for 24 h and infected with metacyclic promastigotes for 2 h. After 24 h of infection, AMIO at different concentrations was added to the cultures and treatment maintained for 48 h, with fresh medium with drug added every 24 h. (c) The cytotoxicity of AMIO was also evaluated in noninfected murine macrophages. After treatment with AMIO for 48 h, the macrophages were incubated with Trypan Blue and 400 cells were counted in randomly chosen fields under light microscopy. The experiments were carried out in triplicate, and the bars represent the standard deviation. Statistical analyses were obtained in Prism Software using 2-way ANOVA. The values of p were obtained comparing the control group with the treated groups: $^{***}P < .001$.

the JC-1 fluorochrome, which is a lipophilic cationic mito-chon-dri-al vital dye that becomes concentrated in the mitochondria in response to $\Delta\Psi m$. The dye exists as a monomer at low concentrations, where the emission is 530 nm (green fluorescence), but at higher concentrations it forms J-aggregates after accumulation in the mitochondrion, where the emission is 590 nm (red fluorescence). Thus, the fluorescence of JC-1 is considered an indicator of an energized mitochondrial state, and it has been used to measure the $\Delta\Psi m$ in *Leishmania* [19–21]. Control and AMIO-treated promastigotes after 48 h of treatment were harvested, washed in PBS, pH 7.2, added to a reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES/K^+ pH 7.2, 2 mM Pi, 1 mM $MgCl_2$, and 500 μM EGTA, and counted in a Neubauer chamber. To evaluate the $\Delta\Psi m$ for each experimental condition, 2.0×10^7 parasites were incubated in 10 μg/mL JC-1 during 30 min, with readings made every 1 min using a Molecular Devices Microplate Reader (a spectrofluorometer SpectraMax M2/M2^e). Cells were incubated in the presence of oligomycin (10 μM), a F0F1-ATP synthase inhibitor, or FCCP (1 μM), a mitochondrial protonophore, during the 30 min of experiment as positive controls of the depolarization of the mitochondrial membrane. FCCP at the concentration of 2 μM was added at the end of all

experiments to abolish $\Delta\Psi m$. This allowed comparison of the magnitude of $\Delta\Psi m$ under the different experimental conditions. The relative $\Delta\Psi m$ value was obtained calculating the ratio between the reading at 590 nm and the reading at 530 nm (590 : 530 ratio). Control and AMIO-treated promastigotes were also observed under a Zeiss Axioplan epifluorescence microscope using different optical filter sets: (1) for J-aggregate alone, we used 546 nm band-pass filter for excitation, with a 580 nm beam splitter, and a 590 nm long-pass for emission; (2) for monomer and J-aggregate, together, we used a 450–490 nm for excitation, with a 510 nm beam splitter, and a 520 nm long pass for emission. Each experiment was repeated at least three times in triplicate, and the figures shown are representative of these experiments.

2.6. Measurements of ROS Levels. Intracellular ROS level was measured in intact control and AMIO-treated promastigotes as described previously [19]. Briefly, cells (3.0×10^7) were washed, resuspended in 500 μL of PBS pH 7.2, and then incubated with the cell-permeable probe green H_2DCFDA at a concentration of 10 μg/mL for 1h at 25°C. After incubation, cells were analyzed at a Molecular Devices Microplate Reader (a spectrofluorometer SpectraMax M2/M2^e, Molecular Devices) using a pair of 507 nm and 530 nm as emission and excitation wavelengths, respectively.

2.7. Evaluation of Membrane Integrity and Nile Red Accumulation. Control and AMIO-treated (6,10, and 15 μM) promastigotes were harvested, washed in PBS, pH 7.2, and counted in a Neubauer chamber. After that, cells (5.0×10^6) were incubated with 1 μM SYTOX Blue and 10 μg/mL Nile Red for 20 minutes. The experiments were made in triplicate, using a black 96-well plate. The final volume in each well was 200 μL of cell suspension in PBS. The reading was done in a Molecular Devices Microplate Reader (a spectrofluorometer SpectraMax M2/M2^e) according to the following wavelengths for excitation and emission, respectively: 444 and 560 nm for SYTOX Blue, and 485 and 538 nm for the Nile Red. After the readings, control and AMIO-treated cells were observed under a Zeiss Axioplan epifluorescence microscope equipped with optical filters to SYTOX Blue (the same of DAPI) and Nile Red (450–490 nm for excitation, and 528 nm for emission). Each experiment was repeated at least three times in triplicate, and the figures shown are representative of these experiments.

2.8. Electron Microscopy. Control and AMIO-treated promastigotes were fixed for 3 h at 4°C in 2.5% glutaraldehyde (Sigma Chemical Co.) in 0.1M cacodylate buffer (pH 7.2). After fixation, cells were postfixed for 30 min in a solution containing 1% OsO_4 and 1.25% potassium ferrocyanide in 0.1 M cacodylate buffer, washed in the same buffer, dehydrated in acetone, and embedded in EPON. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Zeiss 900 electron microscope.

3. Results and Discussion

3.1. Antiproliferative Effects. L. amazonensis promastigotes were exposed to different concentrations of AMIO, and their proliferation was followed during 4 days. Figure 1(a) shows a concentration-dependent inhibition of the growth induced by the treatment. The 50% inhibitory concentration (IC_{50}) was 4.21 μM after 48 h of treatment. Inhibition of around 100% was obtained with a concentration of 15 μM AMIO, which also induced a delayed lytic effect. We also investigated the effects of AMIO on intracellular amastigotes, the clinically relevant form of the parasite. Macrophages were infected with metacyclic promastigotes and treated with different concentrations of the drug. The IC_{50} obtained was 0.46 μM after 48 h of treatment, with a total elimination of amastigotes when macrophages were treated with 6 μM for 48h (Figure 1(b)). Detailed inspection of treated cultures using light microscopy confirmed the potent antiproliferative effect against intracellular amastigotes, as no intact parasites were seen after treatment with concentrations $\geq$6 μM of AMIO for 48 h (Figure 2).

Comparing our results with those published for *L. mexicana* [9], another species of the same *Leishmania* subgenus but that is not relevant for the epidemiology of leishmaniasis in Brazil, the IC_{50} values found in this study were higher for both stages (4.21 μM for promastigotes and 0.46 μM for intracellular amastigotes compared with 900 nM and 8 nM, respectively, for *L. mexicana*), indicating a lower susceptibility of our *L. amazonensis* strain to the drug. These results are consistent with the known fact that patients infected with *L. amazonensis* are less responsive to the available anti-*Leishmania* treatments [2]. It is interesting to point out that the clinically relevant intracellular amastigote form of the parasite had a ten-fold higher susceptibility to amiodarone than the extracellular promastigotes, a result similar to those obtained for *T. cruzi* [8] and *L. mexicana* [9].

Using the cytotoxicity trypan blue test, viable cells were evaluated in macrophage cultures exposed to AMIO at concentrations varying between 2 and 50 μM for 48 h (Figure 1(c)). The cytotoxicity concentration to reduce 50% (CC_{50}) of viable macrophages was 12.9 μM (Figure 1), giving a mean selectivity index of 28. Light microscopy showed that macrophages treated with 6 and 8 μM AMIO did not present any significant alteration (Figure 2). In several mammalian cell lines, AMIO has been described to be very toxic [17, 22, 23]. However, the selectivity of AMIO for trypanosomatid parasites over mammalian cells and organisms has been confirmed in experimental models of Chagas' disease and leishmaniasis [8–10] and more recently in clinical studies [24, 25].

3.2. Effects of AMIO on the Neutral Lipid Accumulation and on the Fine Structure of L. amazonensis Promastigotes. In order to evaluate the effect of AMIO on the plasma membrane integrity and the presence of lipid-storage bodies in *L. amazonensis* promastigotes, we performed a quantitative analysis after simultaneous incubation of the cells with SYTOX Blue and Nile Red. The results indicated that at the higher concentration used (15 μM AMIO), the plasma

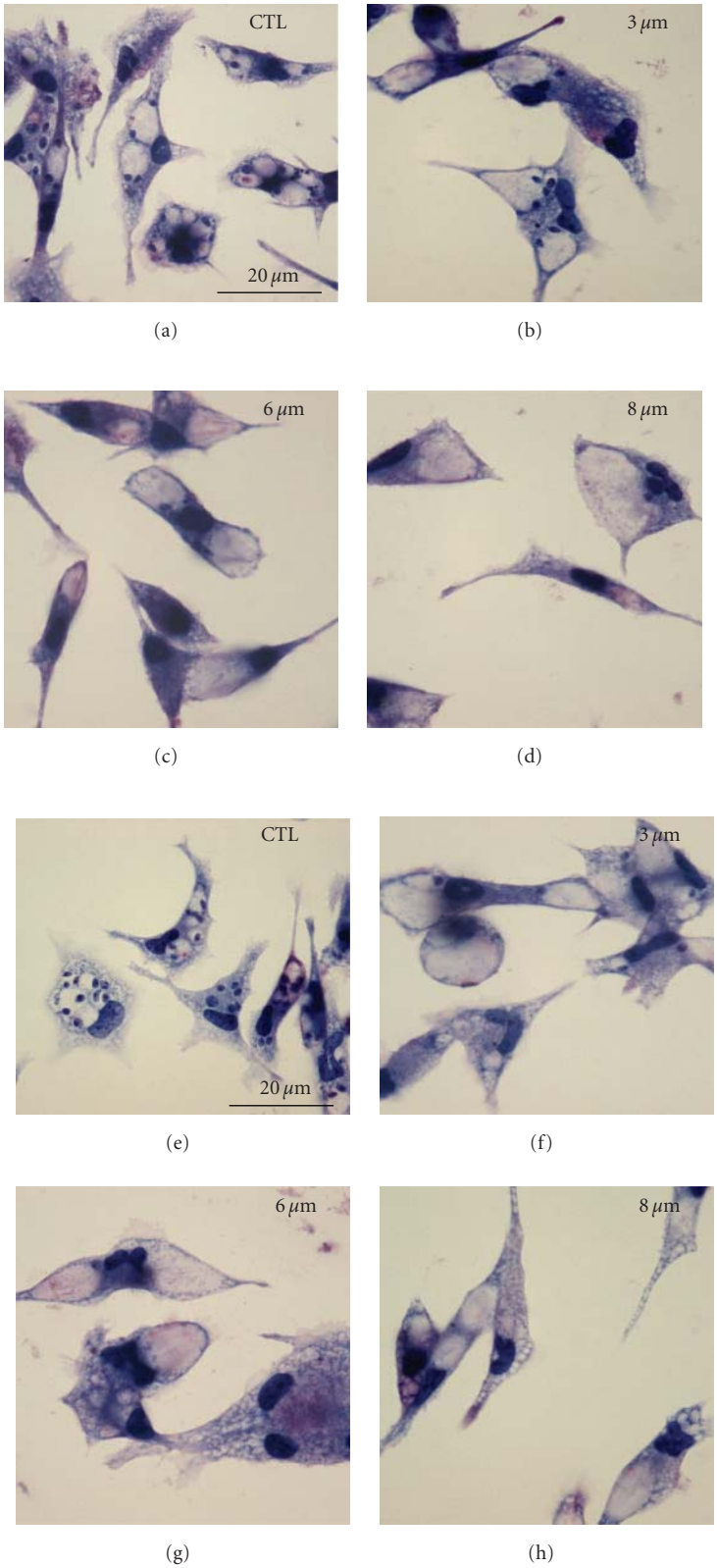

Figure 2: Light microscopy of intracellular amastigote forms of *Leishmania amazonensis* treated with different concentrations of amiodarone (AMIO). (a–d) After 24 h of treatment, it is possible to observe a significant reduction in the parasite number with 6 and 8 μM AMIO (c, d). (e–h) After 48 h of treatment, a significant reduction was observed in the cultures treated with 3 μM AMIO (f). Infection of macrophages was carried out as described in Materials and Methods and Figure 1. For all the images, the scale bars are similar to those observed in the panels (a) and (e). (a) Control parasites/24 h of treatment, which means 48 h of infection; (e) Control parasites/48 h of treatment, which means 72 h of infection.

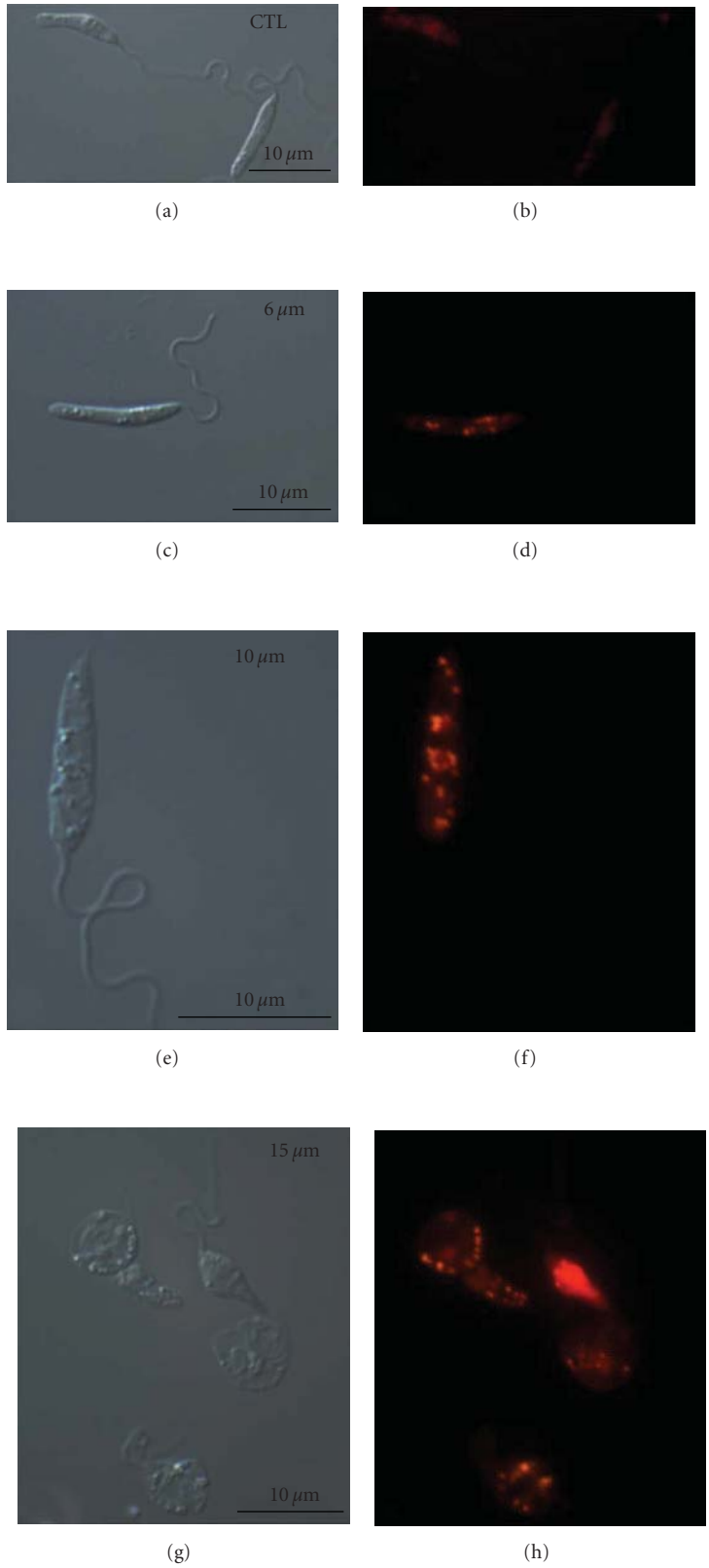

Figure 3: Differential interference contrast microscopy (DIC) and fluorescence microscopy with Nile Red of *Leishmania amazonensis* promastigotes untreated (a-b) and treated with different concentrations of AMIO (6, 10 and 15 μM) for 48 h (c–h, resp.). In the treated promastigotes, images suggest an accumulation of lipid-storage bodies in the cytoplasm, which is concentration dependent. In (g), parasites treated with 15 μM AMIO are completely modified. The conditions used to harvest the parasites, to incubate with Nile Red and to observe under the microscopy using the correct filters sets are described in Materials and Methods.

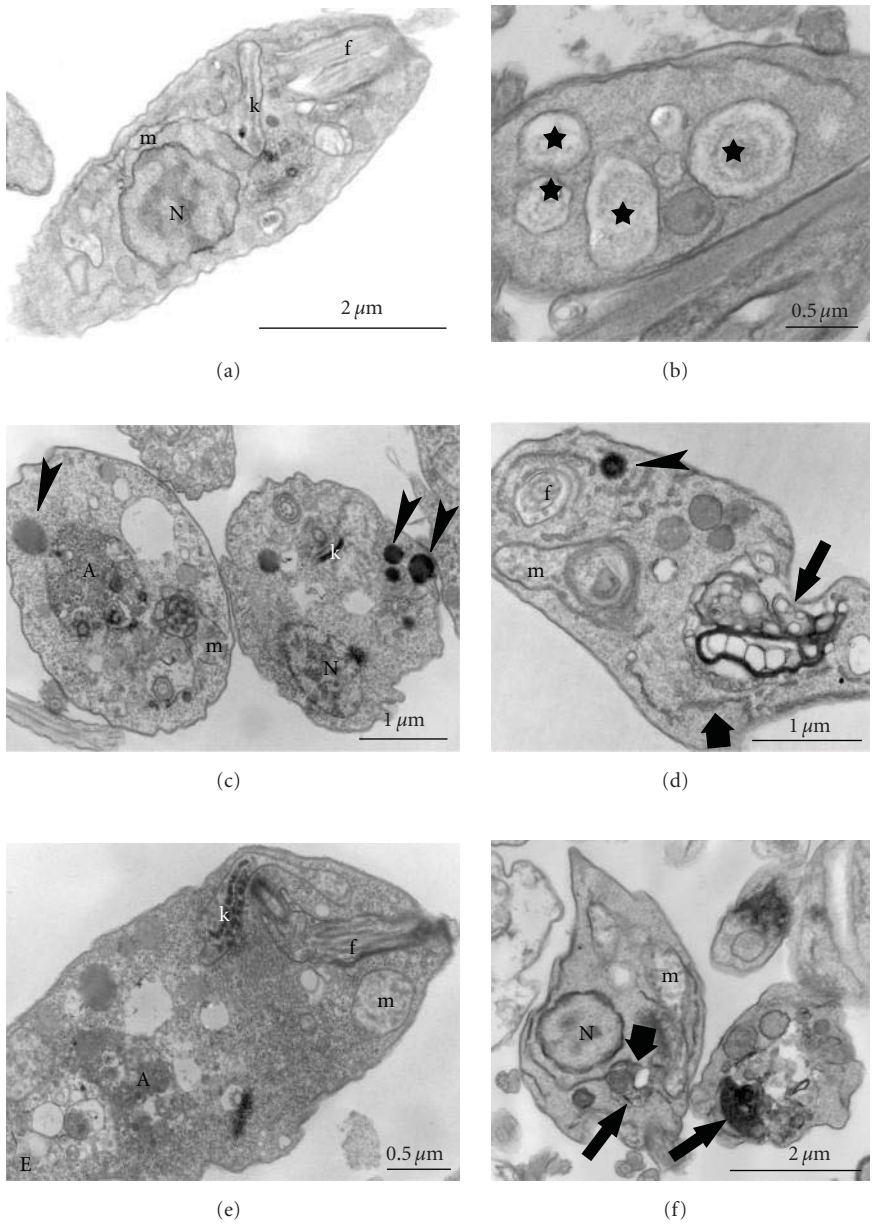

Figure 4: Different ultrastructural alterations on *Leishmania amazonensis* promastigotes induced by the treatment with amiodarone (AMIO). (a) Ultrathin section of *L. amazonensis* promastigotes without treatment, which presents a normal ultrastructure of organelles such as (mitochondrion) m , (kinetoplast) k , (nucleus) N and (flagellum) f . (b) Electron micrograph of *L. amazonensis* treated with 5 μM AMIO for 48 h presenting many vacuoles similar to autophagosomes (stars). (c–e) After treatment with 15 μM AMIO for 24 h, it is possible to observe the presence of large autophagosomes a associated with endoplasmic reticulum profiles (big arrow), lipid bodies (arrowheads), and alterations in the mitochondrion–kinetoplast complex and chromatin condensation. (f) Promastigotes treated with 20 μM AMIO for 24 h presented drastic alterations and destruction of the cytoplasm, where it is possible to observe the presence of autophagosomes (arrows) sometimes associated with endoplasmic reticulum profile (big arrow). A: autophagosome; f: flagellum; k: kinetoplast; m: mitochondrion; N: nucleus.

membrane permeability was significantly altered (data not shown). This effect was not observed at lower concentrations (6 and 10 μM AMIO).

On the other hand, incubation with Nile Red revealed a concentration-dependent effect on neutral lipids' accumulation, which was quantified by fluorimeter (data not shown). The visualization of AMIO-treated promastigotes under fluorescence microscopy revealed the presence of many lipid bodies positive to Nile Red and randomly distributed throughout the cytoplasm (Figures 3(a)–3(h)). The fluorescence images indicate a concentration-dependent increase in the number of lipid bodies (Figures 3(d), 3(f), and 3(h)), while differential interference contrast microscopy (DIC) revealed an important alteration in the shape of

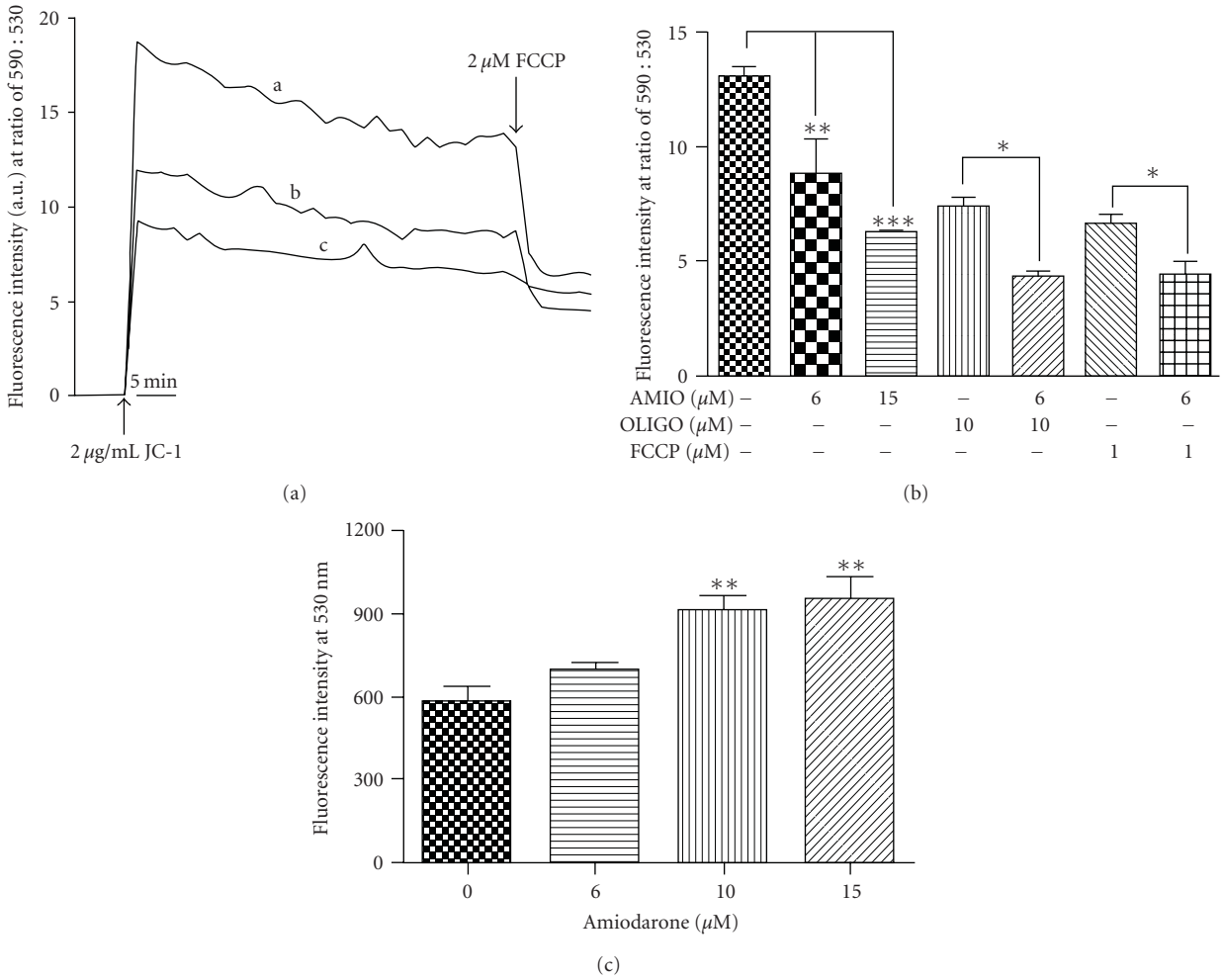

Figure 5: Effects of the AMIO on the mitochondrial function of *Leishmania amazonensis* promastigotes. (a) Values of $\Delta\Psi m$ were evaluated during 30 min, after which the uncoupler FCCP was added to collapse mitochondrial potential. Cells grown in the presence of two concentrations of amiodarone, 6 and 15 μM, for 48 h (curves b and c, resp.) were observed. (b) Effect of oligomycin (10 μM) and FCCP (1 μM) in the $\Delta\Psi m$ was also evaluated. (c) Production of ROS was measured in the same cells. Control and treated cells were incubated with H_2DCFDA, and the fluorescence intensity was measured in a Microplate Reader. Fluorescence intensity is expressed as arbitrary units (A.U.). The experiments were performed three times, each time in triplicate, and the figures shown are representative of these experiments.

promastigotes treated with 15 μM AMIO, which appeared rounded and swollen with a clear evidence of loss of the cytoplasmatic content (Figure 3(g)), confirming the alteration on the plasma membrane integrity indicated by SYTOX Blue staining. These results are consistent with the reported effect of AMIO on the *de novo* sterol biosynthesis in *T. cruzi* [8] and *L. mexicana* [9]. Similar results were obtained with *L. amazonensis* treated with squalene synthase inhibitors [26].

We also evaluated the effect of AMIO on the fine structure of *L. amazonensis* promastigote. Figure 4(a) shows a longitudinal section of a control promastigote presenting different organelles such as mitochondrion, nucleus, kinetoplast, and flagellum with normal ultrastructure. Parasites incubated in the presence of AMIO displayed significant morphological changes, which varied from discrete alterations to a total destruction of the parasite, depending on the drug concentration and length of incubation. The changes began to appear with 5 μM AMIO after 48 h of incubation, when it was possible to observe the presence of some vacuoles similar to autophagosomes (Figure 4(b), stars). With just 24 h of treatment, but using higher concentrations (15 and 20 μM), several alterations could be observed such as presence of lipid-storage bodies (arrowheads), large myelin-like figures (arrow) with presence of endoplasmic reticulum profiles (big arrow), and autophagic structures containing cellular debris (Figures 4(c)–4(f)). These structures could be related to a degradation of damaged organelle induced by the drug treatment. In addition, alterations in the mitochondrion, in the kinetoplast, and in the chromatin condensation can be observed in Figures 4(c)–4(d). We also observed the presence of several small vesicles inside the flagellar pocket (Figure 4(e)) and many cells swollen and completely destroyed after treatment with 15 and 20 μM AMIO for 24 h (Figure 4(f)). All these alterations are characteristic of the

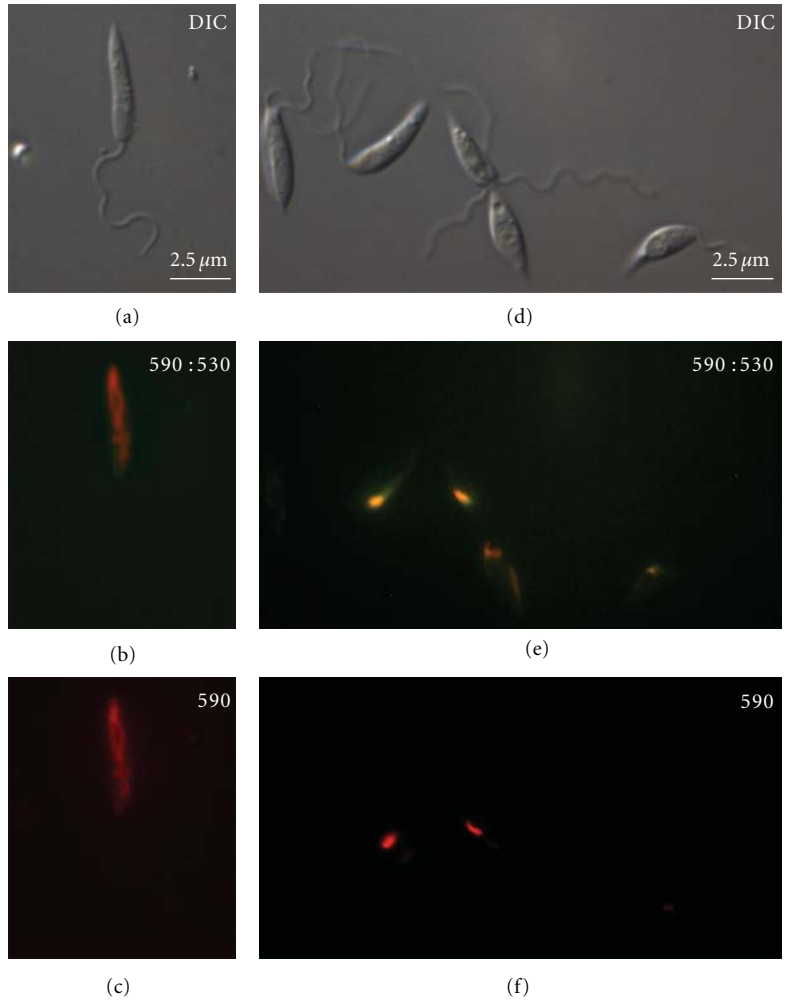

Figure 6: Differential interference contrast (DIC) microscopy and fluorescence microscopy with JC-1 of *Leishmania amazonensis* promastigotes untreated (a–c) and treated with 15 μM AMIO for 48 h (d–f). In panels (b) and (c), the accumulation of aggregated-JC1 is observed in the whole extension of the control mitochondrion. In cells treated with 15 μM AMIO (e-f), the accumulation of J-aggregates occurs in some portions of the mitochondrion, indicating a partial dissipation of the $\Delta\Psi m$. Panels (b) and (e) show an image of the monomers and J-aggregates together , while panels (c) and (f) show the image of only J-aggregates.

three main types of cell death: apoptosis, necrosis, and autophagy (reviewed in [27]).

Some of the lipid bodies appeared near the plasma membrane and the autophagic-like structures, which could be related, respectively, to alterations of the biophysical properties of the plasma membrane and degradation of abnormal lipids that accumulated as a consequence of the treatment. Thus, alterations of lipid composition resulting from treatment with AMIO could interfere with plasma membrane integrity, leading to cell death by necrosis.

3.3. Effects of AMIO on the Mitochondrial Physiology and Ultrastructure of L. amazonensis Promastigotes. We investigated the effect of AMIO on the mitochondrial function and ultrastructure using three criteria: mitochondrial transmembrane electric potential ($\Delta\Psi m$) using JC-1 fluorochrome, production of reactive oxygen species (ROS) using a green H_2DCFDA probe, and transmission electron microscopy. JC-1 is a cell-permeant cationic lipophilic fluorochrome that accumulates in the functional mitochondrion forming red-fluorescent J-aggregates. On the other hand, mitochondrial de-energization leads to an accumulation of green fluorescence monomers. Thus, the decrease in the red/green fluorescence intensity ratio indicates a collapse in the mitochondrial transmembrane potential. There are some advantages of using the JC-1: (1) it is not necessary to permeabilize the cells; (2) it is easy to quantify in a fluorimeter as well as to observe the process under a fluorescence microscopy. Promastigotes were treated with 0, 6, 10, and 15 μM AMIO for 48 h prior to the analysis of the mitochondrial features. Incubation with JC-1 for 30 min indicated that cells treated with 6 and 15 μM AMIO had a very significant reduction of $\Delta\Psi m$ (Figure 5(a), traces b and c) when compared with the control (untreated) parasites (Figure 5(a), trace a). The results indicated a marked reduction in the mitochondrial

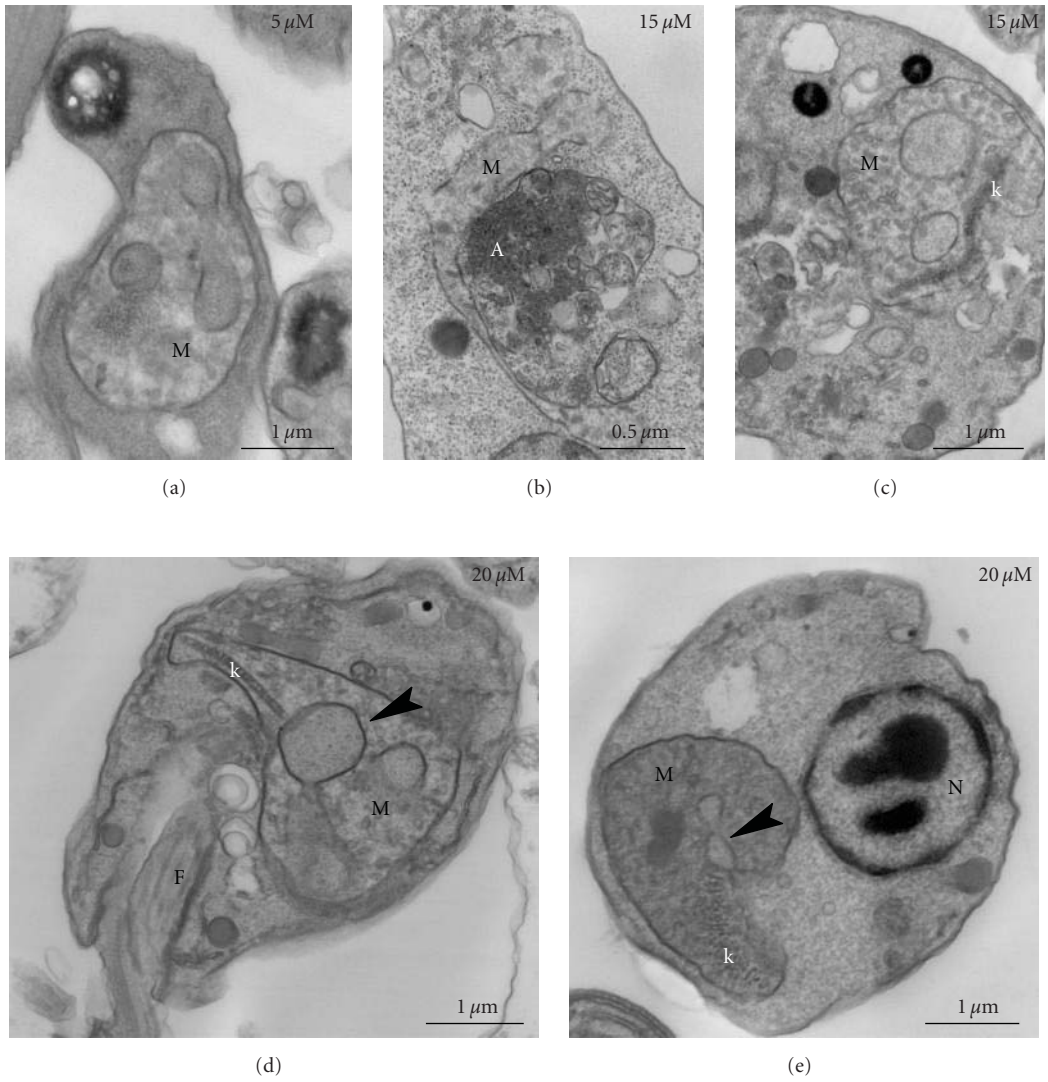

Figure 7: Ultrathin sections of *L. amazonensis* promastigotes treated with different concentrations of AMIO showing several alterations in the mitochondrial ultrastructure such as marked swelling (a, c, d, and e) with loss of the matrix content (a), alterations in the mitochondrial membrane (arrowheads), and presence of autophagic structures near a modified mitochondrion (b). A: autophagic structure; F: flagellum; k: kinetoplast; M: mitochondrion, N: nucleus.

polarization with 6 μM AMIO (Figure 5(a), trace b), and an almost total depolarization of the mitochondrial membrane potential with 15 μM AMIO (Figure 5(a), trace c). After 30 min of JC-1 uptake, 2 μM FCCP was added to fully collapse the $\Delta\Psi m$: it can be seen that in control parasites the release of JC-1 was more prominent than in treated promastigotes. To compare these findings with other situations that can induce depolarization of the mitochondrion, control and treated parasites were incubated with two classical inhibitors of the mitochondrial function: olygomicin, which is an inhibitor of the F0F1-ATP synthase, and FCCP, a classical protonophore uncoupler that dissipates the mitochondrial electrochemical H+ gradient (Figure 5(b)). It can be seen that the reduction of $\Delta\Psi m$ found in cells grown in the presence of 6 and 15 μM AMIO (Figure 5(a)) is similar to those obtained in control cells incubated with olygomicin or FCCP (Figure 5(b)). In addition, when these inhibitors were added in AMIO-treated promastigotes, the decrease of the $\Delta\Psi m$ was more evident.

We also investigated the effect of AMIO on the production of reactive oxygen species (ROS), as it is known that inhibition of oxidative phosphorylation induces an increase in the production of ROS. The results shown in Figure 5(c) indicate that cells grown in the presence of AMIO at the same concentrations used to evaluate the $\Delta\Psi m$, showed very significant and concentration-dependent increase in the production of ROS. The effect was most evident with 10 and 15 μM AMIO.

Alterations of the mitochondrion were also investigated using fluorescence and transmission electron microscopy, confirming the results obtained at the fluorimeter. Visualization of control and AMIO-treated promastigotes incubated with JC-1 under fluorescence microscopy revealed

that cells grown with 15 μM AMIO for 48 h showed reduced accumulation of the fluorochrome, restricted to the kinetoplast region of the single giant mitochondrion (Figures 6(e) and 6(f)), indicating a loss of only $\Delta\Psi m$ in most parts of the organelle. In contrast, in control promastigotes, JC-1 accumulated in the whole extension of the ramified mitochondrion (Figures 6(b) and 6(c)). Images (b) and (e) show the fluorescence for monomers and J-aggregates, together, while the images (c) and (f) show the fluorescence for J-aggregates alone.

Finally, ultrastructural alterations on the mitochondrion were also investigated: in Figure 7 the occurrence of dramatic modifications in different aspects of the organelle can be seen. It is evident that long-term incubation with AMIO induced mitochondrial swelling at all the concentrations tested (Figures 7(a), 7(c), 7(d) and 7(e)), which is followed by alterations in the mitochondrial membranes (Figures 7(d) and 7(e), arrowheads), appearance of circular cristae (Figure 7(a) and 7(c)), and loss of the mitochondrial matrix content (Figures 7(a) and 7(d)). In addition, we also observed an important interaction between the mitochondrion and structures similar to autophagic vacuoles (Figure 7(c)). In this same figure, alterations in the kinetoplast structure are also evident. Ultrastructural alterations on the mitochondrion also predominate in *T. cruzi* and *Leishmania* cells treated with sterol biosynthesis inhibitors, including quinuclidine derivatives, azoles and azasterols, indicating that this organelle is an important target for compounds that interfere with sterol and lipid composition [27–35].

The dose-dependent reduction of $\Delta\Psi m$ induced by amiodarone was correlated with the increase in the production of reactive oxygen species and mitochondrial ultrastructural alterations detected with transmission electron microscopy. Two possible explanations for these combined alterations are: (1) Calcium release from the mitochondrion, which would result from a direct action of AMIO on the organelle and lead to apoptotic cell death [36]; and, (2) Sterol biosynthesis inhibition, as observed in fungi [37] and trypanosomatids [8, 9], leading to an important alteration in the lipid composition of the mitochondrial membranes that should modify their biophysical properties [28, 38, 39], and loss of mitochondrial function.

4. Conclusion

In conclusion, the results of this work support the potential usefulness of AMIO as a chemotherapeutic agent against *Leishmania amazonensis*. Such activity is mainly mediated by profound and selective effects on the ultrastructure and physiology of the parasite mitochondrion, which culminate in cell death by necrosis, apoptosis, or autophagy. More studies are necessary to better characterize the different types of cell death associated with the mechanisms of action of AMIO and the activity of the drug in murine models of cutaneous leishmaniasis by *L. amazonensis*.

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Research Article

Screening the MayBridge Rule of 3 Fragment Library for Compounds That Interact with the *Trypanosoma brucei myo*-Inositol-3-Phosphate Synthase and/or Show Trypanocidal Activity

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Inositol-3-phosphate synthase (INO1) has previously been genetically validated as a drug target against *Trypanosoma brucei*, the causative agent of African sleeping sickness. Chemical intervention of this essential enzyme could lead to new therapeutic agents. Unfortunately, no potent inhibitors of INO1 from any organism have been reported, so a screen for potential novel inhibitors of *T. brucei* INO1was undertaken. Detection of inhibition of *T. brucei* INO1 is problematic due to the nature of the reaction. Direct detection requires differentiation between glucose-6-phosphate and inositol-3-phosphate. Coupled enzyme assays could give false positives as potentially they could inhibit the coupling enzyme. Thus, an alternative approach of differential scanning fluorimetry to identify compounds that interact with *T. brucei* INO1 was employed to screen ~670 compounds from the MayBridge Rule of 3 Fragment Library. This approach identified 38 compounds, which significantly altered the T_m of TbINO1. Four compounds showed trypanocidal activity with ED50s in the tens of micromolar range, with 2 having a selectivity index in excess of 250. The trypanocidal and general cytotoxicity activities of all of the compounds in the library are also reported, with the best having ED50S of ~20 μM against *T. brucei*.

1. Introduction

Human African Trypanosomiasis (HAT), also called African sleeping sickness, is caused by the extracellular protozoan parasite *Trypanosoma brucei*. HAT is a potentially fatal disease with ~200 000 new cases per year in sub-Saharan Africa [1]. Despite this, current drugs are often toxic and difficult to administer, highlighting the urgent need for new, more effective drug therapies. *T. brucei* is able to survive in the hosts' bloodstream due to a dense coat (5×10^6 dimers/cell) of variant surface glycoprotein (VSG) [2, 3]. This coat acts as a diffusion barrier and enables the cell to avoid the hosts' innate immune system by a specialised process of antigenic variation, utilising a repertoire of more than 1000 different VSG genes [4, 5]. Although the VSG coat is systematically changed, it is always attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor [6, 7]. GPI anchors are ubiquitous to eukaryotes and comprise of the basic core structure of $NH_2CH_2CH_2PO_4$H-6Manα1-2Manα1-6Manα1-4GlcNα1-6D-*myo*-inositol-1-HPO_4-lipid (EtN-*P*-Man_3GlcN-PI), with a lipid moiety of diacylglycerol, alkylacylglycerol, or ceramide [8]. Previously, the biosynthesis of GPI anchors in bloodstream form *T. brucei* has been both genetically and chemically validated as a therapeutic drug target [9–11].

The *de novo* synthesis of *myo*-inositol is a ubiquitous process occurring in almost all eukaryotes studied. It is the result of the concerted actions of two enzymes: firstly an D-*myo*-inositol-3-phosphate synthase (INO1) which isomerases glucose-6-phosphate to D-*myo*-inositol-3-phosphate and secondly, an inositol monophosphatase which dephosphorylates the D-*myo*-inositol-3-phosphate to yield *myo*-inositol [12]. Previously, through the creation of a conditional knockout cell line of INO1, it was demonstrated that

the *de novo* synthesis of *myo*-inositol is essential to the survival of bloodstream form *T. brucei* [13, 14]. Surprisingly, the deletion of INO1 could not be overcome by the inclusion of extra *myo*-inositol in the media, which is in striking contrast to all other INO1 null mutants described to date. Intriguingly, further analysis showed that there was no significant decrease in the level of *myo*-inositol in the conditional knockout cells grown under nonpermissive conditions, showing that the cells were not exhibiting the typical "inositol-less" death phenotype described for all other INO1 mutants [12, 15, 16]. *In vivo* labelling and localisation studies of INO1 [13, 14] and the *T. brucei* phosphatidylinositol synthase [17] suggested that the *de novo* synthesised *myo*-inositol is the primary source of *myo*-inositol used in the formation of phosphatidylinositol for GPI anchors and that there was a distinction or compartmentalisation of the *de novo* synthesised *myo*-inositol from that obtained from extracellular sources. The clear dependence by bloodstream form *T. brucei* on *de novo* synthesised *myo*-inositol for GPI anchor biosynthesis has not been described for any other organism to date and shows a unique avenue which could be exploited for future therapeutic drug design.

The MayBridge Rule of 3 Fragment Library (May Ro3) is a relatively small collection of chemical entries that are pharmacophore rich. The rule of 3 refers to compliance to the following criteria: MW $\leq$ 300, cLogP $\leq$ 3.0, H-Bond Acceptors $\leq$ 3, H-Bond Donors $\leq$ 3, Rotatable bonds (Flexibility Index) $\leq$ 3, and Polar Surface Area $\leq$ 60 Å [18]. The library has quantifiable diversity through the application of standard chemometrics, assured aqueous solubility to $\geq$1 mM using LogS and high purity ($\geq$95%).

As *T. brucei* INO1 (TbINO1) is a genetically validated drug target and screening for inhibitors is problematic due to the nature of the reaction and/or the use of a coupled enzyme assay, differential scanning fluorimetry was employed to look for compounds that interact with TbINO1. Thus, ~670 compounds from the May Ro3 fragment library were screened and their trypanocidal and general cytotoxic activities determined.

2. Experimental

2.1. Materials. All materials unless stated were purchased either from Sigma/Aldrich or Invitrogen. Access to the Maybridge Rule of 3 (May Ro3) library, was kindly provided by Dr Rupert Russell (St Andrews). Stock solutions of the compounds (2 M) were prepared in DMSO and kept in master plates at 200 mM in DMSO (100%) by Dr Margaret Taylor (St Andrews). These were replated into daughter (working) plates occupying the central 80 wells of a 96-well plate, at 10 mM in 5% DMSO, allowing the two outside columns for positive and negative controls.

2.2. TbINO1 Protein Overexpression and Purification. Large-scale recombinant expression and purification of TbINO1 was conducted using the construct pET15b-TbINO1 in BL21 Rosetta (DE3) cells, and TbINO protein was purified by Ni affinity chromatography, eluted with 100 mM imidazole, 20 mM Tris pH 7.5, and 300 mM NaCl. The His-tagged protein was then dialysed against 20 mM Tris pH 7.5, 50 mM NaCl, 5 mM DTT and stored with 20% glycerol, at -80°C for up to 12 months without loss of activity. Full details of expression vector construction and purification will be published elsewhere (Martin, K. L. and T. K. Smith unpublished).

2.3. Differential Scanning Fluorimetry with TbINO1. Differential scanning fluorimetry was set up in 96-well PCR plates using a reaction volume of 100 μL. Shifts in TbINO1 T_m with ligand binding were observed when ammonium acetate and NAD^+ were present. Samples contained 2 μM TbINO1, 2 mM Ammonium Acetate, 1 mM NAD^+, 10 mM HEPES pH 7.5, 50 mM NaCl, and 1.25 working stock of Sypro Orange (Invitrogen, as a 5000 times stock). Compounds from the May Ro3 fragment library (1 mM) and positive controls, glucose-6-phosphate as substrate (5 mM) and 2-deoxy-glucose-6-phosphate as inhibitor (4 mM) were added as required.

Differential fluorimetric scans were performed in a real-time PCR machine (Stratagene Mx3005P with software MxPro v 4.01) using a temperature scan from 25°C to 95°C at 0.5°C min^{-1}. Data were then exported to Excel for analysis using "DSF analysis" modified from the template provided by Niesen et al. [19]. T_m values were calculated by nonlinear regression, fitting the Boltzmann equation to the denaturation curves using GraFit.

2.4. Cytotoxicity Studies. The trypanocidal activity of all compounds (final 0.5 mM, 0.5% DMSO) against cultured bloodstream *T. brucei* (strain 427) was determined using the Alamar Blue viability test as described previously [20].

Cytotoxic effects against HeLa and A549 cells were determined in a similar manner. Briefly, the cells were cultured in DMEM supplemented with 10% FCS and 2 mM L-Glutamine. Cells were plated at initial cell concentration of 2×10^4 cells/well and incubated with the compounds for ~65 hours prior to addition of Alamar Blue solution for a further 5 hours.

3. Results and Discussion

3.1. Is TbINO1 Amenable to Differential Scanning Fluorimetry? . Inositol-3-phosphate synthase has previously been genetically validated as a drug target against *T. brucei* [13, 14], and is a prime candidate for chemical intervention as a therapeutic against African sleeping sickness. Unfortunately, no potent inhibitors of INO1 from any organism have been reported; therefore, it was decided to undertake a screen for potential novel inhibitors of TbINO1. Screening for inhibitors of TbINO1 is problematic due to the difficulty in following the reaction, that is, having to directly differentiate between glucose-6-phosphate and inositol-3-phosphate, or alternatively using a coupled enzyme assay, where a compound could potentially inhibit the coupled enzyme [14]. Thus, an alternative approach was taken, using differential scanning fluorimetry. Differential scanning fluorimetry has

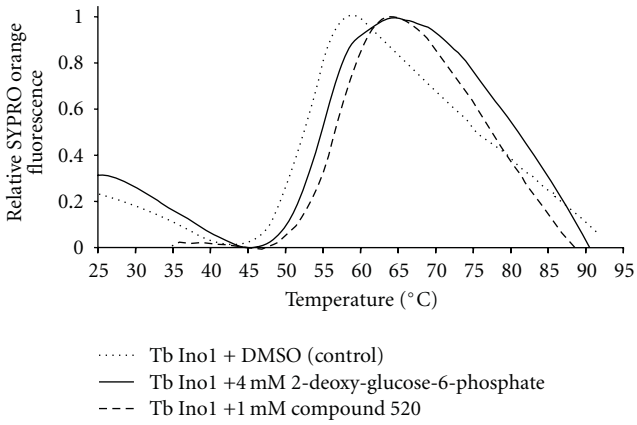


FIGURE 1: Typical differential fluorimetry scans of TbINO1. Differential fluorimetric scans were performed and analysed as described in Experimental. TbINO1 + DMSO (control) dotted line, TbINO1 + 4 mM 2-deoxy-glucose-6-phosphate (positive control) solid line, and TbINO1 + 1 mM compound 520, dashed line.

been used to identify compounds that interact with a protein, either to stabilise or destabilise it, and therefore influencing the protein's T_m (melting point) [21].

TbINO1 was subjected to differential scanning fluorimetry to ascertain if this approach was possible. The T_m for TbINO1 was determined in the presence of NAD^+ and ammonium acetate, both known cofactors and stimuli of INO1 activity [13, 14, 22]; in a typical experiment, T_ms were consistently ~51.4°C (4 samples, range 51.36–51.39°C) (Figure 1, dotted line). The substrate glucose-6-phosphate (5 mM) increased the T_m by 0.70 ± 0.28°C; however, 2-deoxy-glucose-6-phosphate (4 mM), a known inhibitor of INO1s [22–24], increased the T_m value by $+2.84 \pm 0.47$°C (Figure 1, solid line). These encouraging results allowed validation of a screening program; thus, glucose-6-phosphate and 2-deoxy-glucose-6-phosphate were used as positive controls, and DMSO (in which all compounds were dissolved) as a negative control on all subsequent screening plates.

3.2. Screening of the May Ro3 Fragment Library for Compounds That Interact with Tbino1 by Differential Scanning Fluorimetry. As TbINO1 was amenable to screening by differential scanning fluorimetry, ~670 compounds from the May Ro3 fragment library were screened. Additionally, their trypanocidal and general cytotoxicity activities, against bloodstream *T. brucei* and HeLa and A549 cells were assessed (see Supplementary Table 1 in Supplementary Material available online at doi: 10.4061/2011/389364).

From this large amount of data, 38 compounds at 1 mM interacted with TbINO1 with a $\Delta T_m > +1.5$°C (Table 1). Of these compound 520, 2-(2-furyl)benzoic acid, stabilised the protein the greatest with a $\Delta T_m + 4.29 \pm 0.07$°C (Figure 1, dashed line). It is interesting to note that the heterocyclic furan moiety is a common feature in several of the top hits (Table 1, compounds 520, 75, 30, 28, and 383).

Other similarities between the top hits are the presence of a carboxylic acid (Table 1, compounds 520, 75, 28, 513) or a methanol group (Table 1, compounds 186, 30, 383) attached to an aromatic ring. An obvious conclusion is that these moieties form important hydrogen bonds to the protein, in a similar orientation to each other with respect to the aromatic ring to which they are attached.

The trypanocidal and cytotoxicity activities of these 38 compounds (Table 1) revealed that 9 of them killed more than 35% of *T. brucei* at 0.5 mM. These were investigated further, and their ED50s for both *T. brucei* and HeLa cells were determined (Table 1). Of these compound 162, 1H-indol-3-ylmethanol with a ΔT_m $+2.31 \pm 0.06$°C (Table 1), was the most potent with an ED50 of $31 \pm 1.4\,\mu$M, but was also cytotoxic against HeLa cells, ED50 of $103 \pm 6\,\mu$M, thus giving a very poor selectivity index of 3.3. However, another compound 256, 2-quinolinylmethanol, structurally very similar to compound 162, that has an ED50 of $40.1 \pm 1.2\,\mu$M, showed less cytotoxicity against HeLa cells, giving a selectivity index of ~21.

Interestingly, the top TbINO1 differential scanning fluorimetry hit, compound 520, also showed trypanocidal activity, ED50 of $76 \pm 6\,\mu$M, but no cytotoxicity towards HeLa cells at 20 mM, thus giving a selectivity index >256. However, the best selectivity index from these TbINO1 differential scanning fluorimetry hits (Table 1) was compound 239, 2-amino-4-methylthiophene-3-carboxamide, with a *T. brucei* ED50 of $63 \pm 2.5\,\mu$M and a selectivity index >317.

It is interesting to note that compound 520 contains a carboxylic acid and, therefore, should be impervious to membranes by passive diffusion; this seems to be true for the close analogous compound 513, 2-(1H-pyrrol-1-yl)benzoic acid, which also interacts with TbINO1 strongly but has no trypanocidal or general cytotoxicity activity at 0.5 mM (Table 1).

Puzzlingly, this suggests that some carboxylic acid containing compounds, such as 520, may be specifically and actively taken up by *T. brucei*, while other closely related carboxylic acid containing compounds are not.

The TbINO1 differential scanning fluorimetry hits will be investigated further in the future, as outlined in the conclusions.

3.3. Screening of the May Ro3 Fragment Library for Trypanocidal Compounds. The most potent trypanocidal compounds (ED50s < 100 μM) of the ~670 compounds from the May Ro3 fragment library (Supplementary Table 1) were investigated further by determining their ED50s in both *T. brucei* and HeLa cells (Table 2). From first observations, it is clear that the vast majority of these compounds contain a primary amine, with the most potent compounds (ED50s < 40 μM), 269, 270, and 348, containing an aromatic primary amine. Unfortunately, most of the primary amine containing compounds are cytotoxic and thus have poor selectivity indexes (0.5–21). The only exceptions are compounds 520 and 239 (both of which do not contain a primary amine), as discussed earlier, and showed no cytotoxicity at 20 mM (Tables 1 and 2) with selectivity indexes greater than 260.

The structure activity relationship of the most potent trypanocidal compound from the library, compound 269

Table 1: Screening the MayBridge Rule of 3 Fragment Library for TbINO1 differential scanning fluorimetry hits with a $\Delta T_m > +1.5°C$.

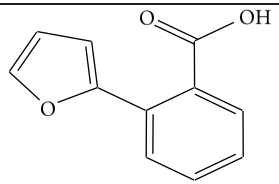
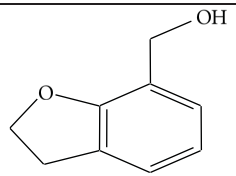
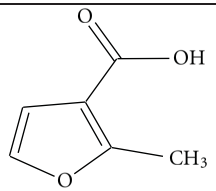
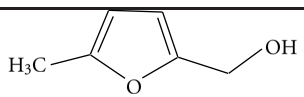
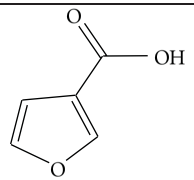
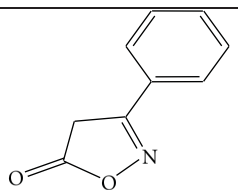
Library number[a]	CAS number[b]	Molecular structure	Product name	TbIno1 T_m shift[c] [°C]	*T. brucei* Alamar Blue (% survival)[d]	*T. brucei* ED50[e] (μM)	HeLa Cells Alamar Blue (% survival)[d]	HeLa ED50[e] (μM)
520	331942-47-3		2-(2-furyl)benzoic acid	4.29 ± 0.07	35.4 ± 8.7	76.1 ± 6.0	**73.0 ± 11.5**	>20 mM
186	151155-53-2		2,3-Dihydrobenzo[b]furan-7-methanol	4.09 ± 0.09	33.8 ± 5.2	370 ± 40	**86.5 ± 14.8**	
75	6947-94-0		2-methyl-3-furoic acid	3.89 ± 0.07	**62.2 ± 10.6**		63.3 ± 5.9	
30	3857-25-8		(5-methyl-2-furyl)methanol	3.79 ± 0.08	45 ± 14.10		23.3 ± 18.6	
28	488-93-7		3-furoic acid	3.72 ± 0.08	**59.7 ± 5.5**		48.2 ± 11.8	
272	1076-59-1		3-phenyl-4,5-dihydroisoxazol-5-one	3.56 ± 0.06	**60.1 ± 21.0**		**89.4 ± 3.9**	

Table 1: Continued.

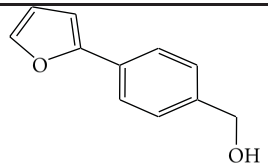
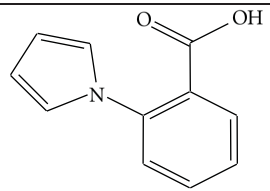
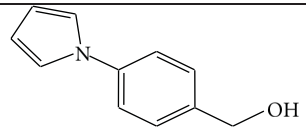
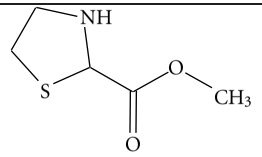
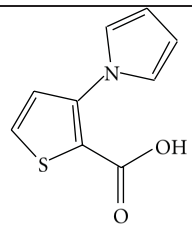
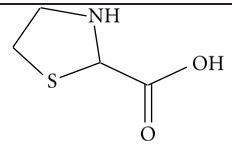
Library number[a]	CAS number[b]	Molecular structure	Product name	TbIno1 T_m shift[c] [°C]	*T. brucei* Alamar Blue (% survival)[d]	*T. brucei* ED50[e] (μM)	HeLa Cells Alamar Blue (% survival)[d]	HeLa ED50[e] (μM)
383	17920-85-3		[4-(2-furyl)phenyl]methanol	3.45 ± 0.09	33.0 ± 7.2	187 ± 17	20 ± 10.6	292 ± 31
513	10333-68-3		2-(1H-pyrrol-1-yl)benzoic acid	3.14 ± 0.09	**104.3 ± 9.6**		**98.5 ± 6.5**	
372	143426-51-1		[4-(1H-pyrrol-1-yl)phenyl]methanol	3.12 ± 0.04	**37.0 ± 14.0**	302 ± 15	**94.9 ± 19.4**	3.3 ± 0.21 mM
44	33305-08-7		methyl 1,3-thiazolane-2-carboxylate hydrochloride	2.81 ± 0.1	86.7 ± 16.4		108.2 ± 15.9	
577	74772-17-1		3-(1H-pyrrol-1-yl)thiophene-2-carboxylic acid	2.78 ± 0.08	**32.2 ± 4.1**	>20 mM	**103.2 ± 2.8**	>20 mM
103	16310-13-7		1,3-thiazolane-2-carboxylic acid	2.63 ± 0.08	77.9 ± 15.6		**103.3 ± 6.4**	

Table 1: Continued.

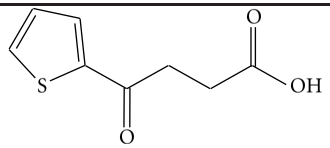
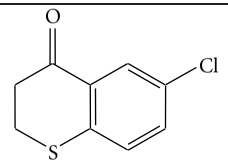
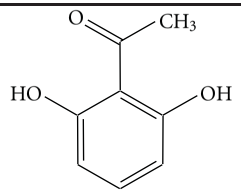
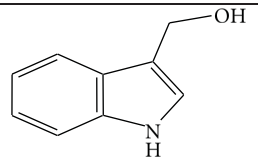
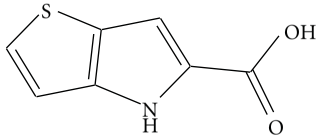
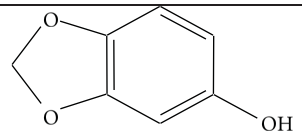
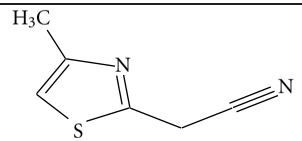
Library number[a]	CAS number[b]	Molecular structure	Product name	TbIno1 T_m shift[c] [°C]	*T. brucei* Alamar Blue (% survival)[d]	*T. brucei* ED50[e] (μM)	HeLa Cells Alamar Blue (% survival)[d]	HeLa ED50[e] (μM)
478	4653-08-1		4-oxo-4-(2-thienyl)butanoic acid	2.58 ± 0.05	**103.8 ± 13.1**		105.8 ± 9.3	
609	13735-12-1		6-chloro-3,4-dihydro-2H-1-benzothiin-4-one	2.49 ± 0.04	**86.3 ± 6.6**		**74.5 ± 13.7**	
204	699-83-2		1-(2,6-dihydroxyphenyl)ethan-1-one	2.35 ± 0.06	45.2 ± 9.7		36.3 ± 12.5	
162	700-06-1		1H-indol-3-ylmethanol	2.31 ± 0.06	32.5 ± 5.9	31.3 ± 1.4	16.3 ± 10.6	103 ± 6
328	39793-31-2		4H-thieno[3,2-b]pyrrole-5-carboxylic acid	2.29 ± 0.10	**51.6 ± 13.3**		105.9 ± 6.9	
256	1780-17-2		2-quinolinylmethanol	2.20 ± 0.06	21.7 ± 6.0	40.1 ± 1.2	99.2 ± 19.5	836 ± 68
126	19785-39-8		2-(4-methyl-1,3-thiazol-2-yl)acetonitrile	2.10 ± 0.09	**75.3 ± 15.4**		69.7 ± 10.3	

Table 1: Continued.

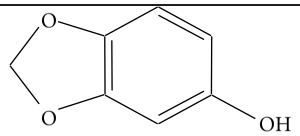
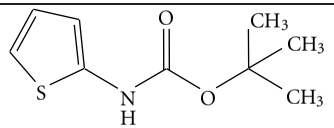
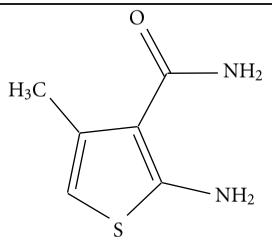
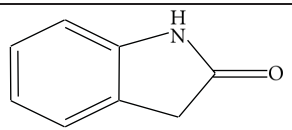
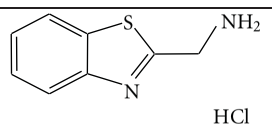
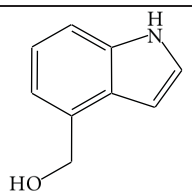
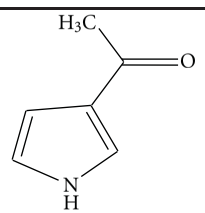
Library number[a]	CAS number[b]	Molecular structure	Product name	TbIno1 T_m shift[c] [°C]	*T. brucei* Alamar Blue (% survival)[d]	*T. brucei* ED50[e] (μM)	HeLa Cells Alamar Blue (% survival)[d]	HeLa ED50[e] (μM)
124	533-31-3		1,3-benzodioxol-5-ol	2.10 ± 0.04	28.2 ± 7.1		39.7 ± 12.5	
620	56267-50-6		tert-butyl N-(2-thienyl)carbamate	2.08 ± 0.1	40.1 ± 17.6		71.3 ± 13.6	
239	4651-97-2		2-amino-4-methylthiophene-3-carboxamide	2.05 ± 0.05	22.9 ± 7.0	63.0 ± 2.5	**95.8 ± 9.3**	>20 mM
99	59-48-3		indolin-2-one	2.04 ± 0.07	37.3 ± 9.2	216 ± 18	66.6 ± 18.7	>2 mM
626	29198-41-2		1,3-benzothiazol-2-ylmethylamine hydrochloride	2.03 ± 0.08	52.9 ± 13.9		**95.1 ± 15.5**	
163	1074-85-7		1H-indol-4-ylmethanol	1.90 ± 0.06	**34.8 ± 0.8**	5.2 ± 0.2 (mM)	**103.3 ± 8.7**	2.5 ± 0.2 mM
20	1072-82-8		1-(1H-pyrrol-3-yl)ethan-1-one	1.88 ± 0.08	**36.8 ± 9.7**	3.6 ± 0.2 (mM)	101.8 ± 4.3	>5 mM

Table 1: Continued.

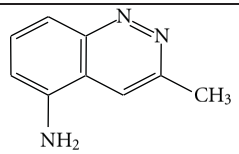
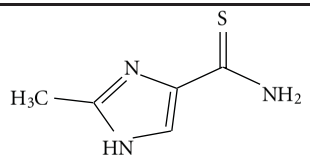
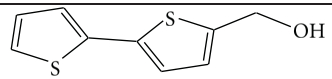
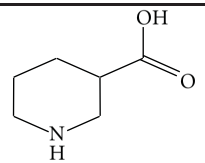
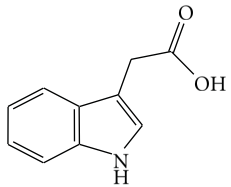
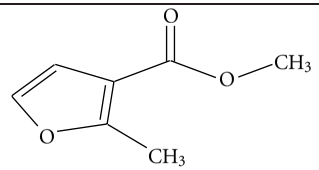
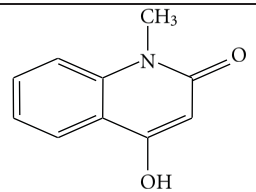
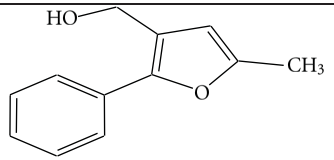
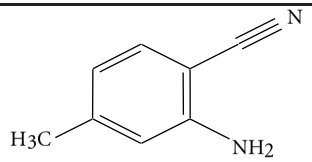
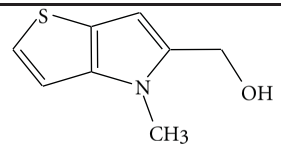
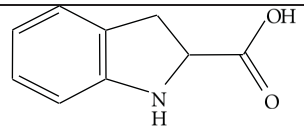
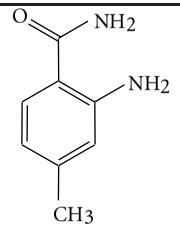
Library number[a]	CAS number[b]	Molecular structure	Product name	TbIno1 T_m shift[c] [°C]	*T. brucei* Alamar Blue (% survival)[d]	*T. brucei* ED50[e] (μM)	HeLa Cells Alamar Blue (% survival)[d]	HeLa ED50[e] (μM)
261	300690-74-8		3-methylcinnolin-5-amine	1.83 ± 0.11	21.7 ± 8.5	148 ± 8	**62.5 ± 14.7**	726 ± 64
135	129486-91-5		2-methyl-1H-imidazole-4-carbothioamide	1.74 ± 0.09	115.5 ± 16.6		90.3 ± 10.9	
598	3515-30-8		2,2′-bithien-5-ylmethanol	1.72 ± 0.06	55.3 ± 15.6		**78 ± 11.6**	
91	498-95-3		3-piperidinecarboxylic acid	1.69 ± 0.10	36.1 ± 11.0		**76.6 ± 29.1**	
391	87-51-4		2-(1H-indol-3-yl)acetic acid	1.67 ± 0.08	38.8 ± 10.1		**42.6 ± 6.4**	
132	6141-58-8		methyl 2-methyl-3-furoate	1.64 ± 0.09	34.3 ± 8.4		71.3 ± 11.3	
395	1677-46-9		4-hydroxy-1-methyl-1,2-dihydroquinolin-2-one	1.62 ± 0.03	54.3 ± 16.4		**93.0 ± 0.3**	

Table 1: Continued.

Library number[a]	CAS number[b]	Molecular structure	Product name	TbIno1 T_m shift[c] [°C]	*T. brucei* Alamar Blue (% survival)[d]	*T. brucei* ED50[e] (μM)	HeLa Cells Alamar Blue (% survival)[d]	HeLa ED50[e] (μM)
522	183210-33-5		(5-methyl-2-phenyl-3-furyl)methanol	1.61 ± 0.06	45.4 ± 7.9		24.3 ± 17.4	
97	26830-96-6		2-amino-4-methylbenzonitrile	1.58 ± 0.07	**72.2 ± 14.4**		81.4 ± 7.4	
331	121933-59-3		(4-methyl-4H-thieno[3,2-b]pyrrol-5-yl)methanol	1.54 ± 0.07	31.0 ± 7.1	121 ± 15	**93.6 ± 14.9**	169 ± 10
292	78348-24-0		indoline-2-carboxylic acid	1.54 ± 0.11	32.9 ± 9.3		37.6 ± 2.5	
190	39549-79-6		2-amino-4-methylbenzamide	1.52 ± 0.06	41.9 ± 11.0		**46.7 ± 23.7**	

[a] Arbitrary Library number.
[b] CAS numbers are unique identifiers assigned by the "Chemical Abstracts Service" to describe every chemical described in the open access scientific literature.
[c] T_m shift in °C, observed for TbINO1 in the presence of compound (1 mM) and value is mean ± SD from the Boltzman curve fitting; see Section 2 for details, mean ± SD ($n = 3$).
[d] For cytotoxicity studies, see Section 2 for details and values are percentage of controls in the absence of compound, either mean ± SD ($n = 3$) or mean ± SE ($n = 2$), the latter being in bold.
[e] For trypanocidal and cytotoxicity activity, see Section 2 for details and values are ED50s ± SD ($n = 4$).

Table 2: Screening for trypanocidal compounds in the MayBridge Rule of 3 Fragment Library.

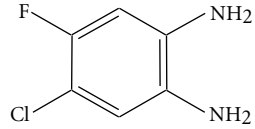
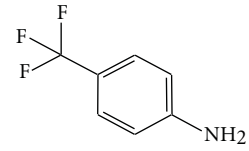
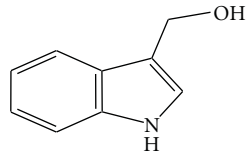
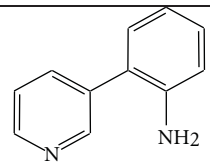
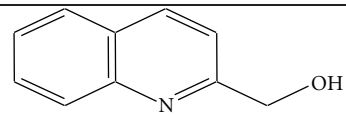
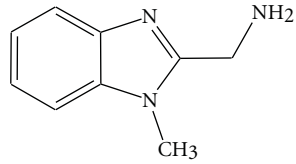
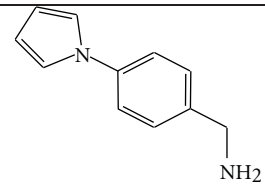
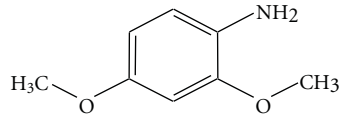
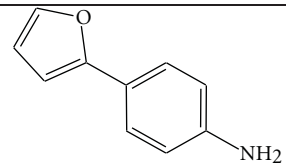
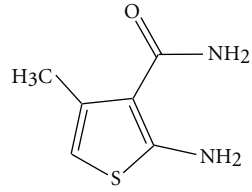
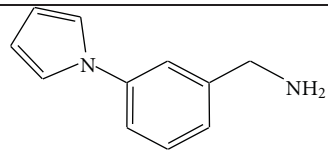
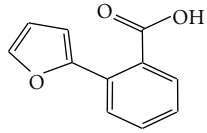
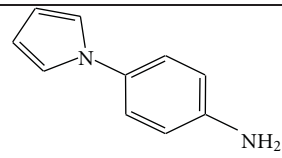
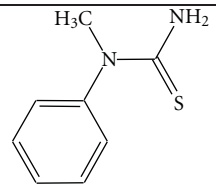
Library number[a]	Molecular structure	*T. brucei* ED50[b] (μM)	HeLa ED50[b] (μM)	Selectivity index
269		20.1 ± 1.0	431 ± 53	21.4
270		30.5 ± 1.6	206 ± 13	6.8
162		31 ± 1.4	103 ± 6	3.3
348		35.0 ± 1.0	21.8 ± 1.8	0.6
256		40.1 ± 1.2	836 ± 68	20.8
281		44.7 ± 1.8	659 ± 65	14.7
365		49.4 ± 2.1	71.8 ± 3.7	1.5
213		55.6 ± 1.3	710 ± 96	12.8
257		56.0 ± 3.6	114 ± 8	2.0
239		63.0 ± 2.5	>20 mM	>317

Table 2: Continued.

Library number[a]	Molecular structure	*T. brucei* ED50[b] (μM)	HeLa ED50[b] (μM)	Selectivity index
364		63.2 ± 1.7	40.4 ± 1.8	0.6
520		76 ± 6	>20 mM	>266
250		81.0 ± 5.1	1.4 ± 0.1 mM	17.1
325		82.2 ± 4.6	415 ± 24	5

[a] Arbitrary Library number.
[b] For trypanocidal and cytotoxicity activity, see Section 2 for details and values are ED50s ± SD ($n = 4$).
[c] Selectivity index: *T. brucei* ED50/HeLa ED50.

(4-chloro-5-fluorobenzene-1,2-diamine), with an ED50 of 20.1 ± 1.03 μM, was investigated further. A series of analogues were purchased and tested in parallel; this included analogues that had the two amino groups, but with only one of the halides or one of the halides replaced by a methyl group or no halides. Other analogues maintained the halides, and reduce the amino groups to one, or by masking both free amines as a benzimidazole.

T. brucei and HeLa cell ED50s values were determined for this collection of compounds (Table 3).

Repurchased compound 269, gave ED50s similar to those obtained earlier (20.1 ± 1.03 and 31.1 ± 1.22 μM). As to the structure activity relationship of the analogues, it is apparent that the two amino groups seem to be important for potent trypanocidal activity, as both compounds 269b and 269d have dramatically increased ED50s.

The presence of two bulky groups opposite to the amine groups seems detrimental as shown by compound 240 (4-chloro-5-methylbenzene-1,2-diamine); a compound from the May Ro3 fragment library (Supplementary Table 1).

The absence of one or both of the halides does not alter the *T. brucei* ED50s dramatically, as demonstrated by compound 269a (benzene-1,2-diamine), with an ED50 of 35.2 ± 3.0 μM (Table 3). The HeLa cell cytotoxicity of various benzene-1,2-diamine analogues varies dramatically, however the selectivity indexes are poor, the best being the original compound 269, 4-chloro-5-fluorobenzene-1,2-diamine, with a selectivity index of ~21 (Table 3)

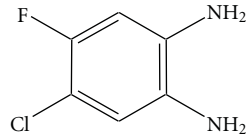
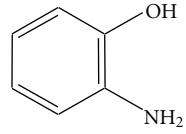
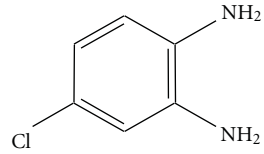
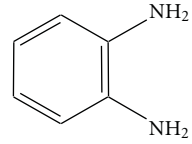
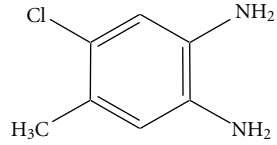
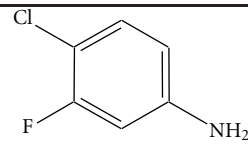
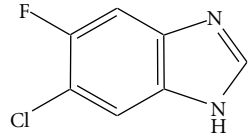
Unfortunately, the benzene-1,2-diamine analogues are thought to be carcinogenic, as they are suspected to be able to interchelate DNA, although direct proof of this is still lacking [25]. These diamines are also well known to form Schiff bases and are often used for derivatisation of natural ketones and aldehydes, such as methglyoxal [26]. Ironically, it is also this Schiff base capability, which has led them to be investigated as possible anticancer, antibacterial, antifungal and antiviral agents [27, 28]. Either or both of the DNA interchelating, or Schiff base capabilities could be the possible mode of action for the trypanocidal activity. To avoid the obvious possible problems associated with carcinogenic compounds, while still maintaining the dual functionality that seems to be important for potency, compound 269e (2-amino-1-hydroxy-benzene or 2-aminophenol), was purchased and tested. This proved to be the most potent trypanocidal agent tested in this study with an ED50 of 20.0 ± 0.3 μM; however, it was cytotoxic to the HeLa cells (Table 3). Despite this, the relatively simple molecule (2-amino-phenol) has been shown to have anti-microbial activity [29, 30].

Considering these are very simple molecules, it is surprising that they are trypanocidal at low-micromolar concentrations, highlighting the importance of screening programs to identify novel pharmacophores.

4. Conclusions

In this work, screening of a comparatively small fragment library for thermal stabilisation of TbINO1 has allowed identification of several novel compounds that interact strongly

Table 3: Structure activity relationship for trypanocidal activity of analogues of the diamine compound 269.

Library number[a]	CAS number[b]	Molecular structure	*T. brucei* ED50[c] (μM)	HeLa ED50[c] (μM)	Selectivity index[d]
269	139512-70-2		20.1 ± 1.2	431 ± 53	21.4
269e	51-19-4 or 95-55-6		20.0 ± 0.3	68.2 ± 3.2	3.4
269c	95-83-0		21.9 ± 1.2	224 ± 35	10.2
269a	95-54-5		35.2 ± 3.0	32.9 ± 2.5	0.9
240	63155-04-4		86.6 ± 4.8	454 ± 55	5.2
269b	367-22-6		150 ± 13	1.4 ± 0.1 mM	9.3
269d	175135-04-3		331 ± 24	520 ± 52	1.6

[a] Arbitrary Library number.
[b] CAS numbers are unique identifiers assigned by the "Chemical Abstracts Service" to describe every chemical described in the open access scientific literature.
[c] For trypanocidal and cytotoxicity activity, see Section 2 for details and values are ED50s ± SD ($n = 4$).
[d] Selectivity index: *T. brucei* ED50/HeLa ED50.

and stabilise TbINO1. Unexpectedly, several of the significant hits are also trypanocidal with ED50 values in the 30–80 μM range, despite being relative simple molecules.

Several other compounds from the May Ro3 library showed low-micromolar trypanocidal activity. The majority of the most potent hits contain primary amines whose mode of action could be via Schiff-base formation, while some of the diamines could also be acting by inter-chelating DNA, thus interfering with cell-cycle progression/cell division.

Unfortunately, some of these compounds are cytotoxic against mammalian cells and thus are unlikely to progress as lead compounds. However, the biological activity of related compounds such as 269e (2-amino-phenol), also known to have anti-microbial activity, will be investigated further.

Future work outside the scope of this study will include the following:

(i) investigating if the lead compounds that interacted with TbINO1 inhibit its activity, in an *in vitro* coupled enzyme assay,

(ii) investigating the mode of killing by the lead compounds that interacted with TbINO1 by undertaking various *in vivo* labelling experiments to ascertain if they are inhibiting TbINO1, thus causing a lack of *de novo* synthesised *myo*-inositol, required for PI synthesis for the essential GPI pathway,

(iii) the direct interactions of compounds with TbINO1 will be investigated by protein crystallography studies.

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Research Article

Peptide Inhibition of Topoisomerase IB from *Plasmodium falciparum*

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Control of diseases inflicted by protozoan parasites such as *Leishmania, Trypanosoma*, and *Plasmodium*, which pose a serious threat to human health worldwide, depends on a rather small number of antiparasite drugs, of which many are toxic and/or inefficient. Moreover, the increasing occurrence of drug-resistant parasites emphasizes the need for new and effective antiprotozoan drugs. In the current study, we describe a synthetic peptide, WRWYCRCK, with inhibitory effect on the essential enzyme topoisomerase I from the malaria-causing parasite *Plasmodium falciparum*. The peptide inhibits specifically the transition from noncovalent to covalent DNA binding of *P. falciparum* topoisomerase I, while it does not affect the ligation step of catalysis. A mechanistic explanation for this inhibition is provided by molecular docking analyses. Taken together the presented results suggest that synthetic peptides may represent a new class of potential antiprotozoan drugs.

1. Introduction

Protozoan parasites, such as *Leishmania*, *Trypanosoma*, and *Plasmodium* species are the cause of a large array of diseases hampering the lives of people all over the world [1]. Control of such diseases depends on a rather small number of prophylactic or therapeutic antiparasite drugs, many of which are highly toxic and/or inefficient [2–5]. In addition, an increasing number of parasites develop resistance towards several of the frontline drugs [6–9]. This has created an urgent need for novel compounds to prevent and cure diseases caused by protozoan parasites. Species-specific inhibition of parasitic enzymes has been suggested as one promising approach in the development of new therapeutics [10]. One family of enzymes that have attracted considerable interest as potential targets for antiparasitic therapeutics are the DNA topoisomerases (Topos) [1, 11] of which the human counterparts are well-known targets in anticancer treatment [12].

DNA Topos are ubiquitous enzymes needed to overcome the topological stress arising in DNA during replication, transcription, recombination, and repair [13]. This is achieved by the enzymes introducing transient breaks in the DNA in a reaction that restores the energy of the broken phosphodiester bond in a covalent phosphotyrosyl cleavage intermediate. Based on their mechanism of action Topos are divided into two main classes [13, 14]. The type I Topos are with few exceptions monomers and relax DNA by breaking only one strand of the double helix, while type II Topos are mainly homodimers or heterotetramers and break both strands of the DNA simultaneously. Type I Topos are further classified into two structurally unrelated families denoted the type IA and type IB Topos, defined by the polarity of their strand cleavage. The type IA Topos are prevalent in prokaryotic species and create a 5′-phoshotyrosyl linkage and a free 3′-OH DNA end during cleavage. Type IB Topos are mainly found in eukaryotic species and generate a 3′-phosphotyrosyl linkage and a free 5′-OH DNA end during

cleavage. The class of type II Topos are subdivided into the type IIA and type IIB families, of which all members are structurally related and characterized by the formation of a 5′-phoshotyrosyl linkage and a free 3′-OH DNA end during cleavage. The type IIA Topos are found both in eukaryotic and prokaryotic species. Typically the eukaryotic members of this group are homo-dimers while the prokaryotic enzymes are heterotetramers [14]. The type IIB group encompasses TopoVI of extreme thermophilic archaebacteria [15].

Besides their important biological functions, DNA Topos from the various groups are well-known targets of both antibacterial and anticancer therapeutic agents. Hence, bacterial type IIA Topos, such as DNA gyrase and TopoIV, are targets of clinically important antibiotics active against a wide spectrum of bacterial pathogens [16, 17]. Human type IB and IIA Topos are targets of several anticancer compounds, exemplified by camptothecin and etoposide, respectively, of which synthetic derivatives are routinely used in systemic treatment of different cancer types [12, 18]. Of relevance for the treatment of protozoan-caused infectious diseases, structural and/or subtle mechanistic differences between protozoan and host Topos can be exploited for the rational design of novel therapeutic compounds. Indeed, the unusual heterodimeric TopoIB of kinetoplastid parasites, such as *Leishmania donovani* gives hope for the development of drugs targeting parasite TopoIB without interfering with the function of the monomeric TopoIB in the human host [11, 19, 20]. As another example, the apicomplexan parasite *Plasmodium falciparum* contains apicoplast DNA, which requires bacterial-type DNA gyrases (type IIA Topo) for replication, thus providing a unique drug target absent in the host [21, 22]. In addition, the high expression rate of TopoIB and TopoIIA in rapidly growing parasites, compared to the expression levels of these enzymes in the host, may be exploited for the development of Topo-targeting protocols that specifically kill the parasites.

Synthetic peptides have been prophesied to be the ideal inhibitors of enzyme activity either alone or in combination with small-molecule drugs [10, 23]. However, high synthesis costs and great challenges regarding delivery, intracellular targeting and clearance half-life of peptides have until recently hampered the interest of most pharmaceutical companies in developing peptide-based drugs. New efficient synthesis strategies and low monomer prices have led to a renewed interest in therapeutic peptides. Indeed, compared to small-molecule drugs, which are currently dominating the pharmaceutical market, peptide-based therapeutics offer several advantages, such as high specificity, lower accumulation in tissues, lower toxicity, and biological diversity [24–27].

The potential for synthetic peptides as efficient species-specific inhibitors even of discrete steps of Topo catalysis is highlighted in several studies by Nagaraja and co-workers describing the identification and characterization of species-specific antibodies with inhibitory activities against particular steps of *Mycobacteria* DNA gyrase or TopoI catalysis [28–30]. Peptides with similar inhibitory activities and potential in future antituberculosis treatment [29] are likely to be derived from such antibodies [31–34]. Relevant for the potential development of peptide-based drugs targeting eukaryotic Topos, almost a decade ago, Segall and co-workers identified a series of hexapeptides inhibiting various catalytic steps of the tyrosine recombinases (bacteriophage λ-Int and Cre) [35, 36]. Tyrosine recombinases share so important structural and mechanistic features with the type IB Topos that they can be considered a subbranch of the type IB Topo family [37, 38]. It was therefore not surprising, that several of the hexapeptides selected on basis of λ-Int inhibition also inhibited DNA relaxation by the type IB Topo of *Vaccinia virus* (vvTopoI), although less potently [39]. A rescreening of the peptide combinatorial library (used for selection of the above-mentioned peptides) specifically against vvTopoI resulted in the identification of three new peptides, WYCRCK, KCCRCK, and WRWYCRCK with high activity against this enzyme. Of these, WRWYCRCK was the most potent inhibitor of the type IB Topos tested. This peptide inhibited vvTopoI with an IC_{50} value of 0.1–0.25 μM and λ-Int with an IC_{50} value of 0.015 μM, while the structurally unrelated type IA Topo, *E. coli* TopoI was inhibited only to a limited extent (IC_{50} value of 5.5 μM) [40]. Using these peptides as a starting point, it may in longer terms be possible to develop peptide-based TopoI targeting inhibitors with therapeutic activity against protozoan pathogens.

As an initial investigation of this possibility, we address, in the present study, the effect of the peptides WYCRCK, KCCRCK, and WRWYCRCK on the activity of the recently cloned and purified recombinant TopoI (pfTopoI) from the malaria-causing parasite *Plasmodium falciparum*. We find that WRWYCRCK inhibits DNA relaxation and cleavage by pfTopoI whereas neither WYCRCK nor KCCRCK have any effect on pfTopoI activity. Molecular docking of the three peptides in the noncovalent pfTopoI-DNA complex shows WRWYCRCK to be located in the minor groove of the DNA in proximity of the enzyme active site, while WYCRCK and KCCRCK are positioned far from the enzyme active site.

2. Methods

2.1. Expression and Purification of pfTopoI. The plasmid, pPFT100 (the cloning of pfTopoI is to be published elsewhere), containing the pfTopoI gene (PlasmoDB accession number PFE0520c) [41] (codon optimized for expression in *S. cerevisiae* (GENEART, Germany)), was transformed into the yeast *S. cerevisiae* top1Δ strain RS190 (a kind gift from R. Sternglanz, State University of New York, Stony Brook, NY, USA) according to standard procedures, and pfTopoI enzyme was expressed and purified as previously described for human topoisomerase I (hTopoI) [42]. hTopoI was expressed and purified as previously described [42].

2.2. Unit Definition. 1 U is the amount of enzyme needed to fully relax 200 fmol of negatively supercoiled pBR322 plasmid DNA at 37°C in 30 min in 10 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 5 mM $MgCl_2$ and 5 mM $CaCl_2$.

2.3. Synthetic Peptides. WYCRCK, KCCRCK, and WRWYCRCK were purchased from GenScript USA Inc., USA. The lyophilized peptides were dissolved in H_2O.

2.4. Relaxation Assays. DNA relaxation reactions included 1 U pfTopoI in the absence or presence of peptide (WYCRCK, KCCRCK, or WRWYCRCK) at the following concentrations: 1.3 μM, 2.5 μM, 5 μM, 7.5 μM, 12.5 μM, 25 μM, or 50 μM and 200 fmol negatively supercoiled pBR322 plasmid in 20 μL of 10 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 5 mM $MgCl_2$ and 5 mM $CaCl_2$. The plasmid was preincubated with the peptide for 5 min at 37°C prior to addition of enzyme. Reactions were incubated at 37°C for 30 min before being stopped by addition of 0.2% (w/v) SDS and proteolytically digested with 0.5 μg/mL proteinase K for another 30 min at 37°C. Samples were subjected to gel electrophoresis on 1% agarose gels in TBE buffer, and DNA bands were stained with ethidium bromide and visualized by illumination with UV light.

2.5. Synthetic DNA Substrates. Oligonucleotides for assembly of DNA suicide cleavage substrates and DNA ligation substrates were purchased from DNA Technology, Denmark and purified by denaturing polyacrylamide gel electrophoresis. The sequences of the substrates are as follows: OL19: 5′-GCC TGC AGG TCG ACT CTA GAG GAT CTA AAA GAC TTA GA-3′, OL27: 5′-AAA AAT TTT TCT AAG TCT TTT AGA TCC TCT AGA GTC GAC CTG CAG GC-3′, and OL36: 5′-AGA AAA ATT TTT-3′. The oligonucleotide representing the scissile strand (OL19) was 5′-radiolabeled by T4 polynucleotide kinase (New England Biolabs, USA) using [γ-^{32}P] ATP as the phosphoryl donor. To prevent ligation of the 5′-OH from the bottom strand (OL27), these ends were 5′-phosphorylated with unlabeled ATP. The oligonucleotides were annealed pairwise with a 2-fold molar excess of the bottom strand over scissile strand as previously described [43].

2.6. Cleavage/Ligation Assays. The cleavage reactions were carried out in 20 μL reaction volumes by incubating 20 nM of the duplex OL19/OL27 with 500 fmol of pfTopoI or hTopoI enzyme at 37°C, in 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 10 mM $CaCl_2$. The DNA substrate was preincubated with peptide WRWYCRCK at concentrations varying from 0 to 75 μM for 5 min at 37°C prior to addition of enzyme. After 30 minutes of incubation, the reactions were stopped with 0.1% (w/v) SDS. For the ligation reactions, 20 nM OL19/OL27 was incubated with 500 fmol of pfTopoI for 30 min at 37°C in 10 mM Tris (pH 7.5), and 5 mM $MgCl_2$, 5 mM $CaCl_2$. After preincubation of the cleavage samples with the peptide at concentrations varying from 0 to 12.5 μM for 5 min at 37°C, ligation was initiated by the addition of a 200-fold molar excess of oligonucleotide OL36 over the duplex OL19/OL27. Samples were incubated at 37°C for 60 min, and reactions were stopped with 0.1% (w/v) SDS. Cleavage and ligation samples were precipitated with ethanol, resuspended in 10 μL of 1 mg/mL trypsin, and incubated at 37°C for 30 min. Reaction products were analyzed by gel electrophoresis on 12% denaturing polyacrylamide gels, and radioactive bands were visualized by Phosphorimaging.

2.7. Quantification. Densitometric quantification of gel bands was performed using Quantity One v4.6.3 software (Bio-Rad, USA). The relative cleavage was calculated by the following equation: relative cleavage = (IC − BI)/(IC − BI + IS − BI), where IC denotes the intensity of the band(s) representing the cleavage product(s), IS denotes the intensity of the band representing the substrate, and BI denotes the background intensity.

2.8. Restriction Digestion of Plasmid in the Presence of Peptide. Restriction digests were performed in 20 μL reaction volumes by incubating 3 μg pUC19 plasmid with EcoRI or PvuII (both from New England Biolabs) in the reaction buffers provided by the manufacturer in the absence or presence of peptide WRWYCRCK (12.5 μM, 25 μM, or 50 μM). The plasmid was preincubated with the peptide for 5 min at 37°C prior to addition of enzyme. For both restriction endonucleases, the lowest amount of enzyme, able to fully digest the plasmid within the timeframe of the experiment, was used. Reactions were incubated at 37°C for 30 min before being stopped by addition of 0.2% (w/v) SDS and proteolytically digested with 0.5 μg/mL proteinase K for another 30 min at 37°C. Samples were subjected to gel electrophoresis on 1% agarose gels in TBE buffer, and DNA bands were stained with ethidium bromide and visualized by illumination with UV light.

2.9. Docking Experiment. The three-dimensional structure for residues Pro140-Phe839 of pfTopoI was obtained through homology modeling using the SwissModel server [44] and the crystal structure of hTopoI (1K4T and 1A36 PDBs) as a template [45, 46]. The alignment was performed with the TCOFFEE server [47], using the sequences having the SwissProt code Q26024 and P11387 for the pfTopoI and hTopoI protein, respectively. The 22-base-pair DNA present in the noncovalent hTopo1-DNA complex crystal structure 1K4S [46] was fitted into the putative pfTopoI active site in the 3D protein model to obtain the pfTopoI-DNA noncovalent complex that was used for the docking experiment. The bases are numbered from 1 to 22 starting from the 5′ end of the scissile strand and from 23 to 44 starting from the 5′ end of the intact strand. The structure of the octapeptide WRWYCRCK and of the two hexapeptides WYCRCK and KCCRCK was designed with the Sybyl v. 6.0 program (TRIPOS, http://www.tripos.com/) creating a disulphide bond between the two Cys3 and Cys5 cysteines in all the peptides (this was done since the experimental data confirmed that disulfide bridging was necessary for the inhibitory effect of the peptide). The structure of the peptides was minimized in vacuum using the Powell algorithm [48] implemented in the Sybyl program and then simulated in a rectangular box filled with water molecules using the Gromacs 4.0 Package [49] for 2 ns in order to regularize the structure. 250 docking runs were performed using the Autodock 4.2 program [50] using the Lamarckian genetic algorithm [51]. The structures of the ligands (WRWYCRCK,

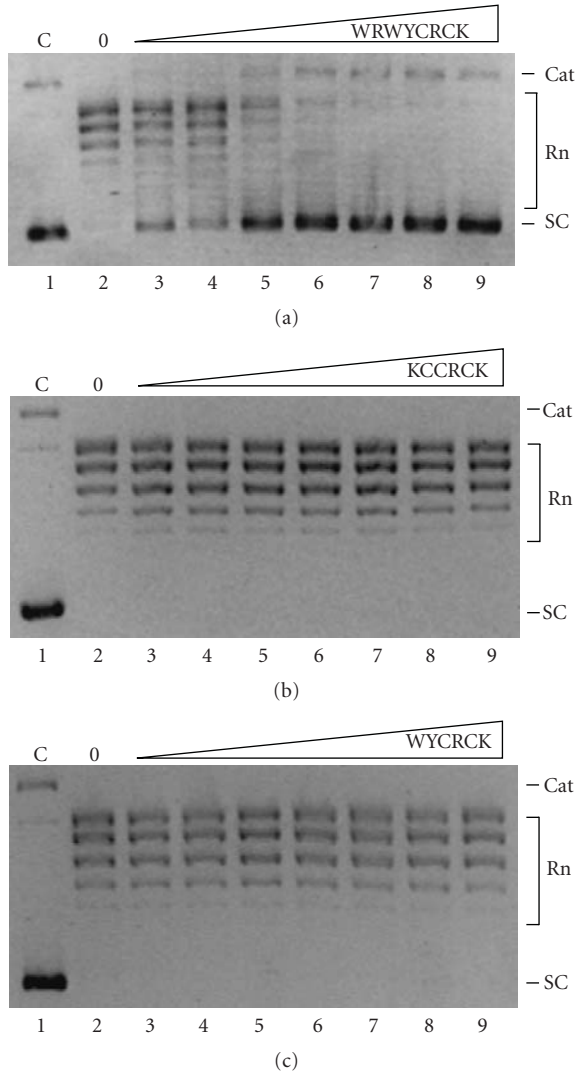


Figure 1: Effect of peptides on pfTopoI-mediated DNA relaxation. The effect of peptides on relaxation was assayed by incubating 200 fmol supercoiled plasmid with enzyme and peptide WRWYCRCK, KCCRCK, or WYCRCK at the following concentrations: 1.3 μM, 2.5 μM, 5 μM, 7.5 μM, 12.5 μM, 25 μM, or 50 μM. (a) Representative gel picture showing the relaxation activity of pfTopoI in the presence of increasing concentrations of peptide WRWYCRCK. (b) Representative gel picture showing the relaxation activity of pfTopoI in the presence of increasing concentrations of peptide KCCRCK. (c) Representative gel picture showing the relaxation activity of pfTopoI in the presence of increasing concentrations of peptide WYCRCK. C: negative control lane without any enzyme added; 0: positive control lane with pfTopoI but no peptide added; SC: supercoiled plasmid; Rn: relaxed topoisomers; Cat: supercoiled catenated plasmid.

WYCRCK, or KCCRCK) and the receptor (pfTopoI-DNA complex) were first prepared using the AutodockTools v. 1.5.2 suite [50], building a cubic box able to contain the cap and cat domains of the protein and the DNA bases. The contacts between the ligand and the receptor were identified using a cutoff of 3.5 Å applying a modified version of the g_mindist tool present in the Gromacs 3.3.3 package for Molecular Dynamics analysis [52]. The images were created with the program VMD [53].

3. Results

3.1. Inhibitory Potency of Peptides WYCRCK, KCCRCK, and WRWYCRCK in Relaxation by pfTopoI. The inhibitory potency of the peptides WYCRCK, KCCRCK, and WRWYCRCK on pfTopoI activity was investigated in a standard plasmid relaxation assay. The assay was performed with the minimum amount of pfTopoI that sufficed to fully relax the plasmid DNA (i.e., convert fast-mobility supercoiled plasmid to slow-mobility relaxed plasmid forms) in the absence of added peptide within the timeframe (30 min) used in the experiment (data not shown). As evident from Figure 1, the peptide WRWYCRCK inhibited pfTopoI relaxation activity in a dose-dependent manner, with an IC_{50} of 2.5–5 μM. The peptides WYCRCK, KCCRCK had no effect on the relaxation activity of pfTopoI, even at concentrations up to 50 μM. Moreover, consistent with previous reports of inhibition of vvTopoI by WRWYCRCK,

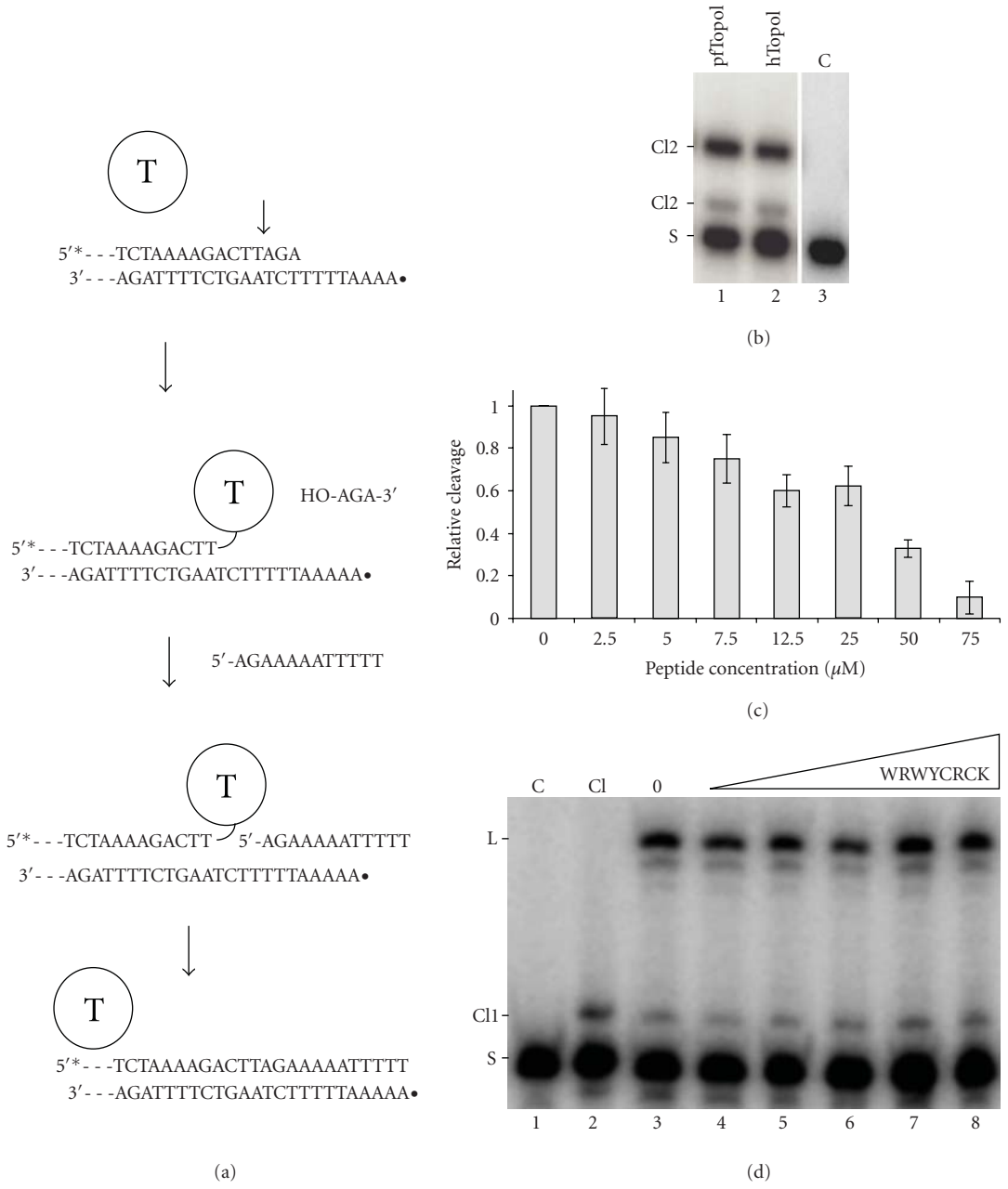


Figure 2: Effect of peptide WRWYCRCK on pfTopoI-mediated DNA cleavage. (a) Schematic depiction of the cleavage and religation reactions. The substrate (OL19/OL27) used for assaying cleavage allows covalent attachment of the enzyme to the 3′ end of the 5′-radiolabeled scissile strand (OL19) by cleaving off a trinucleotide. Ligation is prevented by diffusion of the trinucleotide. To initiate ligation, the ligator strand (OL36) is added to covalent cleavage complexes generated by incubating pfTopoI with radiolabeled OL19/OL27. (b) Gel picture showing the cleavage products obtained by incubating 5′-radiolabeled OL19/OL27 with pfTopoI (lane 1) or hTopoI (lane 2). (c) Graphical depiction of the cleavage activity of pfTopI plotted as a function of peptide WRWYCRCK concentration. The cleavage activity was calculated as described in Section 2. (d) Representative gel picture showing the ligation activity of pfTopoI in the presence of peptide WRWYCRCK at the following concentrations: 1.3 μM, 2.5 μM, 5 μM, 7.5 μM, or 12.5 μM. T: topoisomerase I; asterisk: 5′-radiolabel with [γ-32P]; filled circle: 5′-cold phosphorylation; S: substrate; Cl1: cleavage product resulting from cleavage at the black arrow in the schematic depiction; Cl2: cleavage product resulting from cleavage two nucleotides upstream of the black arrow in the schematic depiction; C: negative control lanes without any enzyme added; Cl: cleavage control lane without ligator strand added; 0: positive control lane with pfTopoI but no peptide added.

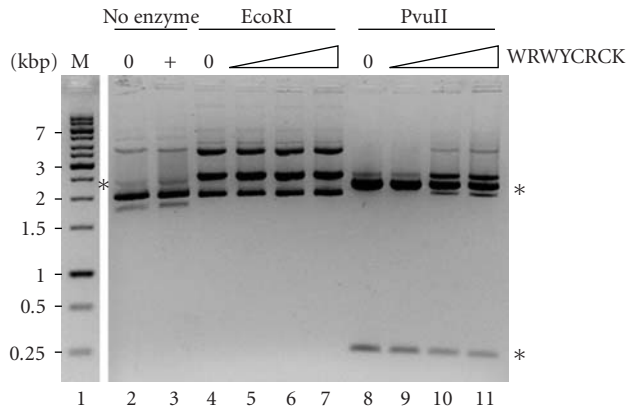


Figure 3: Effect of peptide WRWYCRCK on restriction digestion by restriction endonucleases. Representative gel picture showing the result of incubating pUC19 plasmid with EcoRI (lanes 4–7) or PvuII (lanes 8–11) in the presence of peptide WRWYCRCK at the following concentration: 12.5 μM, 25 μM, or 50 μM. The sizes, kbp, of the DNA marker (lane 1, labeled M) are shown to the left of the gel picture. 0: control lanes with no peptide added; +: control lane with 50 μM peptide added; asterisks indicate the gel electrophoretic mobility of the digested plasmid, for EcoRI, 2.7 kbp, and for PvuII, 0.3 kbp and 2.4 kbp.

the peptide only retained its inhibitory effect in the absence of DTT (data not shown), suggesting that the active form of the peptide involves disulfide bridging.

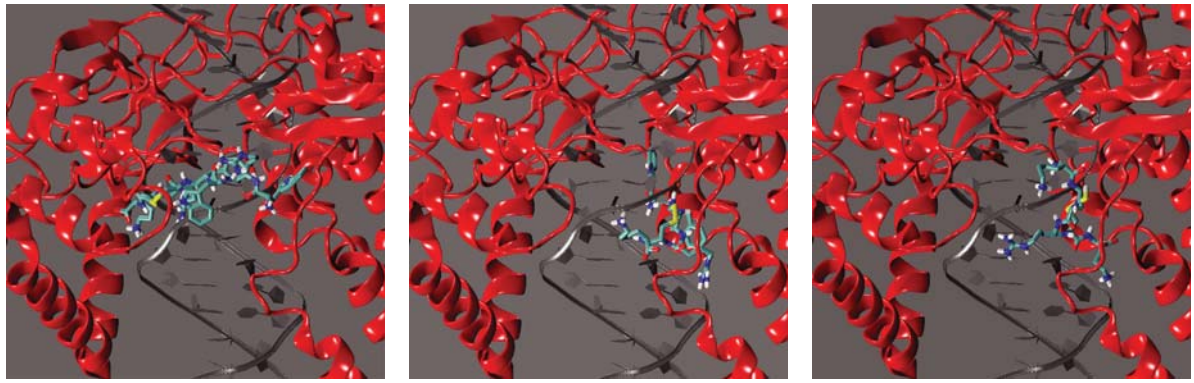
3.2. Inhibitory Potency of Peptide WRWYCRCK in Cleavage/Ligation by pfTopoI. DNA relaxation by type IB Topos involves two discrete transesterification reactions that is, a cleavage reaction, in which the active site tyrosine attacks the phosphodiester bond of the DNA backbone to generate a 3′-phosphotyrosyl cleavage intermediate and a free 5′-OH end, and a ligation reaction in which the 5′-OH acts as a nucleophile on the phosphotyrosyl bond to restore intact DNA. It was previously demonstrated that the inhibitory effect of the peptides WYCRCK, KCCRCK, and WRWYCRCK on DNA relaxation by vvTopoI and λ-Int could be ascribed to a specific inhibition of the cleavage and not the ligation step of catalysis [39, 40]. To address which steps of pfTopoI catalysis are affected by peptide WRWYCRCK, that inhibited relaxation by this enzyme we used a synthetic partially single-stranded suicide DNA substrate containing a preferred type IB TopoI cleavage sequence. This substrate, that was originally developed to investigate cleavage by hTopoI, acts as a mechanism-based inactivator of nuclear type IB Topos by allowing DNA cleavage, while the subsequent religation reaction is prevented due to diffusion of the generated 5′-OH end (see Figure 2(a)). Prevention of religation, however, is only conditional and this step of catalysis can be initiated by the addition of a surplus of a 5′-OH-containing ligator strand with a sequence matching the protruding noncleaved strand of the generated cleavage complexes (Figure 2(a)).

First, the ability of pfTopoI with cleave the suicide DNA substrate was tested in comparison to cleavage by hTopoI. The two enzymes were incubated with substrate radiolabeled at the 5′-end of the cleaved strand (to allow visualization of the cleavage products), the products were ethanol precipitated, trypsinated, and separated on a denaturing polyacrylamide gel prior to visualization by PhosphorImaging. As evident from Figure 2(b), pfTopoI cleaved the substrate and gave rise to cleavage products (marked Cl1 and Cl2) with approximately the same gel electrophoretic mobilities as those of cleavage products generated by hTopoI (compare lanes 1 and 2). These products were retained in the slot of the gel if trypsin digestion was omitted (data not shown), confirming their identity as covalent pfTopoI-DNA or hTopoI-DNA complexes. As previously reported in [54], even after trypsin digestion, the cleavage products of both pfTopoI and hTopoI were retarded in the gel due to the covalent attachment of short protease-resistant peptides to the radiolabeled strand of the DNA substrate. For hTopoI, the major cleavage product Cl1 was previously demonstrated to result from cleavage at the preferred site (indicated by an arrow in Figure 2(a)), while the minor Cl2 product arises from cleavage two nucleotides upstream to the cleavage site [54]. The gel electrophoretic mobility of cleavage products generated by pfTopoI suggests that this enzyme cleaves the utilized substrate at the same positions as does hTopoI.

To test the effect of peptide WRWYCRCK on pfTopoI mediated cleavage, increasing concentrations of the peptide were incubated with the above-described suicide DNA substrate prior to addition of pfTopoI. The reactions were performed essentially as described above and the percentage of substrate converted to cleavage product shown as a function of peptide concentration (Figure 2(c)). As previously reported for vvTopoI and λ-Int, peptide WRWYCRCK inhibited DNA cleavage by pfTopoI in a dose-dependent manner, although the observed cleavage inhibition was less potent than that observed for DNA relaxation.

Using the suicide substrate system, the effect of peptide WRWYCRCK on pfTopoI-mediated religation was investigated. In this experiment, preformed cleavage complexes were incubated with increasing concentrations of WRWYCRCK prior to addition of the ligator strand shown in Figure 2(a). Consistent with previous results obtained for vvTopoI and λ-Int the peptide did not affect ligation by pfTopoI (Figure 2(d)).

3.3. Peptide Specificity. The three peptides tested for activity against pfTopoI in the present study were previously demonstrated to inhibit vvTopoI and λ-Int activity with IC_{50}'s of 0.015–2.3 μM, while more unrelated enzyme activities such as *E. coli* type IA Topo and restriction endonucleases were hardly affected by any of the peptides. Although far from being species-specific, the peptide inhibitors appear rather sensitive to even subtle structural differences between the different target enzymes. This is evident from the different inhibition pattern of pfTopoI observed here (only WRWYCRCK inhibits pfTopoI) relative to that of the above mentioned TopoIB type enzymes (inhibited by WYCRCK, KCCRCK, and WRWYCRCK) [40]. To further address the specificity of the pfTopoI active inhibitor WRWYCRCK, we tested the effect of this peptide on the two restriction endonucleases EcoRI and PvuII. Increasing concentrations of



(a) (b) (c)

Figure 4: Docking of peptides into the noncovalent pfTopoI-DNA complex. Side view of the ternary pfTopoI-DNA-peptide complex. The structure represents the best complex obtained from the docking experiment. The protein is shown in red cartoon, the DNA in grey ribbon and the peptide in licorice, with the atoms coloured with the following code: carbon: cyan, nitrogen: blue, oxygen: red, sulphur: yellow. (a) Octapeptide WRWYCRCR, (b) hexapeptide WYCRCR, and (c) hexapeptide KCCRCR.

the peptide were incubated with the test plasmid (pUC19) before addition of either of the restriction enzymes. As evident from Figure 3, and consistent with previously published results [40], the peptide had no or only very modest effect on the cleavage activity of these enzymes, confirming the specific action of WRWYCRCK.

All three peptides, WYCRCK, KCCRCK, and WRWYCRCK have previously been shown to possess an unspecific DNA binding capacity, which was confirmed in the present study (data not shown) [40]. However, the lack of inhibition of endonuclease activity and the inhibition of pfTopoI activity only by WRWYCRCK and not by WYCRCK and KCCRCK even at very high concentrations argues against peptide inhibition being the result of a simple competition for noncovalent DNA binding. Indeed, for vvTopoI and λ-Int, all peptides were demonstrated not to affect noncovalent DNA interaction and it was suggested that inhibition was a result of the peptides preventing the transition from noncovalent to covalent binding, that is, DNA cleavage, by interfering with the enzyme-DNA interphase [40].

3.4. Prediction of the Interaction Mode between the Peptide and the pfTopoI-DNA Complex. Docking experiments have been carried out to identify the preferential binding site of the WRWYCRCK octapeptide on the noncovalent pfTopoI-DNA complex. 250 docking runs were done and the best complex, having a free energy value of −14.0 Kcal/mol, was selected and analyzed. This complex shows that the peptide is located in the minor groove cavity in front of the active site (see Figure 4(a)), establishing many contacts with both the protein and the DNA bases, as reported in Table 1. Concerning the DNA contacts, interesting interactions occur between the peptide and Gua12-Ade15 and Thy32-Thy34 on the scissile and intact strand, respectively. The optimal positioning of the octapeptide in the minor groove is due either to a good geometrical fitting between the two molecules, or to the high number of electrostatic interactions between the positively charged residues of the peptide and the negatively charged DNA phosphates. As far as the protein is concerned, interesting interactions occur between Trp3 and Cys5 of the peptide and Arg310 of pfTopoI and between Tyr4 and Asp513 of pfTopoI (see Table 1). Residues Arg310 and Asp513 of the *Plasmodium* protein correspond to residues Arg364 and Asp533 of the human enzyme, which are known from the 3D structure of the ternary drug-DNA-enzyme complex to directly interact with the camptothecin drug [55, 56]. The peptide then, positioned in the minor groove of the DNA just in front of the protein active site, exerts an inhibition of the cleavage process thus providing an explanation for the experimental results reported in Figure 2(c).

An identical docking experiment has been performed also for the two hexapeptides WYCRCK and KCCRCK, not having any inhibitory effect on pfTopoI relaxation. The best docked complexes, having a free energy value of −11.36 and −11.04 Kcal/mol, are reported in Figures 4(b) and 4(c) for the WYCRCK and KCCRCK peptide, respectively. Both peptides are found in a region different from the one found for the octapeptide. The two hexapeptides are located in proximity of the major groove in a region far from the enzyme active site and, in contrast to what was observed for the octapeptide, they are not able to interact with Arg310 and Asp513, providing a structural explanation for their lack of inhibition.

4. Discussion

During recent years, bioactive peptides have been suggested as an alternative or complement to traditional small-molecule drugs in the combat against protozoan parasites [10, 24, 55, 57, 58]. One of the suggested advantages of peptide drugs in antiparasite treatment relies on the ease by which such drugs can be selected or modified to target desired biological pathways using nature's own selection mechanisms or large throughput *in vitro* screening and/or

Table 1: Contacts between the peptide WRWYCRCK, and the noncovalent pfTopoI-DNA complex calculated for the best docked complex.

Peptide	Protein	DNA
Trp1	—	—
Arg2	Lys208, Gly313, Glu314, Ser514	Ade13
Trp3	Tyr205, Lys208, Arg310, Arg312, Gly313	Ade14
Tyr4	Arg312, Gly313, Asp513	Gua12, Ade13, Ade14
Cys5	Tyr205, Arg310, Gly311	Ade14
Arg6	Arg312	Gua12, Ade13, Ade14, Ade15, Thy32, Cyt33
Cys7	Lys322	—
Lys8	—	Ade15, Cyt33, Thy34

directed evolution setups. Another advantage relies on the relatively large interphase between peptide drugs and their target, possibly facilitating an increased specificity of peptide drugs compared to small-molecule drugs [25, 59]. Until recently, high synthesis costs have hampered the possibilities of developing peptide-based drugs against various relevant targets. However, with new synthesis strategies and lowered monomer costs the interest in developing peptide drugs has markedly increased [24–27]. One of the very promising strategies was first presented by Nagaraja's research group, who had taken advantage of antibodies raised by the natural immune response of mice injected with the desired target, in the reported cases, *Mycobacteria* DNA gyrase or TopoI [28–30]. As a result different antibodies with specific inhibitory effects on either target have been identified. Remarkably, these antibodies appear extremely specific and show no activity against the *E. coli* counterparts of the *Mycobacteria* topoisomerases. Hence, these antibodies hold great promise for the further development of *Mycobacteria*-specific peptide drugs based on the amino acid sequence of the active parts of the antibodies [28]. Indeed, several studies highlight the feasibility in deriving active peptides with specificity retained from the antibodies from which they originate [31–34].

Another reported strategy was based on selecting peptides with activity against the TopoI related λ-Int from a large library [35]. As a result of this study, a number of peptides with inhibitory effect on the recombinase were identified. Some of these, WYCRCK, KCCRCK, and WRWYCRCK, also inhibited the relaxation activity of vvTopoI [39, 40]. In the present study, we demonstrate that of these peptides, WRWYCRCK but not WYCRCK, or KCCRCK inhibits DNA relaxation mediated by pfTopoI. As previously reported for the peptide inhibition of λ-Int and vvTopoI, it is specifically the cleavage reaction of pfTopoI that is inhibited by WRWYCRCK, while ligation is largely unaffected by the peptide, possibly due to the peptide being unable to bind to the covalent pfTopoI-DNA cleavage complexes. The inhibition on cleavage appears to be dependent on cysteine bridging since the addition of DTT counteracts the peptide effect. For λ-Int and vvTopoI it was demonstrated that although the peptide does bind DNA unspecifically, the inhibitory effect of active peptides on DNA cleavage could not be ascribed to a simple competition preventing noncovalent DNA interaction of the Topos [40]. Rather the peptides were suggested to prevent the transition from noncovalent to covalent binding. Although this was not addressed experimentally for pfTopoI the inhibition of this enzyme by only one of the peptides, WRWYCRCK, argues for a specific inhibition rather than merely an unspecific competition for DNA binding. Note that all three peptides bind DNA in an unspecific manner [40].

This notion is further supported by molecular docking experiments in which the molecular mechanism for the inhibition exerted by the octapeptide was analyzed. This analysis allowed us to predict the preferential interaction interface between the noncovalent pfTopoI-DNA complex and the peptide itself. This is in agreement with the peptide being able to prevent the transition from noncovalent to covalent binding. Hence, the complex with the lowest free energy, that is, the best complex, is represented by the peptide inserted in the DNA minor groove, near the active site (Figure 4), where it impedes the catalytic tyrosine to produce the nick on the scissile strand, as demonstrated by the cleavage assay (Figure 2). Indeed, the peptide interacts with two residues in proximity of the active site, Arg310 and Asp513, which are the plasmodial counterpart for the human residues Arg364 and Asp533 that in the 3D structure of the human enzyme are in direct contact with the camptothecin drug [56]. The peptide is stabilized by numerous contacts to either the protein or the DNA, confirming that it represents an efficient inhibitor of the enzyme. Docking of the two noninhibiting peptides, WYCRCK and KCCRCK, into the noncovalent pfTopoI-DNA complex revealed that these peptides were located far from the active site of pfTopoI, which may explain why they do not inhibit pfTopoI.

Although, until now, no species-specific peptide inhibitors of parasitic Topos have been reported, we believe that the presented studies demonstrate the feasibility of inhibiting Topos relevant in antiparasite treatment and that molecular docking may pave the road for the rational development of species-specific inhibitors.

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Research Article

Bioinformatic Analysis of *Leishmania donovani* Long-Chain Fatty Acid-CoA Ligase as a Novel Drug Target

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Fatty acyl-CoA synthetase (fatty acid: CoA ligase, AMP-forming; (EC 6.2.1.3)) catalyzes the formation of fatty acyl-CoA by a two-step process that proceeds through the hydrolysis of pyrophosphate. Fatty acyl-CoA represents bioactive compounds that are involved in protein transport, enzyme activation, protein acylation, cell signaling, and transcriptional control in addition to serving as substrates for beta oxidation and phospholipid biosynthesis. Fatty acyl-CoA synthetase occupies a pivotal role in cellular homeostasis, particularly in lipid metabolism. Our interest in fatty acyl-CoA synthetase stems from the identification of this enzyme, long-chain fatty acyl-CoA ligase (LCFA) by microarray analysis. We found this enzyme to be differentially expressed by *Leishmania donovani* amastigotes resistant to antimonial treatment. In the present study, we confirm the presence of long-chain fatty acyl-CoA ligase gene in the genome of clinical isolates of *Leishmania donovani* collected from the disease endemic area in India. We predict a molecular model for this enzyme for *in silico* docking studies using chemical library available in our institute. On the basis of the data presented in this work, we propose that long-chain fatty acyl-CoA ligase enzyme serves as an important protein and a potential target candidate for development of selective inhibitors against leishmaniasis.

1. Introduction

Leishmaniasis is a disease caused by protozoan parasites of the *Leishmania* genus. Visceral leishmaniasis (VL), also known as kala-azar, is the most severe form of leishmaniasis (http://www.dndi.org/diseases/vl.html). With no vaccine in sight, treatment for kala-azar relies primarily on chemotherapy [1].

Phylogenetics suggests that *Leishmania* is relatively early branching eukaryotic cells and their cell organization differs considerably from that of mammalian cells [2, 3]. Hence, the biochemical differences between the host and parasite can be exploited for identification of new targets for rational drug design. It is also imperative that the probability of developing drug resistance should be less with these targets. This can be achieved by targeting an essential cellular process, which has the pressure to remain conserved and cannot be bypassed by using alternative pathway.

One interesting target which emerged from our microarray experiments [4] was long-chain fatty acid-CoA ligase (EC 6.2.1.3) (GenBank Accession No. XM_001681734), a key enzyme involved in the metabolism of fatty acids in all organisms [5–9]. Fatty acyl-CoA has multiple roles involved in protein transport [10, 11], enzyme activation [12], protein acylation [13], cell signaling [14], transcriptional regulation [15], and particularly β-oxidation and phospholipid biosynthesis. Especially in *Leishmania*, long-chain fatty acids are predominant precursors of total lipid composition (the combination of phospholipids, sphingolipids, and ergosterol). Long-chain fatty acyl-CoA ligase is critical enzyme processing long-chain fatty acid acylation which is essential for lysophosphatidylinositol (lyso-PI) incorporation into glycosyl phosphatidylinositols (GPIs) [16, 17]. These GPIs-anchors are the major surface virulent factors in *Leishmania* and have received considerable attention [18]. *De novo* sphingolipid biosynthesis starts with the condensation of serine and the product of long-chain fatty acyl-CoA ligase. *L. major* preferentially incorporates myristoyl-CoA (C14) over palmitoyl-CoA (C16) into their long-chain base [19, 20]. This selection of specific long-chain fatty acyl-CoA reflects

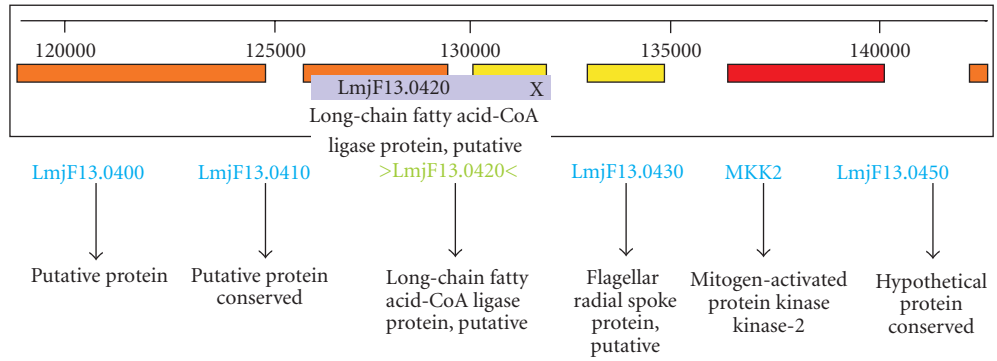


Figure 1: Graphical representation of long-chain fatty acyl-CoA ligase (LCFA) gene (in Artemis) on chromosome 13 of *Leishmania major*.

the presence of myristoyl-specific long-chain fatty acyl-CoA ligase in *Leishmania* [21].

Gaining new knowledge on fatty acid metabolism will not only provide fundamental insight into the molecular bases of *Leishmania* pathogenesis but also reveal new targets for selective drugs. Enzymes involved in fatty acid and sterol metabolism have been shown to be important pharmaceutical targets in *Leishmania* and other kinetoplastida [22]. Triacsin C, a specific inhibitor of long-chain fatty acyl-CoA synthetase, was shown to have an inhibitory effect on the growth of *Cryptosporidium parvum in vitro* [23].

Four fatty acyl-CoA synthetases have been described previously in *Trypanosoma brucei*, displaying different chain-length specificities [24, 25]. The whole genome sequence of three *Leishmania* spp. (*L. major*, *L. infantum,* and *L. braziliensis*) has been sequenced, and the availability of putative long-chain fatty acyl-CoA ligase genes was present in all three *Leishmania* spp. at chromosome 13, which would be required for initiation of β-oxidation and fatty acid metabolism.

In the present study we confirm the presence of long-chain fatty acyl-CoA ligase gene in *Leishmania donovani* clinical isolate collected from, the state of Bihar India [26–29], which alone accounts for 50% of the total burden of visceral leishmaniasis worldwide [30]. Further progress in the understanding of this enzyme is likely to be achieved through the whole genome sequence (WGS) project of these clinically important isolates [26–29], underway in our laboratory (http://www.leishmaniaresearchsociety.org/).

2. Material and Methods

2.1. Collection of Clinical Isolates. The clinical isolates of *L. donovani* were collected from two kala-azar patients selected from Muzaffarpur, Bihar. The criterion for visceral leishmaniasis diagnosis was the presence of Leishman-Donovan (LD) bodies in splenic aspirations performed, which was graded to standard criteria [30]. Response to sodium antimony gluconate (SAG) treatment was evaluated by repeating splenic aspiration at day 30 of treatment. Patients were designated as antimonial responsive (*L. donovani* isolate 2001) based on the absence of fever, clinical improvement with reduction in spleen size, and absence of parasites in splenic aspirate while patients who showed presence of parasites in splenic aspiration were considered to be antimonial unresponsive (*L. donovani* isolate 39) [26–29].

2.2. Sample Collection and Nuclear DNA Isolation. *L. donovani* isolates 2001 (SAG-sensitive) and 39 (SAG-resistant) used in the present study, were maintained in culture as described previously in [26–29]. For nuclear DNA isolation 10–15 mL log-phase culture was taken and centrifuged at 5,000 rpm for 8 min at 4°C. The supernatant was decanted; cell pellet was resuspended in 3–6 mL NET buffer and centrifuged at 5,000 rpm for 8 min at 4°C. The supernatant was discarded, and the pellet was redissolved in 750 μL NET buffer, 7.5 μL proteinase K (10 mg/mL stock) (MBI, Fermentas, Cat No. EO0491), and 50 μL of 15% sarkosyl. Sample was incubated at 37°C overnight for proteinase K activity. The cell lysate was centrifuged at 18,000 rpm for 1 hr at 4°C. The supernatant containing nuclear DNA was transferred to a fresh tube and given RNase treatment (20 μg/mL) (MBI, Fermentas, Cat No. EN0531) at 37°C for 30 min. DNA was extracted first with one volume phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and finally with chloroform. Nuclear DNA was precipitated with 2.5 volumes of prechilled absolute ethanol, dissolved in nuclease-free water and stored at 4°C for future use.

2.3. Primer Design, PCR Amplification, and Sequencing of Long-Chain Fatty Acyl-CoA Ligase Gene. PCR amplification was carried out using *Pfu* DNA polymerase (MBI, Fermentas, Cat No. EP0501). Reactions were carried out in a Perkin Elmer GeneAmp PCR system with 2001 nuclear DNA (10–50 ng) as template. The following oligonucleotide primers were designed on the basis of available gene sequence of *L. major* (GenBank Accession No. XM_001681734): forward primer: 5′GGGCCATATGCTGCAGCG 3′ (18 mer) and reverse primer: 5′GGCCTCGAGCTAAAACAAATCATCG3′ (25 mer). The amplification conditions were initial denaturation at 95°C for 10 min, denaturation at 95°C for 30 sec, annealing at 65°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 10 min; 30 cycles. The PCR product was purified from agarose gel using MBI Fermentas DNA Extraction kit (MBI, Fermentas, Cat No. K0513) and further for DNA sequencing by Bangalore Genei, India.

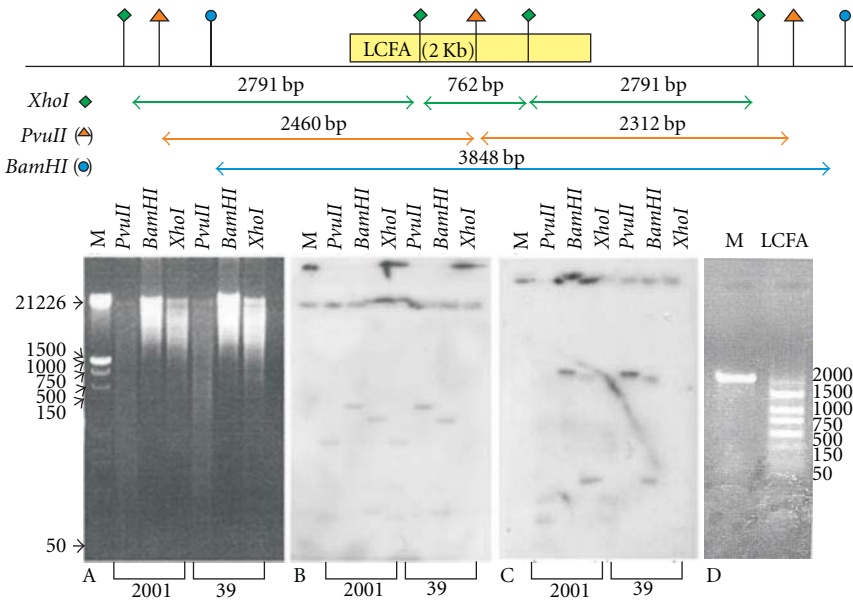
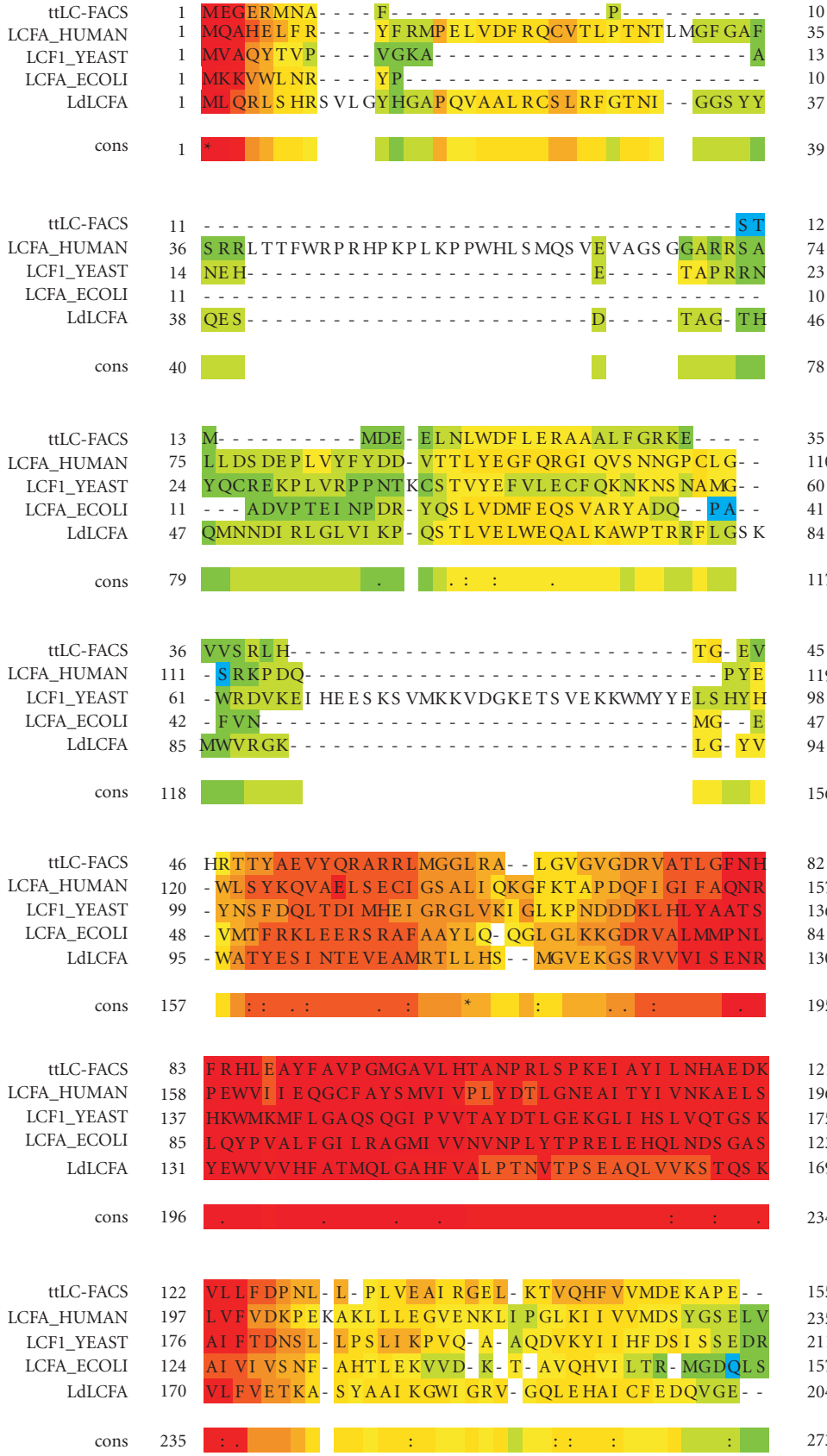


Figure 2: Determination of long-chain fatty acyl-CoA ligase gene copy number. The nuclear DNA of *L. donovani* 2001 and 39 promastigotes was isolated and 16 μg was digested with different restriction enzymes. (A) Nuclear DNA digest stained with ethidium bromide (B) Southern blot of "A" with α-tubulin gene probe. (C) Southern blot of "A" with long-chain fatty acyl-CoA ligase probe. (D) PCR amplification of long-chain fatty acyl-CoA ligase gene (M: Marker, LCFA: 2010 bp of long-chain fatty acyl-CoA ligase gene).

Table 1: Selected ortholog for *Leishmania donovani* long-chain fatty acyl-CoA ligase gene in kinetoplastida: ORTHOMCL4080 (http://www.genedb.org/).

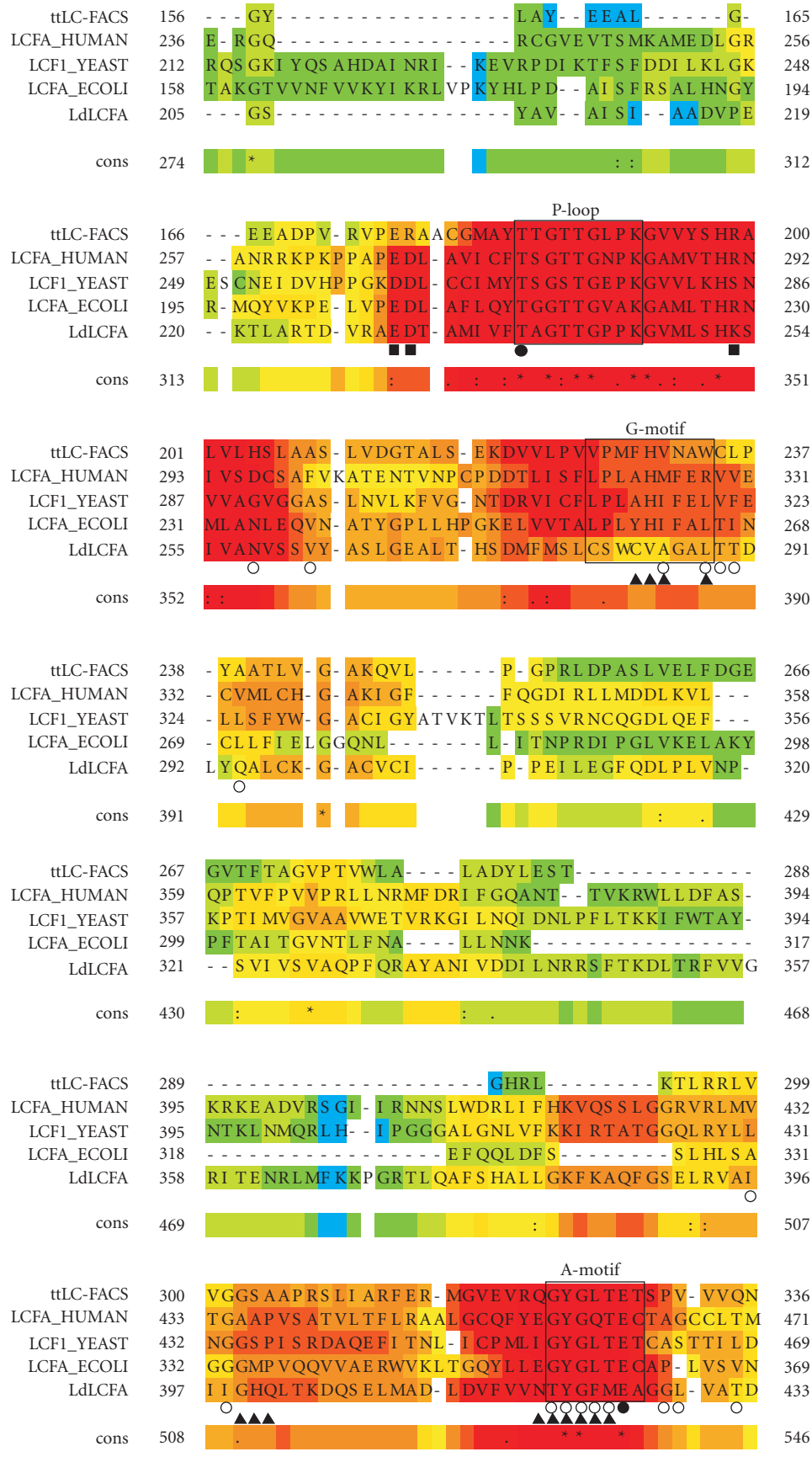
Systematic ids	Organism	Product
LbrM13_V2.0240	*L. braziliensis* MHOM/BR/75/M2904	Fatty acid thiokinase (long chain), putative; acyl-CoA synthetase, putative; long-chain-fatty acid-CoA ligase protein, putative
LinJ13_V3.0300	*L. infantum* JPCM5	Fatty acid thiokinase (long chain), putative; long-chain-fatty acid-CoA ligase protein, putative; acyl-CoA synthetase, putative
LmjF13.0420	*L. major strain* Friedlin	Long-chain fatty acid-CoA ligase protein, putative; acyl-CoA synthetase, putative; fatty acid thiokinase (long chain), putative
Tb11.02.2070	*T. brucei* 927	Long-chain fatty acid-CoA ligase protein, putative; fatty acid thiokinase (long chain), putative; acyl-CoA synthetase, putative
Tc00.1047053504089.40	*T. cruzi*	Long-chain fatty acid-CoA ligase protein, putative; acyl-CoA synthetase, putative; fatty acid thiokinase, long chain, putative
TvY486_1104610	*T. vivax*	Long-chain fatty acid-CoA ligase protein, putative

2.4. Characterization of Long-Chain Fatty Acyl-CoA Ligase Gene. *L. donovani* nuclear DNA (16 μg for each reaction) of two different clinical isolates, drug (SAG) sensitive 2001 and drug (SAG) resistant 39, were digested with 40-unit three different restriction enzymes (*Pvu*II, *Bam*HI, and *Xho*I), which were cut overnight and separated on 0.8% agarose gel by electrophoresis at 50 V. In order to improve transfer efficacy, DNA in agarose gel was treated with 0.25 N HCl for 15 min (partial depurination), rinsed with autoclaved water 3x, and treated with 0.4 N NaOH (breaking backbone at depurinated region) for 30 min. DNA was transferred to nylon membrane by conventional downward capillary transfer method for 5 h using 3 mm Whatman paper wick [8]. The efficiency of transfer was assessed by visualizing DNA by methylene blue staining. After transfer on nylon membrane the DNA was neutralized in 0.5 M Tris (pH 7.4), 1.5 M NaCl, 2x for 5 min at room temperature. The membrane was then washed in 2X SSC, 2x for 15 min. Nylon membrane was incubated with 2.5 mL of prehybridization buffer (0.6 M NaCl, 0.5 M Tris-HCl (pH 7.5), 0.008 M EDTA, 1% sodium pyrophosphate, 0.2% SDS, and 50 μg/mL heparin) and incubated in a hybridization oven at 65°C for 2 h. Radioactive probe was prepared by labeling 25 ng of the DNAs with [α-^{32}P] dCTP by random priming method (BRIT/BARC, India) and purified using a desalting column (sephadex G-50). The radioactivity was checked with a Geiger Muller Counter (dosimeter) and stored at −20°C. The probe was added to the prehybridization buffer and incubated at 65°C overnight in hybridization oven. Membrane was washed twice with 2X SSC, 0.1% SDS (15 min each) at 65°C and then washed with 2X SSC, 0.1% SDS for 30 min at 65°C to reduce background signals. Hybridized



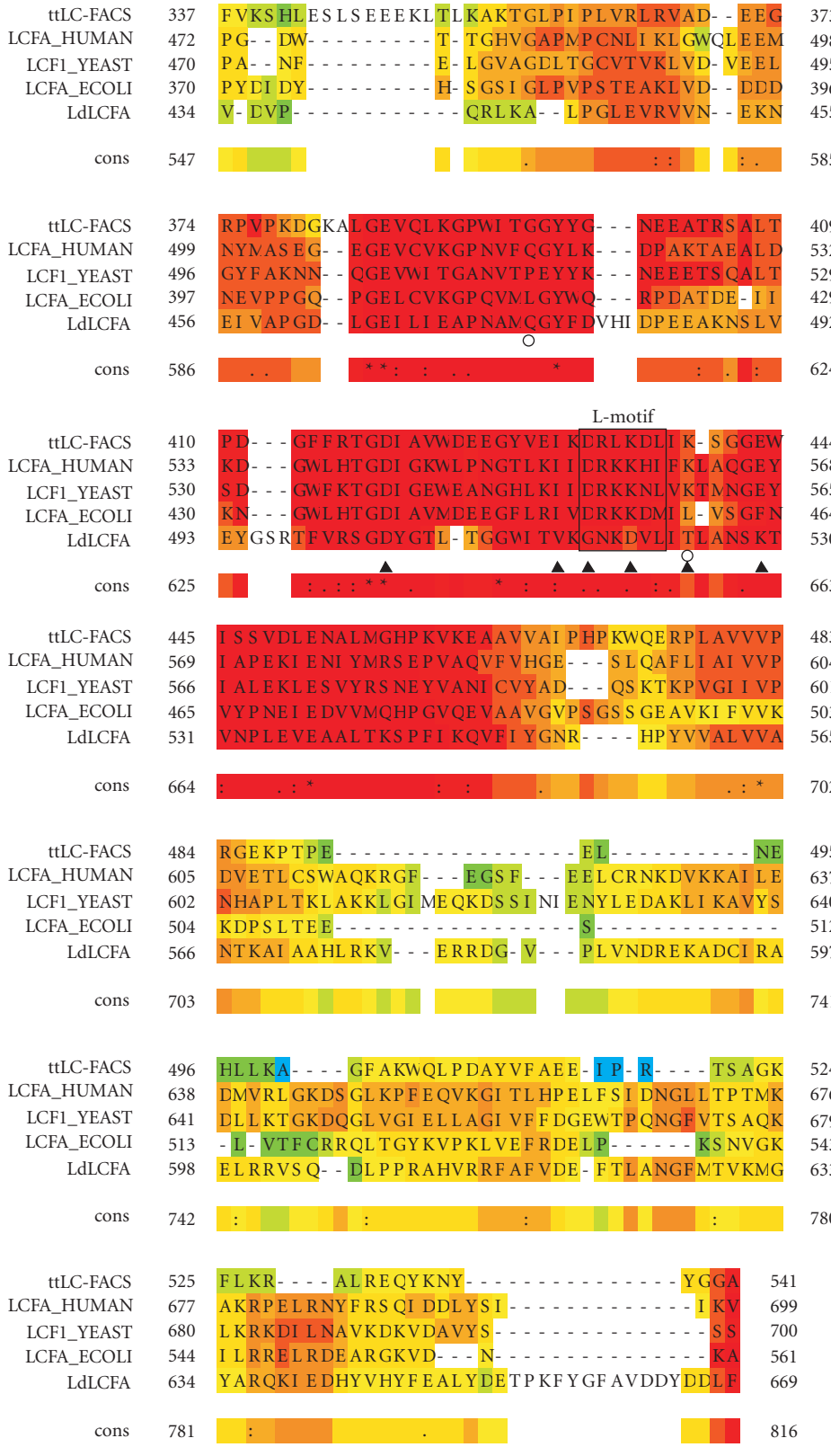
(a)

Figure 3: Continued.



(b)

Figure 3: Continued.



(c)

Figure 3: Amino acid sequence alignments of long-chain fatty acyl-CoA synthetases. The amino acid sequence of *Leishmania* LCFA (LdLCFA) was aligned with LC-FACS from *T. thermophilus* (ttLC-FACS, Q6L8FO), human (LCFA_HUMAN, P41215), yeast (LCF1_YEAST, P30624), and *E. coli* (LCFA_ECOLI, P29212). The *boxed areas* denoted with *bold letters* correspond to conserved motifs of long-chain fatty acyl-CoA ligase: G, A, and L motifs as well as the P-loop. *Filled squares*, *open circles*, *filled circles*, and *filled triangles* indicate residues believed to be involved in dimer formation, fatty acid binding, magnesium ion binding, and adenylate binding, respectively.

membrane was layered over a wet Whatman paper sheet to soak extra solution and covered with Saran Wrap (cellophane paper) and exposed to X-ray film. After 4–18 h exposure in an exposure cassette at −70°C, X-ray film was developed for analysis.

2.5. Phylogenetic Analysis. The amino acid sequence of *Leishmania* long-chain fatty acyl-CoA ligase, obtained from our microarray experiments [4], was compared with sequences available in GeneDB ORTHOMCL4080 database (http://www.genedb.org/) to identify the nearest ortholog of this sequence in kinetoplastida. Multiple sequence alignments were performed using Clustal W version 1.8 (http://www.ebi.ac.uk/clustalw) and T-cofee [31]. To calculate evolutionary distances of kinetoplastida long-chain fatty acyl-CoA ligases with human acyl CoA synthetases (ACSs) [32], phylogenetic dendrograms were constructed by neighbor-joining method and tree topologies were evaluated by performing bootstrap analysis of 1000 data sets using MEGA 3.1 (Molecular Evolutionary Genetics Analysis) [33]. All 26 human ACSs amino acid sequences were selected [32], along with their transcript variants which are aligned with different long-chain fatty acyl-CoA ligase ortholog present in kinetoplastida family, to define the clade difference with *Trypanosome* and *Leishmania* long-chain fatty acyl-CoA ligase, and human acyl-CoA synthetases.

2.6. Homology Modeling of Leishmania Long-Chain Fatty Acyl-CoA Ligase. The amino acid sequence of *Leishmania* long-chain fatty acyl-CoA ligase was retrieved from the NCBI database (GenBank Accession No. XM_001681734). It was ascertained that the 3D structure of *Leishmania* long-chain fatty acyl-CoA ligase protein was not available in Protein Data Bank (PDB); hence, the present exercise of developing the 3D model of this protein was undertaken. cBLAST (http://www.ncbi.nlm.nih.gov/Structure/cblast/cblast.cgi) and PSI-BLAST search was performed against PDB with the default parameter to find suitable templates for homology modeling. The sequence alignment of *Leishmania* long-chain fatty acyl-CoA ligase and respective templates was carried out using the CLUSTALW (http://www.ebi.ac.uk/clustalw) and MODELLER9V8 programs [34, 35]. The sequences that showed the maximum identity with high score and lower e-value were used as a reference structure to build a 3D model.

The retrieved sequences of *Thermus thermophilus* (PDB Accession Code: 1ULT, 1V25, 1V26) [36] and *Archaeoglobus fulgidus* (PDB Accession Code: 3G7S) long-chain fatty acyl-CoA ligases served as template for homology modeling based on its maximum sequence similarity to *Leishmania* long-chain fatty acyl-CoA ligase. The alignment was manually refined at some loops region of the templates. The resulting alignment was used as an input for the automated comparative homology modeling for generating 3D model structure of *Leishmania* long-chain fatty acyl-CoA ligase. The academic version of MODELLER9V8 was used for model building. The backbones of core region of the protein were transferred directly from the corresponding coordinates of templates. Side chain conformation for backbone was generated automatically. Out of 50 models generated by MODELLER, the one with the best DOPE score, minimum MOF (Modeller Objective Function), and best VARIFY 3D profile was subjected to energy minimization. In order to assess the stereochemical qualities of 3D model, PROCHECK analysis [37] was performed and Ramachandran plot was drawn.

3. Results

3.1. Metabolism of Long-Chain Fatty Acyl-CoA Ligase Enzyme. Three types of fatty acyl CoA ligase have been defined with respect to the length of the aliphatic chain of the substrate: short (SC-EC 6.2.1.1), medium (MC-EC 6.2.1.2), and long-chain (LC-EC 6.2.1.3) fatty acyl-CoA ligase. These utilize C2-C4, C4-C12, and C12-C22 fatty acids as substrates, respectively [9]. Fatty acid activation step involves the linking of the carboxyl group of the fatty acid through an acyl bond to the phosphoryl group of AMP. Subsequently, a transfer of the fatty acyl group to the sulfhydryl group of CoA occurs, releasing AMP [38–40]. This magnesium-dependent two-step acylation of fatty acid by fatty acyl CoA synthetases was defined as unidirectional Bi Uni Uni Bi Ping-Pong mechanism [36, 39].

Genome analysis suggests that *L. major* oxidizes fatty acids via β-oxidation in two separate cellular compartments: the glycosome and mitochondria [41]. An argument for the involvement of glycosome in lipid metabolism is the fact that in each of three trypanosomatid genomes three genes called half ABC transporters (GATI 1-3) have been found identical with peroxisomal transporters involved in fatty acid transport. In *T. brucei*, it was conformed that these transporters are associated with glycosomal membrane [42]. These transporters might be coupled with fatty acyl-CoA ligase in glycosome, which can provide activated form of fatty acids to these transporters like oleoyl-CoA, and also other acylated fatty acids.

In *T. brucei,* little β-oxidation was observed in mitochondria. However, *T. brucei* contains at least two enzymes involved in β-oxidation of fatty acid (2-enoyl-CoA hydratase and hydroxyacyl-dehydrogenase, encompassed in a single protein) with glycosome localization [43]. The presence of a PTS (Peroxisomal Targeting Sequence) on *T. brucei* and *T. cruzi* carnitine acetyl transferase, catalysing the last peroxisomal step in fatty acid oxidation, suggests that the major β-oxidation processes are situated in glycosomes [44]. In *L. donovani*, one of the β-oxidation enzyme 3-hydroxyacyl-CoA dehydrogenase has been localized to glycosomes [45]. The hypothetical localization of *Leishmania* long-chain fatty acyl-CoA ligase was predicted in mitochondria or glycosome but, with the reference of other organisms, the specialized localization of specific long-chain fatty acyl-CoA ligase family protein needs to be taken into account in future.

As mentioned in a previous study β-oxidation has been found to be unregulated in *Leishmania*'s amastigotes then in promastigote stage [46–48]. This specialized increase was described so that, in infectious stage, energy requirement

Table 2: Results of protein structure by PROCHECK and VERIFY 3D.

	Leishmania long-chain fatty acyl-CoA ligase	*T. thermophilus* long-chain fatty acyl-CoA synthetase (1ULTA)
Residues in most favoured regions	521 (87.9%)	405 (90.0%)
Residues in additional allowed regions	62 (10.5%)	43 (9.6%)
Residues in generously allowed regions	7 (1.2%)	2 (0.4%)
Residues in disallowed regions	3 (0.5%)	0 (0.0%)
Number of nonglycine and nonproline residues	593	450
Number of end-residues (excl. Gly and Pro)	2	155
Number of glycine residues (shown as triangles)	48	48
Number of proline residues	26	34
Total number of residues	669	687
Residues with Verify 3D Score >0.2	52.24%	96.63%
Errat overall quality factor	44.154	89.655

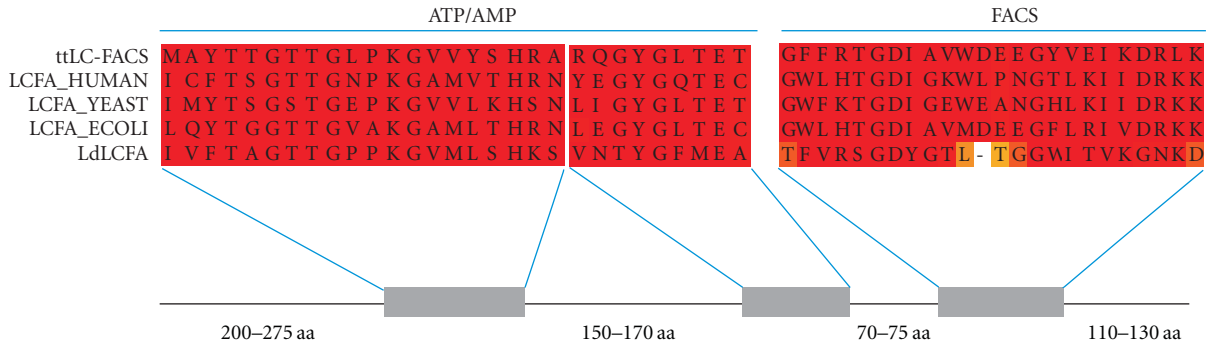


Figure 4: Domain organization of amino acid sequence alignments of ATP-AMP and fatty acyl CoA synthetase (FACS) motif from *T. thermophilus* (ttLC-FACS), human (LCFA_HUMAN), yeast (LCF1_YEAST), *E. coli* (LCFA_ECOLI), and *Leishmania* (L LCFA).

was supplemented to utilize fatty acid as carbon and energy source rather than glucose [47]. Long-chain fatty acyl-CoA ligase is the key enzyme involved in β-oxidation of fatty acids, and its compartmentation in glycosome supports a strong evidence of the involvement of this enzyme in cellular biogenesis and its importance at particular stage of *Leishmania* life cycle. In the same way upregulation of long-chain fatty acyl-CoA ligase with combination of other enzymes involved in fatty acid catabolism might play a crucial role in cell survival at infectious stage of *Leishmania,* and these analyses must be supplemented with experimental biology.

3.2. Characterization of Leishmania Long-Chain Fatty Acyl-CoA Ligase Gene. The presence of *L. donovani* long-chain fatty acyl-CoA ligase gene in the clinical isolates was ascertained by PCR amplification. The putative long-chain fatty acid-CoA ligase gene of *L. major* is present in the *Leishmania* Genome Databank (http://www.genedb.org/) on chromosome 13 (Figure 1). Specific 2010 bp size amplified product was obtained, showing the presence of long-chain fatty acyl-CoA ligase gene in the *L. donovani* clinical isolate (Figure 2(D)). The amplified product was sequenced and confirmed to be long-chain fatty acid-CoA ligase gene by performing NCBI-BLAST identity with *L. major* gene. NCBI-BLAST result showed 96% sequence similarity and 1% gaps with *L. major* long-chain fatty acyl-CoA ligase gene (GenBank Accession No. XM_001681734). The starting 18 nucleotides and 19 nucleotides from the end sequence were missed due to direct amplified product sequencing. These nucleotides were collected from its maximum similar *L. major* long-chain fatty acyl-CoA ligase sequence (GenBank Accession No. XM_001681734).

For the determination of long-chain fatty acid-CoA ligase gene copy number, nuclear DNA from the *L. donovani* clinical isolates (2001, 39) was digested with various restriction enzymes. The restriction map was designed from the complete putative long-chain fatty acyl-CoA ligase gene and the flanking region present in chromosome 13 of *L. major* (Figure 2). Southern hybridization was performed using the 2010 bp long-chain fatty acid-CoA ligase gene PCR product as probe (Figure 2(C)). The same blot was also probed with alpha tubulin gene probe as an internal control, showing equal loading (Figure 2(B)). Complete digestion resulted in a single copy within the *L. donovani* genome, as *Bam*HI enzyme showed only one band of approximately 3848 bp, except *Pvu*II which was cut once into the gene sequence and *Xho*I which was cut twice into the gene sequence, which

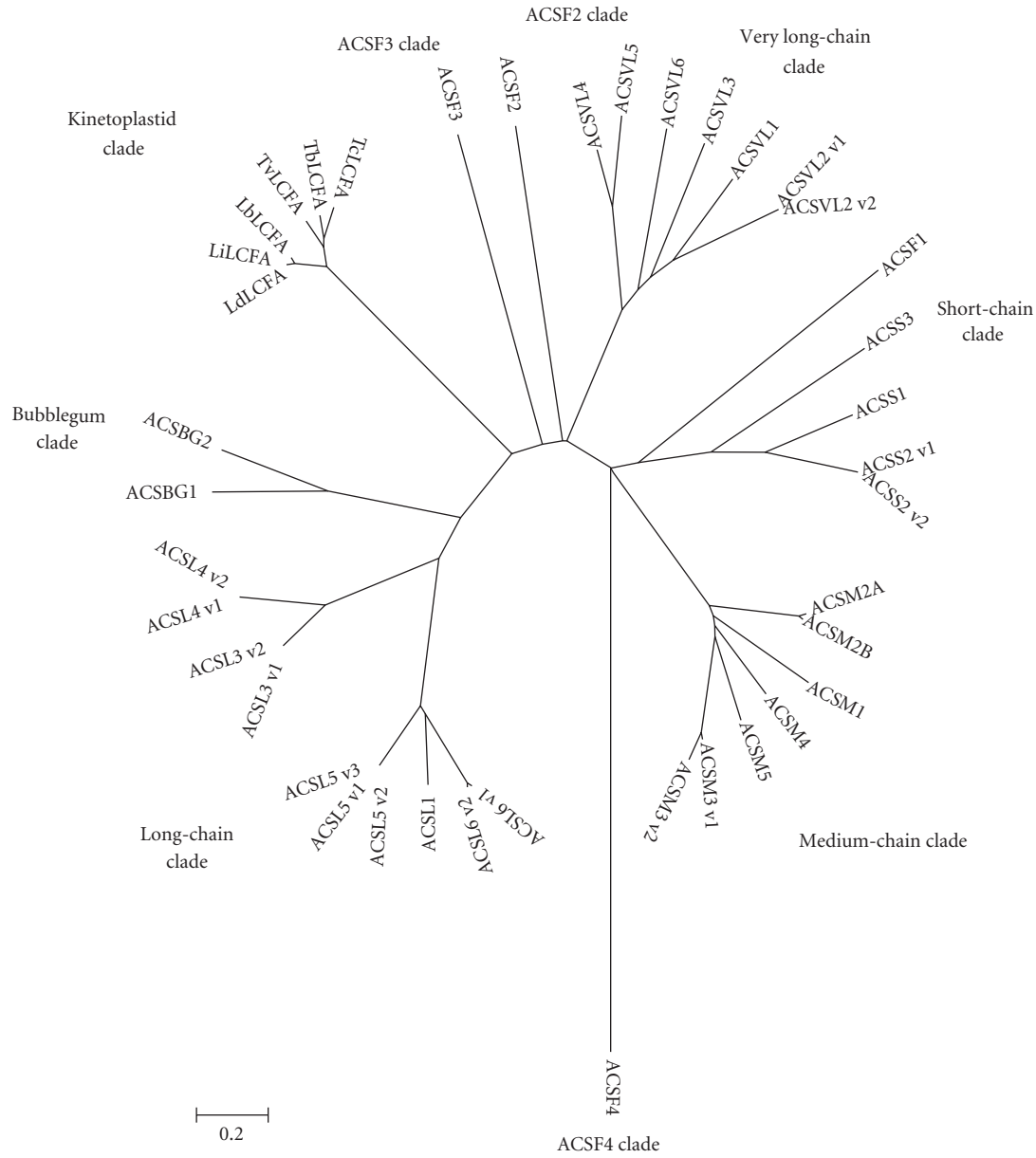


Figure 5: Phylogenetic trees based on human acyl CoA synthetases (ACSs) gene sequences [38] showing the relationship of all *Leishmania* long-chain fatty acyl-CoA ligase orthologs (Table 1), with their nearest phylogenetic relatives. Phylogenetic trees were constructed by the neighbour-joining method as well as the maximum likelihood method as implemented in MEGA4 software. Numbers at nodes are bootstrap values (ML/NJ; xx represents no bootstrap value in NJ tree where nodes differ in both dendrograms;—represents value <50). The bar represents 0.02 substitutions per alignment position. The bar represents substitutions per alignment position.

exhibited two and three hybridizing bands, respectively. The results showed that long-chain fatty acid-CoA ligase is present as a single copy gene in the *L. donovani* genome. The restriction pattern also verifying the restriction pattern of *L. donovani* and *L. major* long-chain fatty acyl CoA ligase coding region is almost the same.

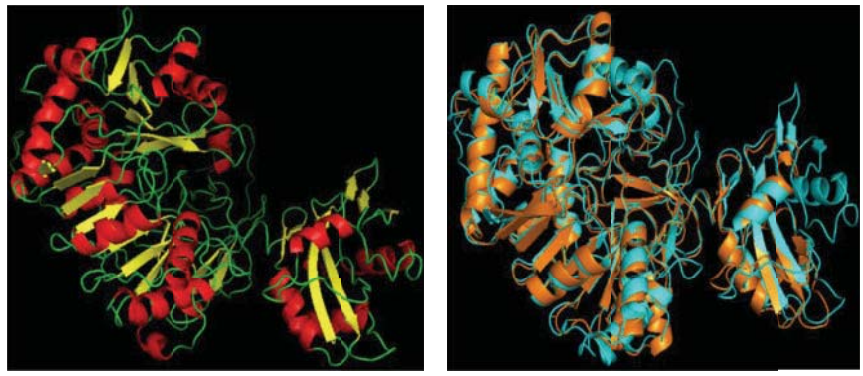
3.3. Identification of Conserved Domains and Structure-Function Correlation in Leishmania Long-Chain Fatty Acyl-CoA Ligase. Leishmania long-chain fatty acyl-CoA ligase gene was translated from full length ORF on the basis of its nucleic acid sequence. Long-chain fatty acyl-CoA synthetase from *T. thermophilus*, yeast, and *E. coli* and all 26 distinct human acyl-CoA synthetases were subjected to phylogenetic analysis to facilitate the evaluation of conserved motif with relationship of reference *Leishmania* long-chain fatty acyl-CoA ligase amino acid sequence. The amino acid sequence of *Leishmania* long-chain fatty acyl-CoA ligase (*Ld*LCFA) was aligned with LC-FACS from *T. thermophilus* (*Tt*LC-FACS,1ultA), human (LCFA_HUMAN, P41215), yeast (LCF1_YEAST, P30624), and *E. coli* (LCFA_ECOLI, P29212) on the basis of PSI-BLAST (Figure 3). However the overall similarity of *Leishmania* long-chain fatty acyl-CoA ligase (*Ld*LCFA) with other

fatty acyl-CoA synthetases family proteins is low, about 17% with *Tt*LC-FACS, 15% with LCFA_HUMAN, 14% with LCF1_YEAST, and 13% with LCFA_ECOLI. Based on the crystal structure of *Tt*LC-FACS and alignment with other long-chain fatty acyl-CoA synthetases [36], the amino acid sequence of *Leishmania* long-chain fatty acyl-CoA ligase shows conserve region corresponding to the linker (L), adenine (A), and gate (G) motifs as well as the P-loop, the phosphate-binding site. Previous studies [32, 36] put forward different motifs which can give insight to enhance our understanding of predicted structure-function relationships in *Leishmania*. P-loop is the Motif I which is also known as AMP-binding domain found in a close proximity to the adenosine moiety and helps to maintain the substrate in the proper orientation. The consensus sequence of Motif I, [Y,F]TSG[T,S]TGXPK shows high level of conservation with respect to *Leishmania* long-chain fatty acyl-CoA ligase, that is, 237-FTAGTTGPPK-246. Motif II contains the L-motif (432-DRLKDL-437) that acts as a linker between the large N-terminal domain and the smaller C-terminal domain in TtLC-FACS. The linker region is thought to be critical for catalysis function as it facilitates a conformational change upon ATP binding that permits subsequent binding of the fatty acyl and/or CoA substrates. In *Leishmania* long-chain fatty acyl-CoA ligase, this linker region (517-GNKDVL-522) is less similar compared with other organisms and is likely to be critical in enzyme activity. Motif III was found to be in all acyl CoA synthetases and a part of A-motif (adenine motif). This region has been described as an ATP/AMP-binding domain in other acyl-CoA synthetases [49–51]. The conserved consensus sequence of A-motif is YGXTE, a highly conserved motif with respect to *Leishmania* long-chain fatty acyl-CoA ligase region, that is, YGFME. From the crystal structure of *Tt*LC-FACS, it was proposed that Y324 was an adenine-binding residue [42] and also conserved throughout all organisms including *Leishmania*. The crystal structure of *S. enterica* acetyl-CoA synthetase revealed that the glutamate residue of A-motif is positioned near oxygen O1 of the AMP phosphate [52]. This region was predicted to be involved in substrate binding or stabilization, conserved in *Leishmania* long-chain fatty acyl-CoA ligase also. Motif IV comprises the first five residues of the nine-amino acid G-(or gate) motif (226-VPMFHVNAW-234) of ttLC-FACS (36), showing less sequence similarity with *Leishmania* long-chain fatty acyl-CoA ligase (281-CSWCVAGAL-289). From the crystal structure of *Tt*LC-FACS, it was proposed that the indole ring of W234 acts as a gate and blocks the entry of fatty acids into its substrate binding tunnel unless ATP is first bound, resulting in a conformational change that swings the gate open (36). However, a tryptophan residue corresponding to W234 was not found in any *Leishmania*, human, yeast, and *E. coli* fatty acyl-CoA synthetase sequences. In contrast, although no highly conserved sequences were identified, a corresponding gate residue may be located elsewhere in the structure of *Leishmania* long-chain fatty acyl-CoA ligase.

The fatty acyl-CoA synthetases are part of a large family of proteins referred to as the ATP-AMP-binding proteins. A common feature of enzymes in this family is that they all form an adenylated intermediate as part of their catalytic cycle. This group of enzymes is diverse in catalyzing the activation of a wide variety of carboxyl-containing substrates, including amino acids, fatty acids, and luciferin. Sequence comparison of members of the ATP-AMP-binding protein family has identified two highly conserved sequence elements, [53] Y[T]S[GTTG]X[PKGV]$\cdots$G[YG]XT[E] (the bracket shows the conserved sequence in *Leishmania* long-chain fatty acyl-CoA ligase), which encompass the ATP-AMP signature motif (Figure 4).

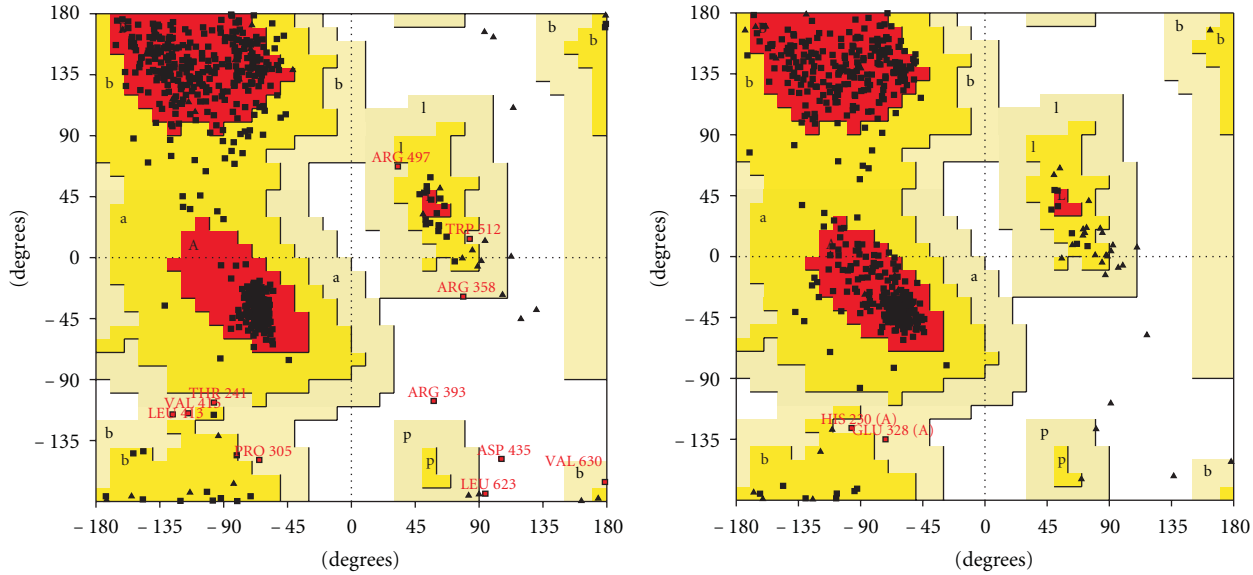
In fatty acyl-CoA synthetases family proteins, there was a third sequence element defined as FACS signature motif that was less conserved and partially overlaps the FACS signature motif, which is involved in both catalysis and specificity of the fatty acid substrate [54]. There are a number of notable features within the FACS signature motif: (i) this region contains two invariant glycine residues (at positions 2 and 7) and a highly conserved glycine at position 16, *Leishmania* long-chain fatty acyl-CoA ligase shares glycine residue with other FACSs at position 7 and 16 but Tyr instead of Gly was found in position 2. (ii) This region contains additional six residues that are invariant in the fatty acyl-CoA synthetases: W[3], T[6], D[8], D[22], R[23], and K[25], but in *Leishmania* long-chain fatty acyl-CoA ligase these residues are F[3], S[6], D[8], G[22], N[23], and D[25]. (iii) The residue in the fourth position is hydrophobic and is a leucine, a methionine, or phenylalanine. However, in *Leishmania* long-chain fatty acyl-CoA ligase hydrophobic residue valine was situated in position 4. (iv) This region of enzyme contains hydrophobic residues (leucine, isoleucine, or valine) at positions 4, 9, 18, 20, and 21. These residues, in addition to tryptophan or phenylalanine residues at position 3, may comprise part of a fatty-acid-binding pocket. All of these five conserved regions from FACS signature motif are having similarity among them except *Leishmania* long-chain fatty acyl-CoA ligase, with some variable regions. These less conserved regions in *Leishmania* long-chain fatty acyl-CoA ligase-FACS signature motif were predicted to adopt inconsistent specificity and catalytic activities of the fatty acid substrate compared to other fatty acyl CoA synthetases.

3.4. Phylogenetic Analysis of Leishmania Long-Chain Fatty Acyl-CoA Ligase and Human Acyl-CoA Synthetases Sequences. We performed phylogenetic analysis to infer evolutionary relationships of all available sequences from kinetoplastida long-chain fatty acyl-CoA ligases (Table 1) and human (host) ACSs family sequences. This experiment was performed to validate that the parasite enzyme is unquestionably different from the human enzyme, and this aspect merits further study to validate this enzyme as a drug target. We obtained comparable results using the neighbor-joining distance-based algorithm as well as maximum parsimony. We found 9 clades, including kinetoplastida clade (one set of six kinetoplastida long-chain fatty acyl-CoA ligase protein family) forming a clade with high bootstrap support (Figure 5). kinetoplastida clade was highly dissimilar and distinct from all ancestral nodes with other human ACSs family proteins and showing distinctiveness of kinetoplastida long-chain fatty acyl-CoA ligases, including *Leishmania* long-chain fatty



(a) (b)

Figure 6: *Leishmania* long-chin fatty acyl-CoA ligase model. (a) The larger left hand-side domain is the N-terminal domain and the smaller one is the C-terminal domain which is connected by a linker chain. (b) Superposition of the modeled structure of *Leishmania* long-chain fatty acyl-CoA ligase (Orange) with the crystal structure of the *T. thermophilus* long-chain fatty acyl-CoA synthetase (PDB code: 1ult A) (Blue).



(a) (b)

Figure 7: Ramachandran plot of (a) modeled structure *Leishmania* long-chain fatty acyl-CoA ligase (b) and the crystal structure of the Tt0168 (PDB code: 1ult A).

acyl-CoA ligase. This divergence of *Leishmania* long-chain fatty acyl-CoA ligase with respect to the homologous human enzymes may be an important protein as a potential target candidate for chemotherapeutic antileishmanial drugs.

3.5. Homology Modeling of Leishmania Long-Chain Fatty Acyl-CoA Ligase Protein. The backbone root-mean-square-deviation (RMSD) values between final model and template crystal structure used are 1.04 Å with *Thermus thermophilus* (PDB Accession Code: 1ULT, 1V25, 1V26) and 1.40 Å with *Archaeoglobus fulgidus* (PDB Accession Code: 3G7S) long-chain fatty acyl-CoA ligase. Small RMSD can be interpreted as structures share common structural homology and the generated structure is reasonable for structural similarity analysis (Figure 6). The final modeled structure of *Leishmania* long-chain fatty acyl-CoA ligase was evaluated for overall quality using available analyses procedures. These analysis compare specific properties of the model with those of known high-quality protein structures using programs like PROCHECK, Verify3D, and WHATIF (Table 2). An important indicator of the stereochemical quality of the model is distribution of the main chain torsion angles phi and psi in Ramachandran plot (Figure 7). The plot clearly shows the vast majority of the amino acids in a phi-psi distribution consistent with right α-helices, and the remaining fall into beta configuration. Only three residues fall outside the allowed regions. Plots comparison shows that the structure is reasonable overall because the space distribution

for the homology-modeled structure was similar to the X-ray structure of the *Thermus thermophilus* long-chain fatty acyl-CoA ligase (PDB Accession Code: 1ULTA). The results showed that our modeled structure was reasonably good at that much less sequence identity.

4. Discussion

Earlier during the course of work, microarray analysis was performed on the same clinical *L. donovani* isolates (2001 and 39) in order to identify differential gene expression [4]. Out of all genes found differentially expressed, significant upregulation of long-chain fatty acyl-CoA ligase gene in SAG unresponsive clinical isolate [33] was found to be intracellular amastigote specific and has confirmed the involvement of long-chain fatty acyl-CoA ligase in resistance. Similarly, it has been proven before that the rate limiting enzyme, long-chain fatty acyl-CoA ligase of β-oxidation, was found to be upregulated in amastigotes derived from cloned line of *L. donovani* ISR because, during late stages of differentiation, the parasites shift from glucose to fatty acid oxidation as the main source of energy, and thereby there is increase in enzyme activity associated with β-oxidation capacity [47, 48]. Early *in vivo* studies showed that enzymatic activities associated with β-oxidation of fatty acids were significantly higher in *L. mexicana* amastigotes [47]. Additionally microarray experiments with intracellular amastigotes hybridized onto Affymetrix Mouse430_2 GeneChips showed that several genes involved in fatty acid biosynthesis pathway were found to be upregulated [55]. Presently studies are ongoing in our laboratory on microarray analysis using intracellular amastigotes hybridized to Affymetrix GeneChip human genome U133 Plus 2.0 array which will further yield useful information towards the fatty acid/lipid metabolism within this clinical isolate. A very recent study by Yao et al., 2010, on differential expression of plasma membrane proteins in logarithmic versus metacyclic promastigotes of *L. chagasi* has also identified long-chain fatty acyl-CoA synthetase [56].

As mentioned before, long-chain fatty acid-CoA ligase is present in both prokaryotes and eukaryotes. This divergence of *Leishmania* long-chain fatty acyl-CoA ligase with respect to the homologous human enzymes may be an important protein as a potential target candidate for chemotherapeutic antileishmanial drugs. Many differences exist between host and parasite pertaining to the structure and arrangement of this enzyme. However, *Leishmania* has significant divergence and adaptation to specific environmental conditions between its two life stages, in the insect vector and human host. This can affect the parasites metabolic machinery in terms of presence of certain pathways, their subcellular localization and expression at different developmental stages, and the interplay between scavenging and synthesis of key metabolites. It has been argued previously [57] that successful targets for metabolic intervention are most likely to be found among enzymes exerting strong control of flux through metabolic pathways. These control points are likely to be species and development dependent. Even if a unique or highly divergent enzymatic process is found in the parasites, this does not necessarily mean it can be developed as a target for useful inhibitors. On the other hand, enzymes that are present in both the parasites and their animal hosts will often differ sufficiently in their sequence for inhibitors to be specific. Finally, even orthologous enzymes functioning in the same pathway and in the same subcellular compartment of the parasites may have different inhibitor binding properties, leading to variability in the effectiveness and specificity of inhibitors targeting any particular enzyme.

The detection of the long-chain fatty acid-CoA ligase gene in the genome of *L. donovani* clinical isolate, in the present study, deserves a full exploration with respect to its potential as a drug target. Changes in membrane lipids/deficiency of certain fatty acids and disease association have been documented [34, 58]. Modulation of enzymes involved in lipid synthesis and of others possibly involved in cell wall metabolism may modify access of drug to the plasma membrane. Moreover, our microarray experiment indicated that this enzyme was amastigote specific making it all the more important to study it further and test if it can be exploited as a validated drug target. We have also shown earlier in our laboratory [34] that modification of lipid composition on the plasma membrane of the parasite might have important implications towards generating susceptibility/resistance to antileishmanial drugs. As this enzyme stipulates several important cellular processes in *Leishmania* like stage-specific expression [47, 48], host-parasite interaction [55], cell membrane composition [17, 18], phospholipid biosynthesis [16, 21], and drug resistance [4], the present study proposed further evaluation of *Leishmania* long-chain fatty acyl-CoA ligase as a candidate drug target.

Acknowledgments

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Review Article

Glucose-6-Phosphate Dehydrogenase of Trypanosomatids: Characterization, Target Validation, and Drug Discovery

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In trypanosomatids, glucose-6-phosphate dehydrogenase (G6PDH), the first enzyme of the pentosephosphate pathway, is essential for the defense of the parasite against oxidative stress. *Trypanosoma brucei, Trypanosoma cruzi*, and *Leishmania mexicana* G6PDHs have been characterized. The parasites' G6PDHs contain a unique 37 amino acid long N-terminal extension that in *T. cruzi* seems to regulate the enzyme activity in a redox-state-dependent manner. *T. brucei* and *T. cruzi* G6PDHs, but not their *Leishmania* spp. counterpart, are inhibited, in an uncompetitive way, by steroids such as dehydroepiandrosterone and derivatives. The *Trypanosoma* enzymes are more susceptible to inhibition by these compounds than the human G6PDH. The steroids also effectively kill cultured trypanosomes but not *Leishmania* and are presently considered as promising leads for the development of new parasite-selective chemotherapeutic agents.

1. Introduction

The family Trypanosomatidae, belonging to the order Kinetoplastida, contains a large number of species, distributed over several genera. All known members of the Trypanosomatidae family are parasites, infectious to humans and other mammals, other vertebrates, insects and plants. The human-infective trypanosomatids are grouped in species of two genera, *Trypanosoma* and *Leishmania*. They are responsible for a wide spectrum of diseases in tropical and subtropical countries. Two subspecies of *Trypanosoma brucei*, *T. b. rhodesiense*, and *T. b. gambiense*, are responsible for different forms of Human African Trypanosomiasis or sleeping sickness, an endemic disease in over 250 distinct foci in rural areas of 36 sub-Saharan African countries. The currently estimated number of cases is 50,000–70,000 with 17,000 new infections annually and 60 million people at risk [1, 2]. The parasite is transmitted between human and/or other mammalian hosts by the tsetse fly. The complex life cycle of this extracellular parasite includes a procyclic form present in the midgut of the insect vector and a bloodstream form present in the blood of the mammalian hosts [3].

Trypanosoma cruzi is responsible for Chagas' disease in most countries of Latin America. It is estimated that 11–18 million people are infected; 13,000 deaths are reported annually and about 100 million people are at risk [4]. The parasites are transmitted by blood-sucking reduviid bugs. After infection, the metacyclic trypomastigotes invade host cells where they proliferate as the so-called amastigote forms and, after differentiation into bloodstream trypomastigotes, they infect more cells, notably of heart muscle, and alimentary track tissue [1, 5, 6].

Different species of *Leishmania* cause a variety of clinical symptoms, collectively called Leishmaniases. These diseases

may involve cutaneous and mucocutaneous lesions, often causing severe debilitating wounds, or life-threatening visceral diseases in which vital organs are affected. The diseases threaten about 350 million people in 88 countries in tropical and subtropical parts of the world. An estimated 12 million people are currently infected with about 1-2 million new cases occurring annually. *Leishmania* transmission occurs via the byte of sandflies which inject metacyclic promastigote parasites into the skin. These forms enter macrophages where they reside as multiplying amastigotes within the phagolysosomes [7].

Sleeping sickness, Chagas' disease and visceral leishmaniasis can have a fatality rate as high as 100% if left untreated or not treated properly [1, 8]. But treatment with drugs currently available is highly unsatisfactory [9, 10]. Most drugs have low efficacy and adverse side effects. Moreover the emergence of drug resistance is a continuous concern. Therefore, and because of the lack of efficacious vaccines, the discovery and development of effective drugs, nontoxic, affordable and easy to administer to the affected populations in the resource-poor areas is an urgent need.

Despite the tremendous progress made in recent decades in understanding the biochemistry and molecular biology of trypanosomatid parasites [11–14], chemotherapeutic treatment of the diseases has seen limited progress.

A currently common strategy for drug discovery against any parasitic diseases is to identify essential metabolic pathways associated with the parasites. In trypanosomatids, several enzymes involved in various metabolic processes have been characterized and established as promising drug targets [14, 15]. Among these validated targets is glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), a key enzyme of the pentosephosphate pathway.

2. The Role of the Pentosephosphate Pathway and Glucose-6-Phosphate Dehydrogenase

In most organisms glucose is metabolized through two major pathways: the glycolytic and the pentosephosphate pathway (PPP) [16]. Whereas glycolysis serves for ATP production and to produce metabolites for use in a large variety of anabolic or further catabolic processes, the PPP can be divided in two successive phases with different functions. The first phase, called oxidative branch, generates reducing power under the form of NADPH and the second one, also known as nonoxidative or sugar interconversion branch, involves a series of reversible nonoxidative reactions leading to the conversion of the 5-carbon sugar resulting from the first phase into other metabolites (Figure 1). The oxidative branch comprises three enzymes: G6PDH, 6-phosphogluconolactonase, and 6-phosphogluconate dehydrogenase (6PGDH), whose successive activities convert glucose 6-phosphate (G6P) into ribulose 5-phosphate, with the concomitant production of NADPH by both dehydrogenases and CO_2 release by the decarboxylation of the 6-phosphogluconate by 6PGDH [17]. The NADPH is used by the cell as a reducing agent in a variety of biosynthetic processes as well as for the defense against reactive oxygen species by keeping glutathione in its reduced state [18]. Glutathione is the major endogenous cellular antioxidant, participating directly in the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced, active forms.

The nonoxidative branch comprises ribose-5-phosphate isomerase, ribulose-5-phosphate epimerase, transketolase and transaldolase, which, by isomerase and epimerase reactions and the transfer of two- and three-carbon units, form a variety of intermediates including ribose 5-phosphate, required for nucleotide synthesis, and erythrose 4-phosphate a precursor of aromatic amino acids and vitamins [19].

Depending on the cell's needs, the PPP can function partially or as a whole cycle by which one of the possible products, fructose 6-phosphate, is converted back into G6P, to enter the oxidative branch again. In addition, the PPP and glycolytic pathway are connected by sharing some intermediates such as G6P, fructose 6-phosphate and glyceraldehyde 3-phosphate.

G6PDH is the first committed enzyme of the PPP. Although most organisms have other means to produce cellular NADPH, notably by the enzymes isocitrate dehydrogenase and malic enzyme, the generally important role of G6PDH in it is illustrated by the severity of G6PDH deficiency in humans. The deficiency may lead to nonimmune hemolytic anemia triggered by oxidative stress as a result of infections or exposure to chemicals such as present in medication or certain foods [20]. G6PDH deficiency is the most common human enzyme defect, occurring in more than 400 million people worldwide [21].

3. Glucose-6-Phosphate Dehydrogenase in Trypanosomatids

The presence of G6PDH activity in trypanosomatids was first demonstrated in 1959 by Raw in *T. cruzi* epimastigotes [22] and in 1962 by Ryley in both cultured procyclic and bloodstream form *T. b. rhodesiense* [23]. A few years later, studies with labeled glucose by Mancilla and colleagues [24–26] suggested that the PPP is functional in some *T. cruzi* strains and *Leishmania* species and, much later, Voorheis and coworkers [27] confirmed the presence of a classical PPP in *T. brucei* by determining the specific activities of all its enzymes in both procyclic and bloodstream forms. Interestingly, no activities of ribulose-5-phosphate epimerase and transketolase were detectable in the bloodstream form and only low activity was found for transaldolase, suggesting an important differential repression of the nonoxidative branch in this life-cycle stage [27]. For some of the PPP enzymes, like G6PDH and 6-phosphogluconolactonase, a dual subcellular distribution was later found in both bloodstream and procyclic form trypanosomes. While their main enzymatic activity was present in the cytosol, approximately 40 and 10%, respectively, of their activity was associated with glycosomes [28, 29]. Glycosomes are the peroxisome-like organelles in Kinetoplastida which uniquely harbour the majority of the enzymes of the glycolytic pathway [30]. These organelles,

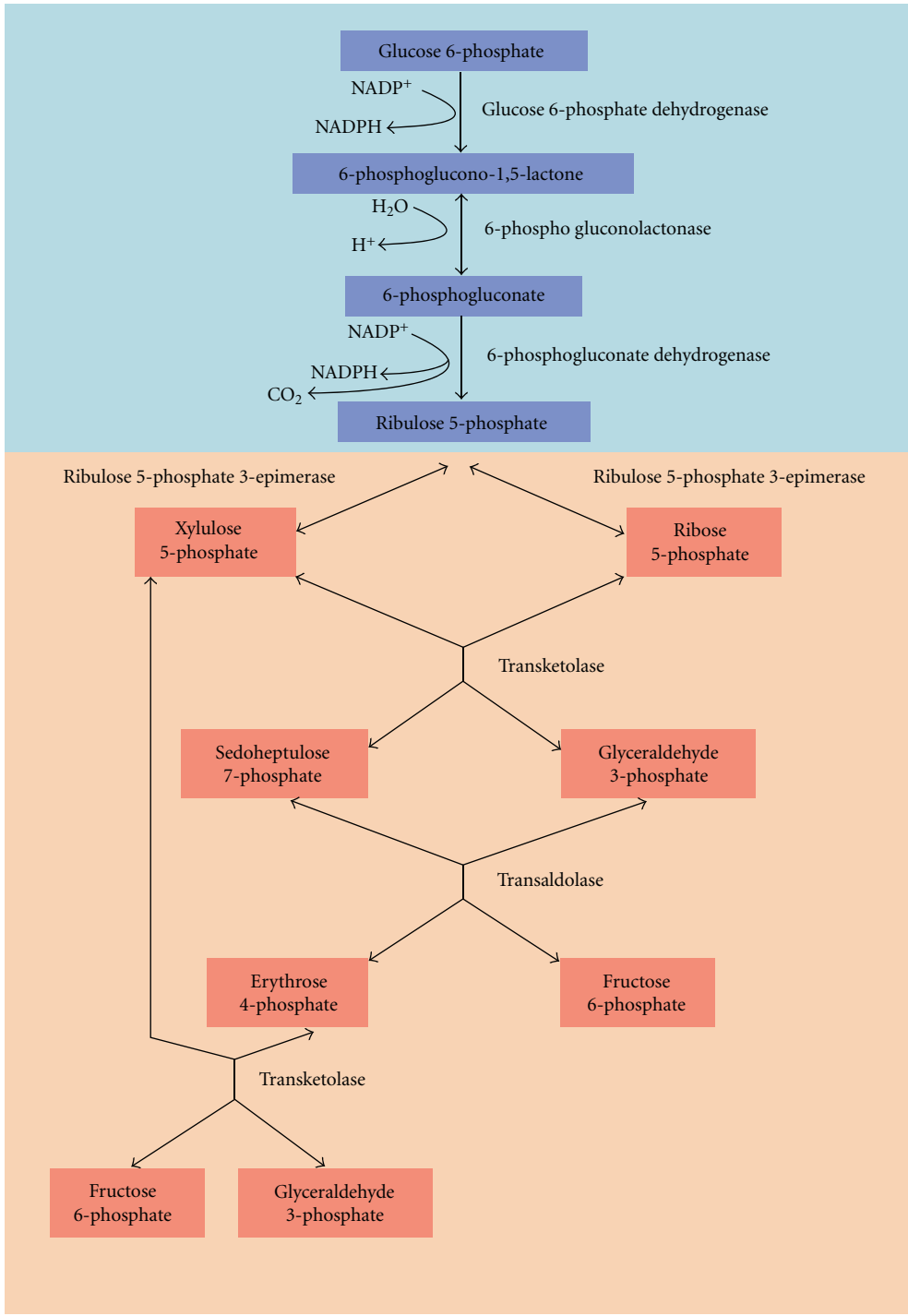

Figure 1: Schematic representation of the pentosephosphate pathway.

which number between 5 and 10 in *Leishmania* amastigotes and approximately 65 in bloodstream form *T. brucei*, may contain enzymes from a variety of different pathways [31–33], with the glycolytic enzymes being most notable and comprising even up to 90% of the organelles' protein content as observed in bloodstream form trypanosomes [33–35].

A functional PPP has also been unambiguously demonstrated in *T. cruzi* epimastigotes by measuring the CO_2 production from radiolabeled glucose [36]. Moreover, the importance of the PPP was demonstrated by a challenge with methylene blue, mimicking oxidative stress, which caused a twofold increase of the flux through the PPP. All enzymes of the pathway were identified by activity measurements in the four major developmental stages of this parasite. The activities were shown to be predominantly cytosolic, with a possible small glycosomal component for most of them.

Table 1: *T. brucei* (Tb), *T. cruzi* (Tc), and *L. mexicana* (Lm) G6PDH kinetic constants for an ordered bi-bi-reaction mechanism.

Source	K_{G6P} (μM)	K_{NADP^+} (μM)	V_1 (nmoles of NADPH·s^{-1})	k_{cat} (s^{-1})	K_{iG6P} (μM)
Tb	57.8 ± 2.4	9.4 ± 0.4	36.2 ± 1.5	16.4 ± 0.6	47.6 ± 1.9
Tc	206.0 ± 4.2	22.5 ± 1.2	77.7 ± 2.5	57.1 ± 1.9	105.3 ± 4.6
Lm	74.5 ± 3.0	12.1 ± 0.5	31.2 ± 1.2	22.2 ± 0.9	86.4 ± 3.5

Data from Cordeiro et al. [45, 46].

In a comparable study, the presence of a functional PPP was also demonstrated in promastigotes of *Leishmania mexicana*, by its twofold stimulation by methylene blue. Each of the classical PPP enzymes was identified and its specific activity measured [37]. Furthermore, it was shown that glucose serves as a source for nucleic acid synthesis, an indication that, in these parasites, the PPP plays also a role in the conversion of glucose into ribose 5-phosphate for nucleotide biosynthesis. Also in *Leishmania*, the PPP has probably a dual subcellular localization, although it has only been demonstrated so far for the transketolase that is predominantly present in the cytosol of promastigotes, but also with a significant component associated with the glycosomes [38]. The presence of the PPP in glycosomes of the three trypanosomatid species is further supported by the presence of peroxisomal-targeting signals (PTSs) in a number of enzymes of both the oxidative and nonoxidative branches [31, 39] and—for *T. brucei*—by the results of proteomic analyses [31, 32].

Whereas in almost all organisms, glutathione, reduced by NADPH produced predominantly in the PPP, is the major intermediate in the defense against reactive oxygen species, in trypanosomatids a different thiol redox component is being used, trypanothione, or N^1,N^8-bis-gluthionyl-spermidine. Several enzymes involved in trypanothione metabolism have been detected both in the cytosol and glycosomes [31].

Preliminary enzymatic studies on G6PDH have been performed with the partially purified enzyme from *T. cruzi* [40, 41] and *T. brucei* [28], but detailed characterization of G6PDH of each of the three trypanosomatid species was only performed after the genes had been identified and used to produce the recombinant proteins. Genomic analysis revealed one gene copy per haploid genome in *T. brucei* [29] and *L. mexicana* [42] and several genes located on three different chromosomes of *T. cruzi* clone CL Brener [43, 44]. Two of them were classified as pseudogenes, while the others were clustered in three groups of nearly identical (98%) coding regions but with considerably different noncoding flanking sequences. The amino acid sequences of the functional G6PDHs of three trypanosomatids are 64 to 69% identical and share about 50% identity with the human enzyme.

The *T. brucei* and *T. cruzi* G6PDH gene sequences both have two possible start codons, 111 bp apart (Figure 2), while in *Leishmania* species only a unique start codon was found corresponding to the first one of the *Trypanosoma* genes. The region between the two start codons codes for a usual 37 amino acid N-terminal extension only present in the trypanosomatid G6PDHs. The long form of the *T. brucei* and *T. cruzi* enzymes code for polypeptides of 557 and 555 residues, respectively, while the *L. mexicana* G6PDH polypeptide encompasses 562 residues. Western blot analysis, performed with an antiserum raised against the N-terminal peptide of the *T. cruzi* G6PDH, showed that mainly the long form of the enzyme is expressed in all four life-cycle stages of this parasite [43]. Whether the long form of *T. brucei* G6PDH is also produced remains to be determined. Duffieux and coworkers [29] proposed that an ORF starting at the first ATG codon would lead to an mRNA with an unusually short 5′ untranslated region.

The *L. mexicana* G6PDH, the short form of the *T. brucei* enzyme and both the long and short form of *T. cruzi* G6PDH have been expressed with an N-terminal His-tag in *Escherichia coli*, purified and kinetically characterized [29, 43, 45, 46]. The kinetic mechanism has been studied, under conditions which were similar for all enzymes, and appeared to follow for each of them a bi-bi-ordered pattern [45, 46]. The values of the kinetic constants are presented in Table 1.

Interestingly, it was shown that the long and short form of *T. cruzi* G6PDH present several differences [43]. Experiments performed independently from those for which the results are presented in Table 1, revealed differences in the apparent K_m values for G6P. While the purified recombinant long and short *T. cruzi* G6PDH had apparent K_m values of 189.9 and 98.4 μM, respectively, that of the partially purified enzyme from parasites was 288 μM. In contrast, the apparent K_m values for $NADP^+$ did not differ. Additionally, both the natural enzyme and the long recombinant G6PDH, but not the short form, were inactivated by reducing agents such as dithiothreitol, β-mercaptoethanol, and reduced glutathione. This effect, reminiscent to the redox-state-dependent activity regulation observed for G6PDH and some other enzymes from chloroplasts and cyanobacteria [47], was attributed to the presence of two Cys residues in the N-terminal peptide [43]. The corresponding N-terminal region of G6PDH of *Leishmania* spp. and the possible extension of the *T. brucei* enzyme do not contain these Cys residues (Figure 2). These observations suggest that among the trypanosomatid G6PDHs studied, only the activity of the *T. cruzi* enzyme is regulated by the redox state of the cell, in a similar way as reported for the enzyme from chloroplasts and cyanobacteria. Indeed, kinetic studies with the two purified recombinant forms showed that the long form was 4-fold more sensitive for inhibition by the product NAPDH than the short one, suggesting that its activity is regulated by

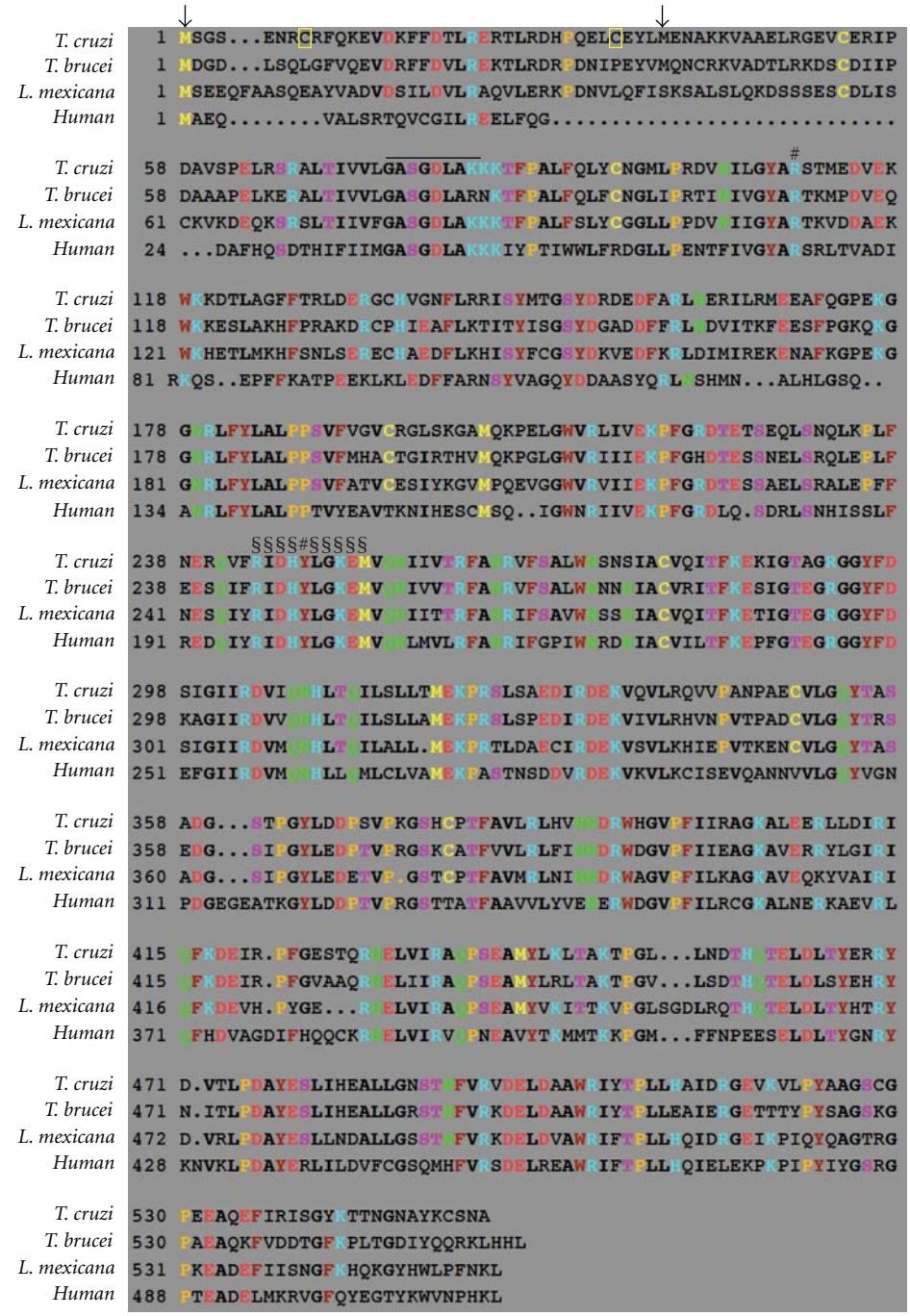

Figure 2: G6PDH amino acid sequence alignment. The predicted amino acid sequences of *T. cruzi* G6PDH-*long* (DQ408239, sharing the highest identity with its *T. brucei* counterpart), *T. brucei* G6PDH-*long* (CAC07816), *L. mexicana* G6PDH (AAO37825), and Human G6PDH (AAL27011) were aligned using the *CLUSTALW* software. The first and second candidate initiator methionines in the *T. cruzi* and *T. brucei* sequences are indicated with arrows. The two regulatory cysteines of the *T. cruzi* G6PDH are boxed in yellow. Overlined, cofactor binding site; §G6PDH signature (residues belonging to the substrate binding site); #residues involved in substrate and cofactor binding.

the $NADP^+$/NADPH ratio in the cell compartment where the enzyme is located, similarly as has been proposed for the enzyme from *Anacystis nidulans* [48].

The importance of G6PDH in the defense against oxidative stress has been demonstrated for both the *T. cruzi* [43] and *T. brucei* enzyme (SG and PM, unpublished; see below). Incubation of *T. cruzi* with H_2O_2 modified the G6PDH expression and specific activity in the cell (Figure 3). In metacyclic trypomastigotes, the life-cycle stage naturally exposed to reactive oxygen species (ROS) produced by the mammalian host, the peroxide exposure enhanced the G6PDH expression as well as the specific activity of the

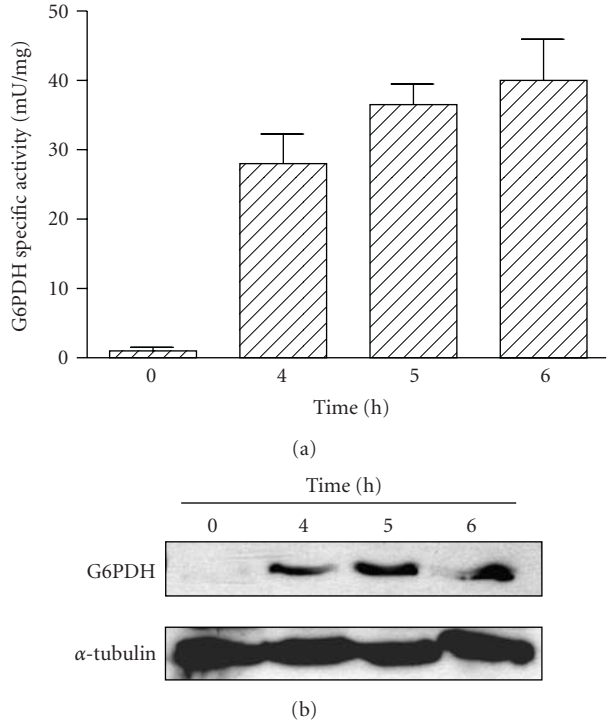

Figure 3: *T. cruzi* G6PDH expression and specific activity are enhanced by oxidative stress in metacyclic trypomastigotes. Parasites were incubated for 4 to 6 h in the presence of 70 μM H_2O_2. Samples for enzymatic activity measurements and western blots were taken before (T0) and after the H_2O_2 addition. (a) G6PDH specific activity. The results are means ± SE of quadruplicates. (b) Autoradiography of a western blot corresponding to metacyclic trypomastigotes cell-free extracts (30 μg of total protein/lane). α-Tubulin was used as loading control. (Figure created with data from [43].)

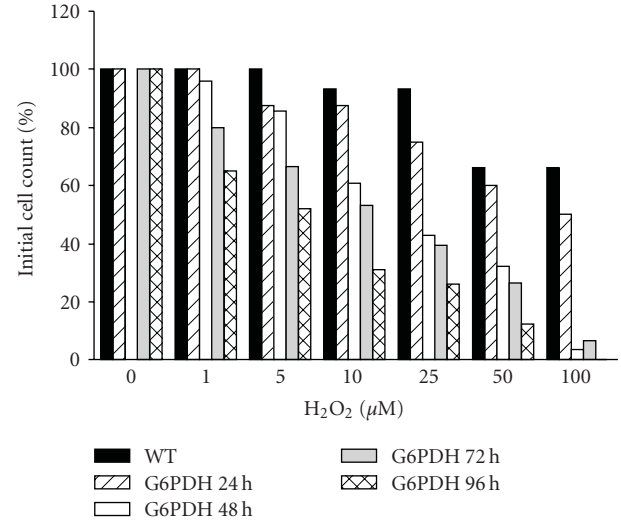

Figure 4: G6PDH is important for defense against oxidative stress in bloodstream form *T. brucei*. Wild-type (WT) trypanosomes and cells in which partial depletion of G6PDH was induced by RNAi were grown in regular HMI-9 medium. After growth for different periods of time as indicated, cells were collected by centrifugation and resuspended in non-reducing medium (i.e., without β-mercaptoethanol and cysteine) and the cell density was determined. Cell suspensions were then incubated for 1.5 h in the presence of different concentrations of H_2O_2 after which the cell density was determined again.

enzyme. Conversely, peroxide treatment in epimastigotes, a life-cycle stage not naturally encountering such a host response, repressed the G6PDH expression and activity. In bloodstream forms of *T. brucei* G6PDH expression has been knocked down by tetracycline-inducible RNA interference (RNAi) [45]. Cells partially depleted from G6PDH showed enhanced sensitivity to different concentrations of H_2O_2 compared to wild-type trypanosomes (Figure 4).

4. Glucose-6-Phosphate Dehydrogenase Is a Genetically Validated Drug Target in Bloodstream Form *T. brucei*

As stated before, most organisms can produce NADPH not only by the action of the two dehydrogenases of the PPP—G6PDH and 6PGDH—but also via isocitrate dehydrogenase and malate dehydrogenase to cope with G6PDH deficiencies. However, trypanosomatids depend only upon malic enzyme [49] and G6PDH for their cytosolic NADPH production. In these parasites the isocitrate dehydrogenase is compartmentalized inside the mitochondrion and, in species of *Trypanosoma* but not *Leishmania*, it has also been predicted as a glycosomal enzyme [39].

As mentioned above, *T. brucei* bloodstream form G6PDH RNAi cell lines have been created [45]. To determine if inhibition of G6PDH (and consequently the flux through the downstream located PPP enzyme 6PGDH) was sufficient to affect the trypanosome growth, a cell line in which RNAi induction led to a considerable reduction in G6PDH expression was studied. In these cells 24 h of RNAi induction led to a mild decrease in G6PDH, while 48 h of knockdown induced a substantial reduction in G6PDH protein, as determined by western blot analysis. The decrease in G6PDH expression was paralleled by an impairment in the trypanosomes' growth rate. This effect was observed between 24 and 48 h after the addition of tetracycline to the culture medium. Beyond 48 h of RNAi induction, G6PDH depleted cells started to die (Figure 5). Parasites containing the RNAi construct, but grown in the absence of tetracycline, showed a similar growth rate as wild-type cells grown in the presence or absence of tetracycline. The decrease of the growth rate correlated with the initiation of the RNAi induction and a simultaneous decrease in the protein level, suggesting that G6PDH plays an important role in growth and survival of the bloodstream form trypanosomes. The possibility that the observed phenotype was a consequence of an RNAi-off-target effect cannot be excluded, but it was considered to be unlikely since the G6PDH sequence is unique and no other highly similar gene was identified in the *T. brucei* genome database.

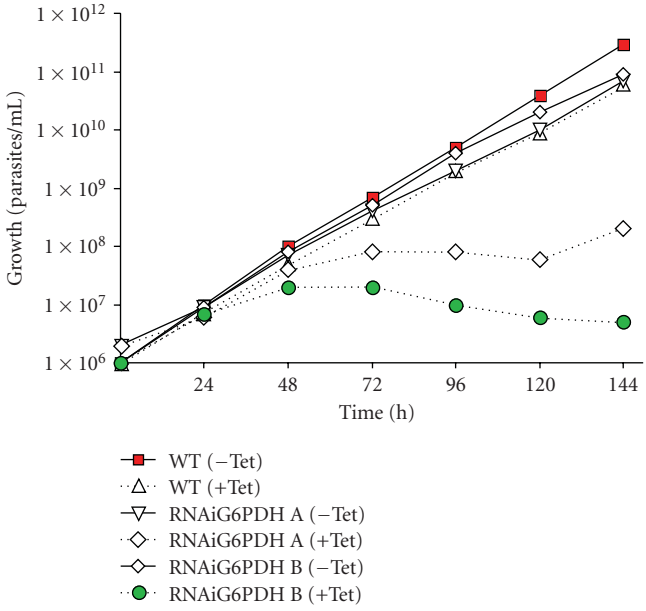

Figure 5: Genetic validation of G6PDH as a drug target in bloodstream form *T. brucei*. Cumulative growth of wild-type (WT) trypanosomes and two independent cell lines (RNAiG6PDH A and B). In the absence of RNAi induction (−Tet), WT and RNAi parasites grow at equal rates; in the presence of the RNAi inducer tetracycline (+Tet), WT cells grow at a normal rate, while trypanosomes of the RNAi-cell lines die after 48 h. (Figure created with data from [45].)

These RNAi experiments genetically validated G6PDH as a drug target in bloodstream forms of *T. brucei* and suggested that the NADPH produced by other enzymes than G6PDH is not sufficient to deal with oxidative stress experienced during normal or stress conditions (Figure 4).

5. Inhibition of Trypanosomatid G6PDH by Steroids

Inhibition of mammalian G6PDH by intermediates of the steroid-hormones biosynthesis was discovered 50 years ago by Mark and Banks [50]. They demonstrated a highly specific and uncompetitive inhibitory effect of dehydroepiandrosterone (DHEA) and derivatives on human G6PDH. These steroid molecules did not inhibit the homologous enzyme of either yeast or spinach. Moreover they did not affect the activity of mammalian 6PGDH and isocitrate dehydrogenase. During the following 35 years, it was a generally accepted notion that only G6PDH from mammalian cells was inhibited by steroid hormone derivatives [51]. As a consequence of such a generalization, the inhibition of G6PDH from lower eukaryotes, like trypanosomatids and other human parasites, was neglected. Observations that DHEA or derivatives of it were effective against infections of *Cryptosporidium parvum* [52], *Plasmodium* species [53], *Schistosoma mansoni* [54], and *T. cruzi* [55] were attributed to a stimulation of the host's immune response by the steroids. Nonetheless, in recent years it was shown that the steroids also affect growth of cultured parasites such as *Entamoeba histolytica* [56] and *Taenia crassiceps* [57], suggesting that they could have a direct effect on these organisms.

Recently, Cordeiro et al. [45] confirmed these findings by showing that steroids kill *in vitro* grown *T. brucei* bloodstream forms with ED_{50} values in the micromolar range. They additionally demonstrated that *T. brucei* G6PDH, the first nonmammalian enzyme, was effectively inhibited by DHEA and epiandrosterone (EA). Similar observations were subsequently made for *T. cruzi* G6PDH [46]. Although, DHEA itself failed to decrease the growth rate of cultured *T. cruzi*, its brominated derivatives 16BrDHEA and 16BrEA (Figure 6), which are more potent inhibitors of G6PDHs, presented ED_{50} values in the micromolar range, comparable to values reported for benznidazole [58], the currently available drug in use for treatment of Chagas' disease. Curiously, DHEA and EA did not inhibit *L. mexicana* G6PDH and had no effect on the growth rate of cultured promastigote parasites. This observation was indirect evidence for the specific inhibition of G6PDH by DHEA in trypanosomes.

6. G6PDH Is the *In Situ* Target of Human Steroids with Trypanocidal Action

Recently, the mechanism by which DHEA and EA kill *T. brucei* bloodstream forms has been evaluated, by exploiting the fact that these compounds showed no inhibitory effect on the recombinant *L. mexicana* G6PDH. Inhibitors were tested on the cell growth of a trypanosome clone that has been created to also express a transgene encoding this *L. mexicana* enzyme [59]. Whereas wild-type bloodstream form *T. brucei* showed a dose-dependent killing by DHEA and EA with ED_{50} values of $41.8 \pm 2.1\,\mu$M and $21.4 \pm 1.6\,\mu$M, respectively, the *T. brucei* (*Lm*G6PDH) transgenic parasites showed no growth inhibition whatsoever by the two compounds, even at concentrations up to $100\,\mu$M [59]. Thus, transfection of *T. brucei* bloodstream form parasites with *Lm*G6PDH could rescue the trypanosomes from being killed by DHEA and EA. This result confirms that the toxic effect of DHEA and EA on the parasites is uniquely due to the inhibition of their G6PDH.

7. Conclusions and Perspectives

Our research has validated the key PPP enzyme G6PDH as a target for new drugs to be developed against trypanosomes. Oxidative insults induce G6PDH expression and enhance its specific activity, while the partial depletion of this enzyme by RNAi makes the parasites vulnerable to oxidative stress. Prolonged depletion of G6PDH from cultured bloodstream form *T. brucei* resulted even in death of the parasites. Whether this was only due to an effect on the redox metabolism or also on the synthesis of metabolic intermediates to be used for biosynthetic processes still needs to be addressed.

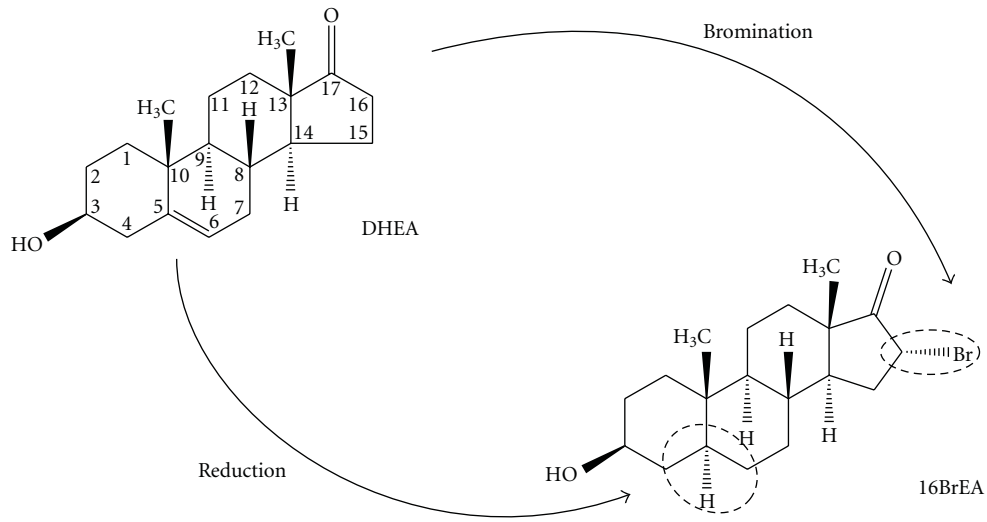

Figure 6: Steroid inhibitors of *Trypanosoma* G6PDH. Two modifications used to increase the inhibitory potency of steroids to G6PDH are indicated: reduction of the double bond between carbons 5 and 6 in dehydroepiandrosterone (DHEA) which leads to epiandrosterone (EA) and the bromination at position 16 which converts DHEA and EA into 16BrDHEA and 16BrEA, respectively.

Steroids derived from DHEA are potent uncompetitive inhibitors of *Trypanosoma* G6PDH, and indeed kill the parasites by *in situ* inactivating this enzyme. The uncompetitive nature of this inhibition is particularly relevant. Contrary to the much more often observed competitive inhibition, the increase of substrate concentration, resulting from this process, will not overcome the inhibition but may rather lead to an increase of metabolic intermediates to toxic levels [60]. The molecular mechanism by which the steroids inhibit the G6PDH of human, *Trypanosoma* and other lower eukaryotes including several human parasites, while they do not affect the activity of the enzyme from plants, yeasts, and *Leishmania* is not yet known. To analyze the differences between the *Trypanosoma* and human G6PDH's steroid binding site and the binding mechanism of these molecules, cocrystallization of the enzymes with steroids and the determination of their crystal structure is needed. To date, no three-dimensional structure of a trypanosomatid G6PDH is available, but crystallization studies are presently ongoing and, when successful, may open new possibilities for the design and synthesis of a different class of molecules with even higher inhibitory potency and selectivity for the parasite G6PDH.

Since these steroids inhibit *Trypanosoma* G6PDHs at much lower concentrations than the mammalian G6PDH, they are promising leads for the development of new drugs for treatment of African sleeping sickness and Chagas' disease. The next steps in the development of drug candidates against these diseases will involve the selection of compounds with potent and highly selective inhibitory activity on cultured parasites *versus* human cells, and the evaluation of their efficacy, bioavailability, and toxicity in infected animal models.

The lack of inhibition of the *Leishmania* G6PDH by DHEA and its derivatives is puzzling. It is feasible that this is due to a single but crucial substitution in the enzyme that prevents the binding of these compounds. Screening of a steroid library against the available recombinant *L. mexicana* G6PDH may help to identify potent uncompetitive inhibitors with *in vitro* anti-*Leishmania* spp. activity.

As mentioned above, steroids also impaired the growth of other parasites, that is, *Taenia crassiceps* and *Entamoeba histolytica*, as well as the parasitaemia of *Plasmodium falciparum* and *P. berghei*, *Cryptosporidium parvum*, and *Schistosoma mansoni*. It is still necessary to determine if G6PDH is also in these cases the main target of the steroid molecules. This will open new perspectives for discovery of drugs also against the diseases caused by these parasites.

Concerning the trypanosomatid-borne diseases, the availability of recombinant G6PDH for each of the three parasites makes also possible an alternative strategy, that is, using these enzymes for high-throughput screening of large libraries of drug-like compounds. This approach may lead to very wide range of inhibitors potentially exploitable for antiparasitic treatment.

Abbreviations

G6P: Glucose 6-phosphate
G6PDH: Glucose-6-phosphate dehydrogenase
6PGDH: 6-phosphogluconate dehydrogenase
PPP: Pentosephosphate pathway
PTS: Peroxisome-targeting signal
RNAi: RNA interference
ROS: Reactive oxygen species.

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Review Article

Glycolysis in the African Trypanosome: Targeting Enzymes and Their Subcellular Compartments for Therapeutic Development

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Subspecies of the African trypanosome, *Trypanosoma brucei*, which cause human African trypanosomiasis, are transmitted by the tsetse fly, with transmission-essential lifecycle stages occurring in both the insect vector and human host. During infection of the human host, the parasite is limited to using glycolysis of host sugar for ATP production. This dependence on glucose breakdown presents a series of targets for potential therapeutic development, many of which have been explored and validated as therapeutic targets experimentally. These include enzymes directly involved in glucose metabolism (e.g., the trypanosome hexokinases), as well as cellular components required for development and maintenance of the essential subcellular compartments that house the major part of the pathway, the glycosomes.

1. Introduction

African sleeping sickness is considered a "neglected tropical disease" yet continues to be a major public health risk to sub-Saharan Africa. A survey from 2005 analyzed by the World Health Organization indicated that African sleeping sickness was still prevalent, with an estimated 50,000 to 70,000 cases occurring (http://www.who.int/mediacentre/factsheets/fs259/en/). A survey from 2009 suggests that the number of cases is falling, but the current level of disease management requires stable social conditions for accurate surveillance and control measures to be effective. Further, the lack of safe and efficacious treatments emphasizes the need for research on new therapies. The current drugs used to treat the disease are often toxic, and their administration typically requires skilled medical care. Additionally, some of the compounds fail to function against certain subspecies, and resistance is a growing concern.

The parasite is transmitted by the bite of the blood-feeding tsetse fly and initially causes fever, headache, and joint pain in humans. Winterbottom's sign, a swelling of the lymph nodes characteristic of early trypanosome infection, has long been recognized in association with African trypanosome infection—slave traders in the 1800s would relocate their operations within Africa upon its appearance in populations destined for slavery [1].

As the disease progresses, parasites enter the brain, and neurological symptoms, such as confusion, disturbed sleep patterns, extreme lethargy (hence, "sleeping sickness"), and coma occur. Left untreated, the disease is invariably fatal. Annual death numbers as a result of African sleeping sickness are difficult to determine, as limited monitoring in rural Africa likely leads to underestimated infection rates.

Human health is also impacted indirectly by the parasite, as animals used for food are also subject to infection. An infected animal experiences fever, listlessness, emaciation, and paralysis, leading the animal to be unfit for use, hence the term "nagana" which is a Zulu word that means "powerless/useless" [1]. It is estimated that 3 million cattle die each year from this disease (Food and Agriculture Organization of the United Nations, http://www.fao.org/). The prevalence of nagana in animals renders much of the African continent inhospitable for livestock production, with an area equal to the continental US unsuitable for beef or dairy production.

Essential lifecycle stages occur in both the vector and mammalian host. In the fly midgut, parasites taken up during a blood meal differentiate into procyclic form (PF) parasites.

These parasites escape the peritrophic membrane and invade the surrounding tissues. Coincident with this behavior, the parasites differentiate into an epimastigote form, which then infects the salivary glands. Once in the salivary glands, parasites develop into nonproliferative metacyclic trypanosomes that are competent for establishing infection in the mammalian host. Delivery of the trypanosome to the mammal occurs when the fly feeds again. Bloodstream form (BSF) parasites develop and grow rapidly in the host blood, with a portion of the population developing into short stumpy parasites that, when taken up by a feeding fly, continue the lifecycle.

Lifecycle stages take advantage of distinct niches to fulfill their metabolic needs. PF parasites utilize the abundant amino acids in their surroundings to generate ATP through mitochondrial-based pathways. While glycolysis is important to the PF parasites, these parasites can thrive in the absence of glucose if adapted to low-glucose conditions, indicating that other metabolic pathways can compensate for the loss of glycolysis [2, 3].

In BSF parasites, glycolysis of host glucose provides the sole source of carbon for ATP production. This dependence on glycolysis for ATP coincides with reduced mitochondrial function, limiting the metabolic options available to the parasite and presenting a series of targets for potential therapeutic development. These include enzymes that participate directly in glycolysis, proteins responsible for enzyme import into glycosomes, and cellular components involved in the regulation of glycosome number and differentiation. Here, we discuss targeting enzymes of glycolysis, with a particular focus on the first enzyme in the pathway, *T. brucei* hexokinase 1 (TbHK1). Additionally, compartmentalization of the pathway is critical to the success of the parasite, so we will consider strategies aimed at disruption of mechanisms the parasite uses during the maturation and development of glycosomes.

2. Glycolysis in the BSF African Trypanosome

Metabolism of host glucose through glycolysis is essential to the success of a BSF parasite mammalian infection, as the pathway is the sole source of ATP production in the mammalian infection lifecycle stage. The pathway is organized into subcellular compartments related to peroxisomes named glycosomes. First characterized in 1977 by Opperdoes and Borst, the single-membrane compartment houses the first seven enzymes of glycolysis [4]. Under aerobic conditions, these enzymes convert glucose to 3-phosphoglycerate, which is then further metabolized to pyruvate with the concomitant production of ATP by pyruvate kinase in the cytosol (Figure 1). The pyruvate is then secreted from the cell.

One key to the presence of compartmentalized glycolysis is related to regulation of energy metabolism. ATP and reducing equivalent depletion and production within the glycosome are balanced. ATP is consumed by the activity of the TbHKs and phosphofructokinase (PFK), while it is regenerated by the activity of the glycosomal phosphoglycerate kinase (gPGK). Additionally, NADH produced by glyceraldehyde-3-phosphate dehydrogenase is balanced by NADH oxidation when glycerol 3-phosphate dehydrogenase (GPDH) metabolizes dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate (Gly-3-p). The resulting Gly-3-p is shuttled from the glycosome to the mitochondria where electrons are ultimately transferred to water through the activity of a glycerol 3-phosphate oxidase complex (consisting of a mitochondrial glycerol 3-phosphate dehydrogenase, ubiquinone, and trypanosomal alternative oxidase). The shuttle returns DHAP to the glycosome, allowing maintenance of the glycosomal redox balance.

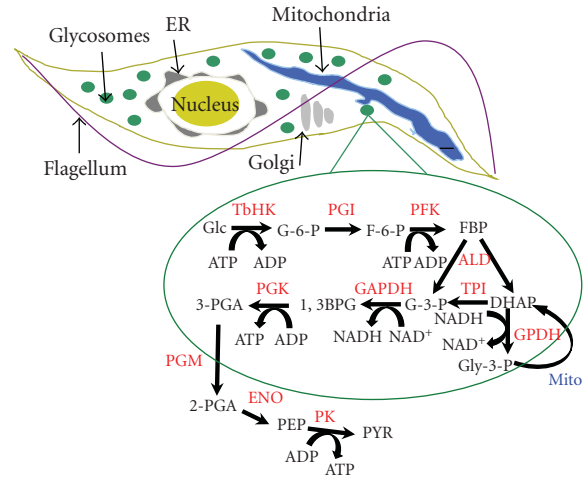

Figure 1: Glycolysis and glycosomes in the bloodstream form African trypanosome. Abbreviations: ALD: aldolase; DHAP: dihydroxyacetone phosphate; 1,3BPGA: 1,3-bisphosphoglycerate; ENO: enolase; F-6-P: fructose-6-phosphate; FBP: fructose 1,6-bisphosphate; G-3-P: glyceraldehyde 3-phosphate; G-6-P: glucose-6-phosphate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Glc: glucose; Gly-3-p: glycerol-3-phosphate; GPDH: glycerol 3-phosphate dehydrogenase; Mito: mitochondrial enzymes; PEP: phosphoenolpyruvate; 2-PGA: 2-phosphoglycerate; 3-PGA: 3-phosphoglycerate; PGI: glucose-6-phosphate isomerase; PGM: phosphoglycerate mutase; PFK: phosphofructokinase; PGK: phosphoglycerate kinase; PK: pyruvate kinase; PYR: pyruvate; TbHK: *T. brucei* hexokinase 1 and/or 2; TPI: triose-phosphate isomerase.

The compartmentalization of a majority of the glycolytic pathway segregates important steps in the path to ATP synthesis and creates what could be considered additional obstacles to efficient energy metabolism. Why does the parasite do this? Bakker and colleagues, through a combination of computational and wet-bench experiments, have found that compartmentalization of glycolytic enzymes that lack allosteric regulation prevents the unchecked consumption of ATP in a "turbo-explosion" of glycolysis [5]. That is, because feedback inhibition does not limit TbHK and PFK activity, these enzymes would generate products (hexose phosphates) at levels beyond the capacity of the downstream enzymes if unchecked by compartmentalization.

3. TbHKs as Targets for Therapeutic Development

In the African trypanosome, TbHK, an activity composed of an unknown ratio of two proteins (TbHK1 and TbHK2), mediates the first step in glycolysis. Because the enzymes have the hallmarks of good targets for therapeutic development, considerable effort has been directed toward the development of TbHK inhibitors as potential antiparasitic compounds. First, both TbHK1 and TbHK2 are essential to the BSF parasite, as demonstrated by targeted gene silencing using RNAi constructs specific to the unique 3′ UTRs of the genes [6, 7]. In both cases, cell toxicity was observed after 3–5 days of RNAi exposure. Second, chemical inhibitors of TbHK1 are toxic to the parasite [7–9]. Third, TbHK1 is likely different enough from host enzymes, sharing only 30–33% sequence identity with mammalian HKs, to suggest that it can be specifically targeted. Last, TbHK1 has unusual properties, including oligomerization into hexamers [10] and is inhibited by compounds distinct from those which inhibit the mammalian enzymes, including fatty acids, to suggest that specific inhibition is possible.

3.1. TbHK1 Inhibitors: Approaches for Discovery. Willson et al. developed structural-based inhibitors of TbHK that were antitrypanosomal through modeling of TbHK1 to known HK structures [9]. These glucosamine derivatives were tested and found to be competitive with respect to glucose, with K_i values similar to the K_M value for glucose [9]. However, the compounds were not particularly toxic to BSF parasites (with LD_{50}s in the range of 5–10 mM, and an LD_{100} for the best inhibitor of 3.6 mM), possibly because the compounds entered the cell by passive diffusion instead of import against a concentration gradient. Alternatively, the compounds may have been imported by facilitated transport through the glucose transporter, again failing to accumulate to sufficient concentrations for toxicity.

TbHK1 inhibitors have also been identified in surveys of chemicals that inhibit HKs from other systems. The activity of molecules identified by this approach is likely the result of conserved structural features of mammalian and trypanosome HKs. For example, the anticancer drug lonidamine (LND, 1-(2,4-dichlorobenzyl)-1,H-indazol-3-carboxylic acid), which inhibits human HK and has been subject to clinical trials in humans also inhibits both recombinant TbHK1 and TbHKs from parasite lysate and is toxic to the parasite [7, 11–13]. Additionally, quercetin (QCN, 3,5,7,3′,4′ pentahydroxyflavone), which inhibits a number of mammalian enzymes including HKs, is toxic to *T. brucei* and inhibits recombinant TbHK1 through binding near the TbHK1 active site [14–16].

Lack of sensitivity of the trypanosome enzymes to other known HK inhibitors, including glucose-6-phosphate, 5-thio-D-glucose, and 3-methoxyglucose, suggests that the TbHKs are sufficiently unique for therapy development [7]. A group of bisphosphonates that are potent inhibitors of *T. cruzi* HK did not inhibit rTbHK1, emphasizing the unique nature of the TbHKs [17, 18]. Notably, rTbHK2, when oligomerized *in vitro* with a catalytically inactive rTbHK1 variant, is active, and the activity is sensitive to PPi inhibition and, to a lesser extent, the bisphosphonate risedronate [10].

The potential arsenal of leads has recently been expanded using two screens to identify specific inhibitors of recombinant TbHK1. The first screen, of a library of pharmacologically active compounds (LOPAC), yielded 18 primary hits (>40% inhibition at 10 μM) from 1280 compounds, including myricetin, a bioflavonoid that is structurally very similar to QCN [19]. In addition to the identification of new lead compounds, the LOPAC screen served to validate the conditions required for automated high-throughput screening (HTS) of a 220,233 compound library.

The HTS campaign initially yielded 239 compounds as primary actives (>50% TbHK1 inhibition at 10 μM), which were then cherry-picked and confirmed as TbHK1 inhibitors. Thirteen compounds with IC_{50} values <50 μM were purchased from commercial sources and ten confirmed with IC_{50} values <50 μM. Of these ten, six clustered into a structurally related group (isobenzothiazolinones), and four were singletons. These compounds had IC_{50}s that ranged from 0.05–41.7 μM, and some of the TbHK1 inhibitors were toxic to BSF *T. brucei*, with EC_{50} values of 0.03–2.9 μM while not exhibiting toxicity towards mammalian cells [19].

In summary, TbHK1 has served as a viable target for therapeutic lead development, with the exciting possibility of the development of potent target-specific inhibitors indicated by recent HTS results. These findings are in agreement with studies that considered the consequences of reduced glycolytic flux through inhibition of the TbHKs on trypanosome growth. Initial *in silico* studies predicted that the TbHKs (and several other glycolytic enzymes) were present in excess, suggesting that significant inhibition would be required to yield a detrimental impact on glycolytic flux and, therefore, parasite health [20]. However, refinement of the model combined with additional experimental assessment revealed that TbHK and PYK were less abundant than initially thought, and that partial inhibition of the enzymes could sufficiently reduce flux to toxic levels in the parasite [6].

3.2. Other Glycolytic Enzymes as Targets. Could other enzymes in glycolysis be targeted for therapeutic development? The other *T. brucei* HK, TbHK2, is 98% identical to TbHK1, so it is likely that compounds that inhibit TbHK1 would also impact TbHK2, though the lack of *in vitro* HK activity has limited studies into this possibility [21]. Downstream, other enzymes have limited identity to human proteins, and several have been validated genetically or chemically as drug targets (Table 1). For a review of the potential of other glycolysis enzymes as therapeutic targets, please see [22, 23].

Mechanisms of regulation of glycolytic enzyme expression may yield interesting targets. In the case of the TbHKs, it has been established that (1) either reduced or excessive expression of TbHK is toxic to the parasite [6, 7], and (2) the environment in which the parasite is grown influences TbHK expression [10]; however, the molecular mechanisms that allow precise yet regulable expression remain unresolved.

Table 1: The *T. brucei* glycolytic enzymes as potential drug targets.

Enzyme[a]	PTS type	% identity to human counterpart	Status of therapeutic development[b]
TbHK1	PTS2 [24]	38% to HKDC1 36% to HXK3	CV [7, 9], GV [7, 9],
TbHK2	PTS2 [24]		GV [6, 7]
PGI	PTS1 [25]	57% to PGI isoform 2	
PFK	PTS1 [25]	27 % to PFK, platelet isoform	CV [26], GV [6]
ALD	PTS2 [26]	49% to brain (C isozyme)	CV [27], GV [28]
TPI	I-PTS [29]	54% to isoform 1	GV [30]
GPDH	PTS1 [25, 31]	38% to GPDH2	
GAPDH	PTS1 [25]	55% to spermatogenic GAPDH-2	CV [32], GV [28]
PGK			
PGKA	I-PTS [33]	42% to PGK 1	
PGKB	N/A	43% to PGK 1	
PGKC	PTS1 [25, 34]	44% to PGK 1	GV [35], CV [36]
PGM	N/A	24% to CAMTA1	GV [6]
ENO	N/A	63% to ENO2	GV [6]
PK	N/A	50% to PKLR	

[a]For enzyme abbreviations, see Figure 1. CAMTA1: calmodulin binding transcription activator 1; HKDC1: hexokinase domain containing protein 1; HXK3: hexokinase type 3; N/A: not applicable because the protein is cytosolic; PKLR: pyruvate kinase, liver, and RBC.
[b]Status: CV: chemically validated target—inhibitors against the target are toxic to parasites; GV: genetically validated target—genetic manipulation of the enzyme leads to growth defects or cell death.

4. Glycosomal Glycolytic Enzyme Import: Targeting the Machinery

Glycosomal resident proteins are encoded by nuclear DNA, translated on cytosolic polyribosomes and targeted to glycosomes as a result of bearing a glycosomal targeting sequence. Proper glycosomal targeting is essential to the parasite because otherwise glucose is toxic to the parasite. RNAi of PEX14, a peroxin required for glycosome protein import, led to accumulation of glycosomal resident proteins in the cytosolic fraction. This condition was tolerated by PF parasites unless they were cultured in the presence of glucose. If grown with glucose, the PEX14-deficient cells accumulated glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-bisphosphate and died [37–39]. Notably, depletion of TbHK in the PEX14-deficient parasites through simultaneous RNAi of the TbHKs and PEX14 yielded cells that were no longer sensitive to glucose, suggesting that the compartmentalization of glycolysis (or the TbHKs) is essential [38]. Additionally, expression of a targeting-deficient HK in *L. donovani* was lethal to parasites in the presence of glucose [40]. While the observed parasite death may have resulted from unchecked ATP consumption, the observation that TbHK1 is regulated by a number of other mechanisms suggests that this may not be the sole explanation for the observed glucose toxicity [10, 21].

Three types of targeting sequences are known to mediate targeting to glycosomes. These sequences, that share similarity with peroxisomal targeting sequences (PTS), include the PTS1, PTS2, and an I-PTS (internal-PTS). Enzymes that participate in glycolysis have PTS1, PTS2, and I-PTS targeting sequences (Table 1).

The PTS1 and PTS2 targeting sequences have been well characterized while less is known about the I-PTS. PTS1 is a C-terminal three amino acid sequence originally identified in firefly luciferase [41]. PTS1-bearing proteins are localized to peroxisomes (and glycosomes) through an interaction with the peroxin protein PEX5, with PTS1 recognition occurring through signal sequence interaction with seven predicted tetratricopeptide repeats in the PEX5 [42].

The PTS2 was first identified when mutations in the N-terminus of the rat peroxisomal 3-ketoacyl-CoA thiolase precursor led to mislocalization of the protein [43]. Mutagenesis studies revealed that the N-terminus of *Saccharomyces cerevisiae* thiolase (which is identical at 6 of 11 amino acids with the rat thiolase N-terminus is necessary and sufficient for protein targeting to the peroxisomes [44]. Contrary to PTS1 and PTS2 signals, the I-PTS sequences lack obvious similarity, sharing only that they are internally located in a polypeptide [45].

4.1. PEX7 and PEX5: Central Participants in Glycosome Targeting. Protein import into the glycosome requires interaction with multiple proteins, including those identified and characterized for peroxisomal import. For example, *S. cerevisiae* PEX7 (originally named PAS7 or PEB1) is involved in transport of PTS2-bearing proteins to the peroxisome [46, 47]. The yeast PEX7 does not require a peroxisomal membrane for binding to the thiolase but binds thiolase in a PTS2-dependent manner. Further, yeast PEX7 does not need a free N-terminus near the PTS2 for binding to occur, and binds thiolase that has already been folded, suggesting that the protein interacts with thiolase in the cytoplasm and acts as a shuttle between the cytoplasm and peroxisome [48].

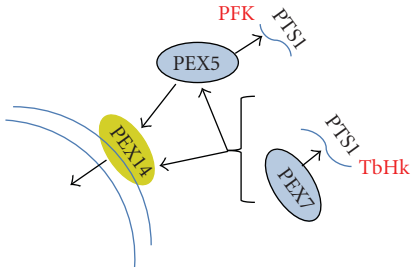

Figure 2: PTS binding proteins participate in delivery of glycolytic enzymes to the glycosome. Fully folded PTS2 harboring proteins expressed in the cytoplasm, like the TbHKs, are targeted to the glycosome through the binding of PEX7 to the PTS2. This complex may or may not interact with PEX5 prior to delivery to PEX14 for transfer to the glycosome matrix. PFK, which harbors an internal PTS1 targeting sequence, is targeted by PEX5.

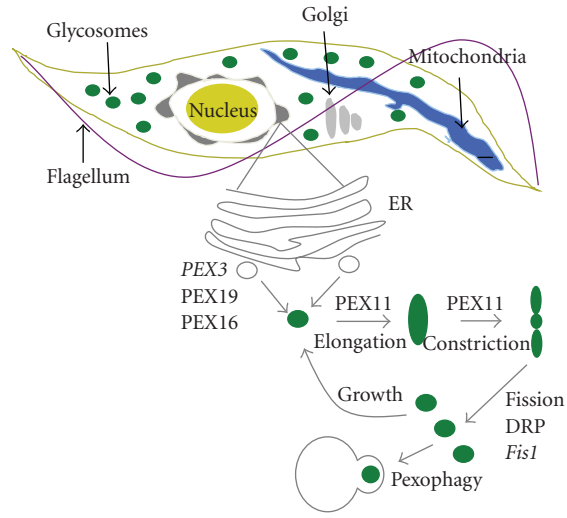

Figure 3: Proposed overview of glycosome biogenesis and remodeling. Proteins without obvious *T. brucei* homologs are indicated in italics.

PEX7 homologs have been identified in three trypanosomatid species, *T. brucei*, *L. major*, and *T. cruzi*. These PEX7 sequences are 65–76% identical to one another and 32–36% identical to the human and *S. cerevisiae* proteins. The trypanosomatid PEX7s contain a C-terminal proline-rich ~40 amino acid extension while the equivalent human and yeast structures have a shorter (5 and 10 residues, resp.) extension that lacks the proline enrichment.

In mammals, PEX7 bound to PTS2 proteins interacts with another peroxin, PEX5, for import into peroxisomes [49, 50]. In 2008, recombinant *L. major* PEX7 was expressed and purified, and this protein was shown to bind to PTS2 sequences [51]. LmPEX7 also binds to a polypeptide derived from *L. donovani* PEX5 (LdPEX5). Other trypanosomatids, including *T. brucei* and *T. cruzi*, also harbor a PEX5 homolog that contains a putative PEX7 binding box located in the N-terminal half of the protein [52]. These findings suggest that the trypanosomatid PEX7 proteins, like the mammalian PEX7 proteins, function through an interaction with PEX5 protein (Figure 2), though RNAi of PEX5 did not alter localization of some PTS2 proteins in *T. brucei*, indicating this relationship may not be an absolute requirement for all PTS2 protein import.

T. brucei PEX5 (TbPEX5) is also involved in the import of PTS1-containing proteins into the glycosome. The PTS1 binding domain of TbPEX5 has been characterized and consists of tetratricopeptide repeats, which typically form super helices that allow protein:protein interactions on both the inner and outer faces [53]. This could allow TbPEX5 to interact simultaneously with multiple proteins [54].

In summary, glycosomal resident proteins are compartmentalized as a result of interactions with peroxins in the cytoplasm. PEX7 binds PTS2-bearing proteins, followed by (in some cases) interaction with PEX5, which may also be loaded with PTS1 harboring proteins. This complex is then delivered to the glycosomal membrane where it docks with a glycosomal membrane protein, PEX14, which participates in import of matrix proteins [38]. The mechanisms of import of PTS1 and PTS2 proteins are slightly different, with PTS1-targeted proteins translocated into the glycosome coincident with the release of their PEX5 binding partner back into the cytoplasm. The PEX7:PTS2 protein complex is translocated *en block* into the glycosome where the PTS2 protein partner is released followed by transport of the PEX7 protein out of the glycosome [51].

Glycosomal resident matrix proteins are expressed from cytosolic polyribosomes as fully folded polypeptides [55]. This creates a potentially dangerous situation for the cell, as inappropriate cytosolic expression of glycolytic enzymes may be toxic to the parasite [40]. While the mechanisms that maintain enzymes in an inactive state in the cytosol are not known, it is tempting to speculate that interaction with peroxisomal targeting proteins may participate in preventing cytosolic activity. With that in mind, targeted disruption of this relationship, through small molecules that interfere with the protein:protein interactions, for example, could ablate regulation and prevent appropriate subcellular localization—with destructive consequences to the parasite.

5. Glycosome Replication and Development as Additional Targets

T. brucei must maintain glycosome number and integrity to maintain homeostasis under normal conditions and remodel glycosomal contents during differentiation and in response to changes in environmental conditions. Components that regulate the dynamics of these essential organelles are potential drug targets.

Glycosome biogenesis involves organelle formation, import of proteins from the cytoplasm (see above), proliferation, and remodeling (Figure 3). Rapid advances in cell biology have facilitated the study of peroxisome dynamics in yeast and other model systems, while less is known about these processes in *T. brucei*. Some peroxisome biogenesis protein gene homologs are readily evident in the *T. brucei*

annotated genome while others either lack sufficient conservation for identification or are absent. In some cases, homology searches may be hampered because the parasites have streamlined glycosome biogenesis and do not carry out all of the processes observed in the regulation of peroxisomes in other systems.

5.1. De Novo Growth of Peroxisomes. Peroxisomes can proliferate through *de novo* budding from the ER and/or by growth and fission of existing organelles. The extent to which process predominates is unclear but appears to vary from organism to organism and is influenced within a given species by growth conditions.

In *S. cerevisiae*, *de novo* peroxisome formation involves the integral membrane protein PEX3, which localizes to the endoplasmic reticulum, forming distinct foci that interact with the peroxisomal membrane protein PEX19. The PEX3/PEX19 vesicles bud from the ER and mature into functional peroxisomes [56]. In support of the ER to peroxisome maturation model, sixteen different peroxisomal membrane proteins were found to localize to the ER in *S. cerevisiae* via traditional ER translocation machinery [57]. In mammalian cells, an additional protein PEX16 (not present in yeast) is involved in formation of peroxisomes from ER in the absence of pre-existing organelles [58, 59].

It is unknown if *de novo* glycosome formation occurs in *T. brucei*. To date, no homologs for PEX3 have been identified in *T. brucei,* although it has been proposed that, through gene displacement, the parasite has developed an alternative replacement activity, as the function of this protein in glycosome biogenesis is likely essential [60].

A PEX19 homolog, on the other hand, has been identified in *T. brucei*. The protein, TbPEX19, exhibits low sequence identity (18–22%) to PEX19 from other organisms and was identified only when relaxed BLAST searches were employed [61]. TbPEX19 is essential in *T. brucei* and is involved in glycosomal protein import with specificity that is similar, though not identical, to that observed for yeast and human PEX19 [62]. Its role in *de novo* formation of glycosomes has not been assessed.

5.2. Growth and Fission of Existing Organelles: The Role of PEX11 in Early Division. In addition to ER-dependent formation of peroxisomes, peroxisome proliferation can also occur through the growth and division of existing organelles. The early process of elongation and constriction of peroxisomes involves PEX11 while the later process of fission involves a set of dynamin-related proteins (DRPs).

PEX11-family proteins, the first proteins to be implicated in peroxisome division, are present in all eukaryotic cells [63, 64]. All PEX11 homologs are ~25 kDa, with isoelectric points greater than 9 and significant sequence similarities at their N- and C-termini. The *S. cerevisiae* PEX11 family includes PEX11, PEX25, and PEX27 [65]. *A. thaliana* contains five PEX11 isoforms (PEXa-e), while mammals have three (PEX11 α, β, γ) [66–68]. *T. brucei* PEX11 family proteins include TbPEX11 as well as two PEX11-like genes, TbGIM5A and TbGIM5B [69, 70].

In *T. brucei*, TbPEX11, TbGIM5A, and TbGIM5B are all associated with the glycosomal membrane via two transmembrane (TM) domains leaving the N- and C-termini exposed to the cytoplasm [69, 70]. TbGIM5A and TbGIM5B are 97% identical with the amino acid differences found within the sequence that links the two TM domains [70]. Like TbPEX11, antiserum that recognizes TbGIM5A and TbGIM5B cross-reacts with proteins that localize to glycosomes, and depletion of this protein results in altered glycosome morphology.

PEX11 proteins undergo a number of posttranslational changes including dimerization and phosphorylation. In *S. cerevisiae* PEX11, homodimers are enriched in mature peroxisomes, and inhibition of this dimerization results in the overproliferation of peroxisomes [71]. TbPEX11, TbGIM5A, and TbGIM5B also form homodimers while TbGIM5A and TbGIM5B form heterodimers with each other but do not interact with PEX11 [70]. The functional significance of this interaction in *T. brucei* is unknown.

S. cerevisiae PEX11 is reversibly phosphorylated at Ser165 and Ser167 [72]. Expressing constitutively dephosphorylated PEX11 results in cells containing fewer, larger peroxisomes while constitutively phosphorylated PEX11 results in enhanced peroxisome proliferation. There is no experimental evidence that TbPEX11 is phosphorylated *in vivo*. Sequence analysis using NetPhos 2.0 (http://www.cbs.dtu.dk/) predicts five potential Ser phosphorylation sites (at residues 42, 50, 154, 159, and 194) and three potential Thr phosphorylation sites (residues 158, 196, and 197).

In fungi, plants, mammals, and *T. brucei*, PEX11 reduction results in cells that contain fewer, larger peroxisomes as compared to wild-type cells [63, 65, 66, 68, 69]. Likewise, increased expression results in the production of smaller peroxisomes in greater abundance than found in normal cells [64–66, 69, 73].

One kinase involved in the phosphorylation of PEX11 is Pho85, a cyclin-dependent kinase. *S. cerevisiae* strains lacking Pho85 had few, larger peroxisomes as compared to parental yeast while cells overexpressing Pho85 had hyperphosphorylated PEX11 [74]. The Pho85 overexpressing yeast also demonstrated increased rates of peroxisome proliferation in comparison with wild-type cells, suggesting that Pho85 plays a role in regulation of peroxisome proliferation [72].

5.3. Growth and Fission of Existing Organelles: The Role of DRPs in Late Division. Peroxisome fission is regulated by a number of dynamin-related proteins (DRPs), which are large GTPases involved in membrane fission and fusion. The peroxisome fission machinery was first identified through studies of mitochondrial fission. In yeast, there are two DRPs, Vps1 and Dnm1, involved in peroxisome fission (for reviews, see [75, 76]). The extent to which each functions is dependent on the organism as well as growth conditions. In *S. cerevisiae*, the Vsp1 dependent system prevails under conditions in which peroxisome proliferation is repressed while the Dnm1 pathway predominates when peroxisome proliferation is induced [77]. *T. brucei* harbors a single DRP, TbDLP, although its role in peroxisome division has not been investigated [78, 79].

DRPs are targeted to the peroxisome membrane through a series of protein-protein interactions. In yeast, Dnm1 is targeted to the peroxisome membrane via interaction with Fis1, a tail anchored protein that has been found to localize to both the mitochondria and peroxisomes [77, 80, 81]. In yeast, Dnm1 is bound to Fis1 through the adaptor proteins Mdv1/Caf4 [80, 82]. In mammals, this adaptor function is likely performed by another set of proteins as no Mdv1/Caf4 homologs have been identified. Instead, mammals target Fis1p to the peroxisome via PEX11β [83]. Vps1 functions independently of Mdv1/Caf4 and Fis1, being targeted to peroxisomal membranes via PEX19 [84].

Peptide antibodies generated against residues 12–25 of TbDLP labeled both mitochondria and glycosomes, though the glycosomal localization may be an artifact of the highly distributed mitochondria [78]. Silencing the TbDLP gene in PF parasites reduced growth rates and resulted in mitochondrial abnormalities with little effect on other organelle morphologies [78]. In another study, silencing TbDLP again resulted in abnormal mitochondrial morphology with no obvious effect on glycosome morphology [79]. The lack of obvious glycosome defects may be a result of the essential nature of the organelle under these conditions. In standard procyclic media containing glucose, glycosome defects are lethal and would not be available for analysis.

5.4. Remodeling of Glycosome Protein Composition: Peroxisome Specific Autophagy. Peroxisomes can be selectively degraded through a conserved mechanism of selective autophagy termed pexophagy. Microscopic observation of *T. brucei* undergoing differentiation of BSF to PF parasites revealed a population of glycosomes that associated with the lysosome. This association is concomitant with changes in the expression of glycosome proteins and suggests that this turnover of glycosomes may occur through a process analogous to pexophagy [85]. Recent bioinformatic analysis has identified trypanosome homologs for about one-half of the known autophagy components from yeast. See [60] for a discussion of the proteins involved in autophagy and their trypanosome homologs.

5.5. Targeting Glycosome Dynamics with Therapeutics: Challenges and the Future. Our understanding of *T. brucei* glycosome dynamics and biogenesis is limited, particularly when compared to what is known about the regulation of peroxisomes from other systems. This is in part due to the unusual properties of the glycosome—it differs functionally from peroxisomes in a number of ways that are not limited to compartmentalization of glycosomes. These differences yield a compartment that is regulated by means distinct from peroxisomes—many of the key proteins involved in these processes lack homologs in other systems. To overcome this obstacle, one could envision applying the power of forward genetics, a tool that has been deployed in the study of the African trypanosome, to identify cellular mechanism that regulate glycosome dynamics [24]. These genes will include many parasite-specific, essential regulators of glycosome biology—which will add to the list of interesting therapeutic targets.

6. Conclusions

Glycolysis and mechanisms required for its compartmentalization remain attractive targets for therapeutic development. Specific inhibitors of parasite glycolytic enzymes have been identified, suggesting that differences, though they may be slight, are sufficient between mammalian and trypanosomal components for development of novel agents. Pathways involved in import of glycolytic enzymes into the glycosomes are being elucidated, and these present interesting targets for development, given the toxicity of mislocalization of these activities. Lastly, resolving mechanisms behind the control of dynamic developmental regulation of glycosomes may yield additional means of disrupting glucose metabolism in the cell, a prospect we look forward to tackling.

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Review Article

Experimental Chemotherapy for Chagas Disease: A Morphological, Biochemical, and Proteomic Overview of Potential *Trypanosoma cruzi* Targets of Amidines Derivatives and Naphthoquinones

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Chagas disease (CD), caused by *Trypanosoma cruzi*, affects approximately eight million individuals in Latin America and is emerging in nonendemic areas due to the globalisation of immigration and nonvectorial transmission routes. Although CD represents an important public health problem, resulting in high morbidity and considerable mortality rates, few investments have been allocated towards developing novel anti-*T. cruzi* agents. The available therapy for CD is based on two nitro derivatives (benznidazole (Bz) and nifurtimox (Nf)) developed more than four decades ago. Both are far from ideal due to substantial secondary side effects, limited efficacy against different parasite isolates, long-term therapy, and their well-known poor activity in the late chronic phase. These drawbacks justify the urgent need to identify better drugs to treat chagasic patients. Although several classes of natural and synthetic compounds have been reported to act *in vitro* and *in vivo* on *T. cruzi*, since the introduction of Bz and Nf, only a few drugs, such as allopurinol and a few sterol inhibitors, have moved to clinical trials. This reflects, at least in part, the absence of well-established universal protocols to screen and compare drug activity. In addition, a large number of *in vitro* studies have been conducted using only epimastigotes and trypomastigotes instead of evaluating compounds' activities against intracellular amastigotes, which are the reproductive forms in the vertebrate host and are thus an important determinant in the selection and identification of effective compounds for further *in vivo* analysis. In addition, due to pharmacokinetics and absorption, distribution, metabolism, and excretion characteristics, several compounds that were promising *in vitro* have not been as effective as Nf or Bz in animal models of *T. cruzi* infection. In the last two decades, our team has collaborated with different medicinal chemistry groups to develop preclinical studies for CD and investigate the *in vitro* and *in vivo* efficacy, toxicity, selectivity, and parasite targets of different classes of natural and synthetic compounds. Some of these results will be briefly presented, focusing primarily on diamidines and related compounds and naphthoquinone derivatives that showed the most promising efficacy against *T. cruzi*.

1. Chagas Disease and Its Treatment

Chagas disease (CD) is caused by the intracellular obligatory parasite *Trypanosoma cruzi*. The life cycle of this parasite involves haematophagous triatomine insect vectors, diverse vertebrate mammalian hosts, and different developmental forms. Briefly, after bloodstream trypomastigotes are ingested by an insect, they are converted to epimastigotes, which proliferate and differentiate into metacyclic forms within the posterior intestine of the triatomine. These infective forms, released within the faeces and urine, can invade vertebrate cells and undergo another round of differentiation into the intracellular amastigote forms, which proliferate and then transform back to trypomastigotes, the form that disseminates the infection. CD is the major cause of infectious cardiopathy and represents an important public

health problem; it is broadly dispersed in 18 developing countries in South and Central America [1]. It affects approximately eight million people in Latin America, of whom 30–40% either have or will develop cardiomyopathy, digestive megasyndromes, or both [2]. CD transmission occurs primarily via the vector (90%) but may also occur through blood transfusion and congenital transmission as well as laboratory accidents [3], organ transplantation [4], and ingestion of contaminated food and beverages [5]. Recently, CD has become a major concern due to globalisation, which results in immigration of infected individuals to nonendemic regions, thus spreading the disease [6]. Although both vectorial and transfusional transmission have sharply declined in the past 20 years due to Southern Cone countries' policies, several challenges still need to be overcome, including those related to sustainable public health initiatives, vector control strategies, and educational approaches [7, 8]. Thus, because epidemiological and transmission control characteristics of CD may vary according to each country's ecological conditions and adopted health policies, continuous epidemiological survey in conjunction with efficient and universal therapy of the infected individuals must be performed to maintain and even reduce the number of new acute cases [9].

CD affects mainly poor, rural and forgotten populations and has two consecutive clinical phases: the acute phase that appears shortly after infection, which ranges from flu-like symptoms to intense myocarditis (in approximately 10% of infected people), and the chronic symptomatic phase, which develops in approximately one-third of infected individuals after an asymptomatic period (indeterminate form) lasting years or decades [10, 11]. The main clinical manifestations of the chronic stage include cardiac and/or gastrointestinal involvement, and the variability in CD outcome has been related to host response and parasite heterogeneity [12]. Although its pathology is poorly understood, growing evidence has shown that parasitic persistence within the target organs associated with an unregulated host immune response is involved in pathogenesis, disease progression, and outcome [1, 13, 14].

Since its discovery by Chagas more than a hundred years ago [15], CD still poses many challenges, including its peculiar epidemiology, characterised by a variety of risk factors (diverse vectors and reservoirs and different forms of transmission and parasite isolates present in domiciliar, peridomiciliar, and sylvatic environments), and the lack of prophylactic therapies and effective chemotherapeutic schemes [10, 16, 17].

Nifurtimox ([3-methyl-4-(5′-nitrofurfurylideneamine) tetrahydro-4H-1,4-tiazine-1,1-dioxide], Nf) and benznidazole (N-benzyl-2-nitroimidazole acetamide, Bz) were empirically introduced into the clinical therapy regime for CD over four decades ago. Neither drug is ideal because they present variable results depending on the phase of the disease (they are only effective in the acute and recent chronic phases of the infection), the dose and duration of the treatment, patient age, and endemic region in addition to showing undesirable secondary side effects [18, 19]. Additionally, differences in the susceptibility and natural resistance of different *T. cruzi* isolates to both nitroderivatives have also been reported [20]. It has been suggested that CD must be treated in all its stages, including acute (acquired or congenital), chronic reactivated (under immunosuppressive conditions), indeterminate, and early chronic phases, as determined by the presence of parasitic DNA using PCR analysis [21]. Additionally, although there is still no criterion of cure for symptomatic late chronic cases because most treated individuals show positive serology, recent data suggest the benefits of Bz therapy for chronic patients through the arrest of cardiac damage and a decrease in serology titres [22, 23]. These results reinforce a goal for identifying parasitic targets that is strengthened by the concept that chagasic cardiomyopathy is related to parasitic persistence within the target organs along with an unbalanced host immune response, which could be useful for a new CD therapy [24].

Since the introduction of Bz and Nf, only allopurinol and a few azoles, such as itraconazole, fluconazole, and ketoconazole, have moved to clinical trials [25–27]. In fact, drug development efforts for CD are almost exclusively in preclinical research, although phase II studies for the antifungal drug posaconazole and a prodrug of ravuconazole are being planned [28]. In addition, clinical data have demonstrated a positive effect of posaconazole in the therapy of a chronic chagasic patient with systemic lupus erythematosus [29]. However, the high costs of posaconazole may impair its use in CD.

The gap between preclinical studies and clinical trials may be associated with the small amount of investments by pharmaceutical industries due to the low monetary return and to the previous mistaken concept that during the later stages of CD, parasitism is absent [30]. In addition, the lack of standardised protocols and the use of epimastigotes for drug screening may represent significant impairments for the discovery of novel anti-*T. cruzi* candidates [31]. Based on current knowledge regarding parasite and host physiology, a promising trypanocidal drug would include the following characteristics: (i) high activity against the parasitic forms present in mammalian hosts (intracellular amastigotes and bloodstream trypomastigotes), (ii) high activity against diverse *T. cruzi* strains for use in different endemic regions, (iii) efficacy against both acute and chronic infections, (iv) oral bioavailability in few doses, (v) low toxicity and improved safety with low potential for genotoxicity and teratogenicity given the potential use in children and women of reproductive age, (vi) low cost and high stability for a long shelf-life in tropical temperatures, (vii) high levels of tissue accumulation and long terminal half-lives, (viii) low risk for cardiotoxicity because the heart is the primary organ affected in chagasic patients, and (ix) low risk for interactions with hepatic cytochrome P450s to avoid drug-drug interactions because many patients use antiarrhythmic drugs and anticoagulants [28, 32].

2. Parasite Targets and Lead Compounds for New Drugs

Advances in proteomics, biochemistry, and in understanding the biological aspects of *T. cruzi* infection have allowed for

the development of new approaches to identify parasite-specific targets and, thus, the design of novel potential drugs [33, 34]. It has been proposed that a rational therapy for *T. cruzi* should be directed against different parasitic metabolic targets, including ergosterol biosynthesis, trypanothione metabolism, cysteine protease, pyrophosphate metabolism, protein or purine synthesis, and DNA [21, 28, 35–37]. The combination of different drugs with the aim of achieving higher efficiency and lower toxicity is also an interesting therapeutic strategy [38]. Recent studies have demonstrated the successful synergism of posaconazole with amiodarone, an antiarrhythmic drug, against *T. cruzi in vitro* and *in vivo* [39]. Another relevant approach is the aetiological therapy of CD using carrier molecules, such as ruthenium complexes, that bind 14a-demethylase inhibitors [40] or benznidazole [41], improving both solubility and parasite specificity [28]. A recent study has shown the successful use of ruthenium complexes to deliver nitric oxide to *T. cruzi*-infected cells [42].

Proteomic approaches have been extensively applied for the evaluation of the expression, structure, and function of proteins on a large-scale, including their physiological role, expression regulation, and validity of genome annotations [43]. In trypanosomatids, the control of gene expression is particularly important because all protein-encoding genes are organised in large polycistronic transcription units, producing the RNA that will be processed by trans-splicing. Furthermore, the modulation of protein expression and, consequently, its function is directly related to posttranslational modifications in these protozoa [44, 45]. Proteomic studies have been performed to evaluate the mechanisms of *T. cruzi* resistance/susceptibility to drugs [44–46].

In the present review, we summarise *in vitro* and *in vivo* results on the efficacy, toxicity, selectivity, and cellular targets of aromatic diamidines and naphthoquinone derivatives, two groups of compounds with promising efficacy against *T. cruzi*. In this framework, morphological techniques, such as light (confocal and fluorescence) and electron (transmission and scanning) microscopy, have been employed. Other cellular (flow cytometry), biochemical (respirometry and fluorimetry), and proteomic (bidimensional electrophoresis and mass spectrometry) approaches have also been employed to identify specific targets in the parasite.

2.1. Aromatic Diamidines and DNA Damages. Diamidines, such as pentamidine, propamidine, and diminazene aceturate, have been successfully used in human and veterinary medicine, and they are the first class of drug extensively employed for early-stage human African trypanosomiasis and for cutaneous leishmaniasis caused by *Leishmania guyanensis* [47–49]. Their major drawbacks are poor oral bioavailability and severe side effects. To overcome these issues, new dicationic analogues and prodrugs have been synthesised by different medicinal chemistry groups and widely assayed *in vitro* and *in vivo* [50]. One of the most promising compounds is an orally effective prodrug of furamidine (DB75) named DB289, which has been in phase III clinical trials for African trypanosomiasis [51]. Unfortunately, recent results at an extended dosage led to the withdrawal of DB289 from human trials due to toxicity issues.

Despite the strong activity of these dicationic compounds against African trypanosomes, few have been assayed as anti-*T. cruzi* candidates [50]. Our team has recently been working on the potential effect of diamidines and congeners against this parasite using both *in vitro* and in vivo models to compare analogues with different structures, cationic centres, and effective motifs [19]. Our data have clearly shown the promising activity of some of these compounds, which displayed high therapeutic windows [52–56].

Although DB75 and its N-phenyl-substituted analogue (DB569) display equivalent DNA-binding properties, DB569 exhibited higher *in vitro* activity against different strains and stages of *T. cruzi*, with IC_{50} values in the low micromolar range [57]. Due to the characteristic fluorescence of these diamidines, their localisation in DNA-enriched organelles was determined due to strong labelling of the kDNA [58]. Flow cytometry and transmission electron microscopy (TEM) analysis also demonstrated that DB75 and DB569 disturb parasite mitochondria and nuclei, leading to morphological characteristics of programmed cell death, such as higher levels of apoptotic-like parasites observed after the treatment with DB569 [57, 59]. These findings stimulated further *in vivo* analysis with this analogue, which showed a reduction in the number of parasites and $CD8^+$ T cells in heart tissues and reversion of electrocardiogram (ECG) alterations in acutely *T. cruzi*-infected mice, leading to an increase in the survival rates [60]. The ECG protection provided by DB569 was also found during the chronic infection of experimental animals, suggesting that the reversion observed in treated animals may be associated with the reduction in cardiac $CD8^+$ lymphocyte infiltration and parasitism, ultimately contributing to their survival [59, 60].

A diarylthiophene diamidine (DB1362) was evaluated against amastigotes and bloodstream trypomastigotes of *T. cruzi* and showed good efficacy *in vitro* at submicromolar concentrations, inducing low host cytotoxicity. This diamidine presented a dose-dependent trypanocidal effect after incubation in the presence of plasma constituents (mouse blood), exhibiting IC_{50} values similar to those found in the absence of blood, pointing to its potential prophylactic application in blood banks. TEM and flow cytometry have shown that in bloodstream parasites the most important alterations were in kinetoplast organisation and mitochondrial membrane potential [54]. In an acute *T. cruzi* experimental mouse model, treatment with two doses of 25 mg/kg DB1362 (at the onset and at the parasitaemia peak) led to a 40% decrease in the circulating trypomastigotes and cardiac parasitism (similar levels to Bz) and protected against ECG alterations, leading to a 100% survival rate [54].

Studies on the biological and ultrastructural effect and subcellular localisation of six novel diamidines in *T. cruzi* confirmed their low toxicity towards mammalian cells ($LC_{50} > 96\,\mu M$) and demonstrated that small linear molecules (DB1627, DB1646, and DB1670) were not effective. However, the other three diamidines (DB1645, DB1582, and DB1651) were active, with IC_{50} values between 0.15 and $13.3\,\mu M$ against bloodstream and intracellular amastigotes [61].

Several potential transporters of diamidines have been described in other parasites, such as African trypanosomes, *Leishmania* species and *Plasmodium falciparum* [62–64]. However, the mechanism of diamidine uptake in *T. cruzi* is unknown and requires further investigation. The intrinsic fluorescence of some of these compounds allows for monitoring their localisation, as previously reported in studies with African trypanosomes [50, 65, 66]. Some of these diamidines, like DB1582 and DB1651, were localised in parasitic nuclei and kDNA (with higher intensity in kDNA) and within punctate non-DNA-containing cytoplasmic organelles usually localised in the anterior portion of trypomastigotes and near the nuclei and kinetoplasts in amastigotes, which are possibly acidocalcisomes, as previously described for *T. brucei* [65]. As previously suggested for African trypanosomes, the localisation of these compounds within these acidic organelles could play a role in their mechanism of action and/or act as storage sites [65, 66], but additional studies are needed to clarify this matter. Batista et al. [61] demonstrated that these diamidines caused striking alterations in the mitochondria and kinetoplasts of *T. cruzi*, and some of them also induced disorganisation of microtubules, with the appearance of multiple axoneme structures in trypomastigotes [52, 61]. No major alterations have been reported in either subpellicular or flagellar microtubules of *T. cruzi* treated with drugs that target microtubules, such as taxol, colchicines, and vinblastine, possibly due to the high content of acetylated tubulin and/or polyglutamylation of tubulin in these parasites [67]. Because these structures are more resistant to microtubule disrupters in trypanosomatids compared to mammalian cells, they may represent interesting targets for drug development and justify further investigations.

Although the exact mechanism of action of diamidines on *T. cruzi* and other trypanosomatids has not been clearly demonstrated, it is likely that multiple modes of action may be responsible and that compound uptake represents a fundamental step in their action and selectivity [50, 68]. One of the long-hypothesised mechanisms of diamidines is related to their selective binding to sequences rich in adenosine and thymine (AT) of kDNA minicircles, leading to kinetoplast destruction and parasite death [58, 69]. Because the kDNA of trypanosomatids contains high numbers of AT-binding sites in thousands of repeated minicircles, it is possible that these structures represent potential specific targets for diamidines [50]. However, although these compounds are excellent minor groove DNA-binders, this interaction itself cannot fully explain their biological activity. Recent reports have suggested that their association with DNA could represent an initial step followed by topological changes leading to molecular instability and destruction and/or modification of DNA-protein interactions, leading to replication errors, DNA degradation, and parasite death [70]. TEM studies have shown that the organisation of mitochondria and kinetoplasts in *T. cruzi* is highly altered by several diamidines and related compounds, such as arylimidamides (AIAs), at concentrations that do not affect mammalian host cells [19, 51, 58]. AIAs, previously known as reversed amidines, have extraordinary activity against both *Leishmania* [71–73] and *T. cruzi* [52, 53, 55, 56, 74]. They differ from other furan analogues because the amidine is bound to the central aromatic linker via a nitrogen atom rather than a carbon atom [72].

Flow cytometry data have confirmed that diamidines and AIAs target the mitochondria-kinetoplast complex of *T. cruzi* through interference with the mitochondrial membrane potential [53, 54]. *In vitro* screening of novel diamidines against *T. cruzi* has shown that these compounds localise to a higher extent within the kinetoplast than in the nucleus, and no correlation was found between trypanocidal activity and higher kDNA accumulation [75, 76], as previously reported in *T. brucei* [65]. Other targets for diamidines that also have been proposed include the inhibition of tyrosyl-DNA phosphodiesterase, topoisomerases, protein kinase A, proteases, and polymerases [51, 77, 78].

To better understand the mechanism of action of these aromatic compounds, a study of the possible correlation between kDNA-binding properties of 13 amidines with their trypanocidal efficacy against *T. cruzi* was performed. Four diamidines (DB75, DB569, DB1345, and DB829), eight arylimidamides (DB766, DB749, DB889, DB709, DB613A, DB1831, DB1852, and DB2002), and one guanylhydrazone (DB1080) were assayed using thermal denaturation (T_m) and circular dichroism (CD) studies using both whole *T. cruzi* purified kDNA and a conserved synthetic parasitic sequence corresponding to the biological activity of each compound [79]. The findings suggest that the strong interaction of amidines with kDNA may not be sufficient to generate and trigger their trypanocidal activity, and other mechanisms of action may be involved and/or associated.

AIAs have potent *in vitro* dose-dependent activity against *T. cruzi*, showing superior trypanocidal activity compared to diguanidino cationic groups and other classical diamidines [52, 56]. Recently, a monoamidine, an arylimidamide, and a guanylhydrazone were evaluated, and the data showed that all compounds exerted, at low micromolar doses, a trypanocidal effect upon both intracellular and bloodstream parasites [74]. However, the potency and selectivity of DB613A, an AIA, towards intracellular parasites (with a selective index >126) corroborated previous results that demonstrated the high promising trypanocidal activity of these compounds.

In vitro and *in vivo* studies conducted with a novel AIA, DB766, showed its strong trypanocidal activity and excellent selectivity for intracellular amastigotes and trypomastigotes (Y strain), the two relevant parasite forms present in mammalian hosts, exhibiting IC_{50} values of 25 and 60 nM, respectively [61]. DB766 also exerted striking effects on a wide panel of different parasite strains, including those naturally resistant to Nf and Bz, displaying higher activity *in vitro* than Bz and gentian violet, which are important requirements for identifying a potential anti-*T. cruzi* agent. It is also important to point that DB766 was active against parasite isolates that circulate in peridomiciliar and sylvatic ecotopes from two different regions in Brazil: (i) the northeast (Jaguaribe Valley, Ceará state) that represents an important area for CD surveillance, where high rates of natural triatomine infection are observed (mostly *T. cruzi*

type I lineage) and vectorial control still requires effort to avoid new cases of human transmission, and (ii) the Amazon region that presents an important new epidemiologic challenge due to the increasing reports of human acute cases, mainly by oral contamination as well as by wild triatomine vectorial transmission [61]. Next, as no major acute toxicity was noted with uninfected mice, this AIA was moved to models of acute and chronic experimental *T. cruzi* infection. DB766 effectively reduced the parasite load in blood and cardiac tissue and presented similar efficacy to Bz in mouse models of acute and chronic *T. cruzi* infection (using Y and Colombiana strains, which are considered modered and highly resistant to Bz), using few oral and intraperitoneal doses up to 100 mg/kg/day given after the establishment of parasite infection. As *T. cruzi* is an obligatory intracellular parasite and reservoirs of amastigotes can be found in quite distinct organs and tissues, the ability of AIAs (including DB766) to traverse host cell membranes possibly by passive diffusion and or transporters associated with their extensive tissue binding in liver, spleen, and heart [80] makes this class of compounds very attractive for CD treatment. In fact, the pharmacokinetic properties of DB766 are especially relevant since the poor activity of the nitroheterocyclic compounds during the chronic stages of CD may be related to short half-lives and limited tissue penetration of Bz and Nf. The efficacy of DB766 upon several strains *in vitro* and *in vivo* is a very important finding since this parasite comprises numerous clonal populations with distinct characteristics such as different sensitivity to Nf and Bz, diverse biological parameters and enzymatic diversity, and strain heterogeneity may also be related to the different clinical manifestations and outcomes in CD. Thus, the broad spectrum of DB766 activity is a desirable characteristic of a novel compound for the treatment of this neglected illness. In acute experimental models of *T. cruzi* infection, DB766 ameliorated heart alterations, reduced hepatic and heart lesions induced by the infection, and provided 90–100% protection against mortality. DB766 also presented high *in vivo* efficacy when given orally at 100 mg/kg/day, showing similar effect to the Bz-treated group [61]. Interestingly, the oral administration of DB766 (at 100 mg/kg/day) leads to reduced circulating and cardiac parasitism besides protecting against mortality without causing major side effects. These results suggest that although not being a prodrug, sufficient quantities of DB766 were absorbed from the mouse gastrointestinal tract, effectively delivering this AIA across the gut mucosa, similar to that reported for the AD prodrug DB289. The bulk of these results demonstrate the promising trypanocidal efficacy of DB766, suggesting that AIA may represent a new lead candidate for CD treatment. Interestingly, DB766 produced a clear dose-dependent decrease in parasitaemia in the liver, spleen and bone marrow in two experimental models of *L. donovani* infection [80]. Additionally, pharmacokinetics, mutagenicity, and toxicity studies revealed that this AIA did not exhibit mutagenicity (AMES test), displayed low acute toxicity, had moderate oral bioavailability, was distributed to different tissues (such as the liver and spleen), presented large areas of distribution, and showed an elimination half-life ranging from one to two days in mice [80].

Six novel aromatic amidinic compounds were tested *in vitro* to determine activity against the infective and intracellular stages of *T. cruzi* and evaluate their selectivity and toxicity towards primary cultures of cardiomyocytes [56]. The data demonstrated that all of the aromatic amidines were active against *T. cruzi in vitro* and that the arylimidamide DB1470 was the most effective compound, presenting IC_{50} values at submicromolar levels and a good selectivity index and maintaining significant trypanocidal activity at 4°C in the presence of blood constituents [56]. Interestingly, AIAs, such as DB1470 and DB766, exhibited potent trypanocidal activity against *T. cruzi* in the presence of blood constituents [52, 55]. This characteristic is highly desirable for new potential trypanocidal agents for use in blood banks in endemic areas. Unfortunately, although transfusional control has led to a decline in the number of new blood bank-related infections, it is not universally performed. The only trypanocidal agent available for chemical prophylaxis of blood in areas of high endemicity is gentian violet, which is a toxic cationic dye that gives a purple colour to the blood and stains the skin and mucosa of the recipients [10, 81]. These limitations encourage the search for new compounds that could be used in blood bank prophylaxis; thus, AIAs such as DB766 represent promising agents for further evaluation for this purpose [55]. In summary, the efficacy of diamidines and congeners, like AIAs, against *T. cruzi* requires further studies to help establish a valuable scheme of therapy for CD.

2.2. Naphthoquinone Derivatives and Mitochondrial Dysfunction. Naphthoquinones are compounds present in different families of plants that serve as vital links in the electron transport chains in the metabolic pathway and participate in multiple biological oxidative processes [82, 83]. They are considered privileged structures in medicinal chemistry due to their biological activities and structural properties [84]. The redox cycling of quinones may be initiated by either a one- or two-electron reduction. The one electron reduction leads to the formation of semiquinones, unstable intermediates that react rapidly with molecular oxygen, generating free radicals. All of these highly reactive oxygen species (ROS) may react directly with DNA or other cellular macromolecules, such as lipids and proteins, leading to cell damage [85]. This reaction results in shunting electrons toward oxygen, an ineffective pathway for reduction equivalents otherwise used for cytochrome P450 reductase-dependent reactions [86–89]. Another alternative is reduction by two electrons, leading to the formation of hydroquinone, mediated by DT-diphorase [90, 91]. This enzyme reduces toxic, reactive, and unstable quinones, bypassing the creation of toxic intermediates (e.g., a semiquinone radical), and sparing the cell from ROS formation. Whether the two-electron reduction of a quinone leads to detoxification or to activation of oxidative stress depends upon the rate of autoxidation of the formed hydroquinone [86]. If this rate is low under physiological conditions, conjugation may occur before oxidation. As a consequence, the two-electron reduction will lead to detoxification, and an increase in the DT-diphorase activity in tissues would be expected to decrease the toxicity of the quinone. If, however, the hydroquinone

is rapidly oxidised, only a minor fraction may be conjugated before oxidation occurs, and hydroquinone formation would constitute an activation reaction. As a result, enhanced tissue levels of NQO1 would be expected to increase the toxicity of the quinone [92]. Quinones are oxidants and electrophiles, and the relative contribution of these properties to both their toxic and therapeutic activities is influenced by their chemical structure [93]. Two major mechanisms of quinone cytotoxicity have been proposed: stimulation of oxidative stress and alkylation of cellular nucleophiles, which are the mechanisms of action of encompass a large range of biomolecules [94]. Cellular damage can also occur through the alkylation of crucial proteins and nucleic acids.

In addition to their widespread presence in nature, the great interest in the study and mechanisms of action of compounds with a quinoidal structure is due to their multiple roles in organisms. Several quinonoids isolated from traditional medicinal plants are being investigated for their anticancer properties [95]. The antiprotozoal activities of naphthoquinones have been reported, and several of them have been identified as possible leads for drug development [96–98].

Lapachol is easily isolated from the heartwood of trees of the Bignoniaceae family abundant in tropical rainforests, while both α-lapachone and β-lapachone are present only in small amounts. In Brazil, more than 46 types of such woods, popularly known by the name "ipes" (*Tabebuia* sp.), have been described. In folk medicine, especially among Indian populations, plants containing naphthoquinones have been employed for the treatment of different diseases, such as cancer [99, 100]. The inner bark of *Tabebuia avellanedae*, commonly known as "pau d'arco" (lapacho, taheebo), is used as an analgesic, an anti-inflammatory, an antineoplastic, and a diuretic by the local people in the northeastern regions of Brazil [101].

Previous reports have shown that against *T. cruzi* epimastigotes, β-lapachone increases the generation of reactive oxygen species through formation of the semiquinone radical, leading to lipid peroxidation and inhibition of nucleic acid and protein synthesis [102–106]. *T. cruzi* is known to be deficient in reactive oxygen and nitrogen species detoxification and for being especially sensitive to oxidative stress conditions [107]. Its single mitochondrion, containing a branched electron transport chain and a specialised kDNA region [77], is an extraordinary drug target [33]. The ultrastructural injuries observed in β-lapachone-treated epimastigotes [108] together with the increase in the generation of hydrogen peroxide clearly demonstrates the mitochondrial susceptibility of *T. cruzi* to naphthoquinones. Unfortunately, no trypanocidal effect was observed in suspensions containing foetal calf serum or rabbit haemoglobin solution, suggesting that β-lapachone could be inactivated by either reduction in the presence of oxyhaemoglobin or interaction with serum proteins [109].

Due to the easy access to natural sources of quinones from Brazilian flora and the synthetic mechanisms developed by the group of Dr. Pinto (NPPN/UFRJ) exploring the electrophilicity of 1,2-quinoidal carbonyls [110–113], naphthoquinones have been used as starting points for medicinal chemistry studies. Since the 90s, our group has been studying the anti-*T. cruzi* activity of this class of chemicals [114]. An initial screening was performed on 60 derivatives obtained through the reaction of several naphthoquinones with common reagents from heterocyclic chemistry, leading to 14 oxazolic, 30 imidazolic, and 10 other related heterocyclic compounds [115–119]. Comparing the activity of the original naphthoquinones and their derivatives, we concluded that minor structural features involved with an increase in lipophilicity, such as the furane moiety, the presence of a methoxyl group, and an aliphatic side chain, led to an increase in the effect on *T. cruzi*. It is possible that a lipophilic character allows better penetration of the compound through the plasma membrane of the parasite. The activity of the synthesised compounds on *T. cruzi* showed no uniform behaviour and was in some cases higher, lower, or similar to the activity of the original naphthoquinones from which they were obtained. For the naphthooxazoles assayed, there was no correlation between biological activity and the type of the mono-oxygenated ring (pyrane *versus* furane). As shown for naphthoquinones, a lipophilic characteristic, introduced by this appendage, and the presence of a methoxyl or a phenyl group increased the trypanocidal activity. A characteristic of the synthesised naphthoimidazoles was that most of them had aromatic groups containing electron-releasing or electron-withdrawing groups attached to the imidazole ring, and the most active compounds against *T. cruzi* were obtained from β-lapachone (see Section 2.3) [115, 119].

Another group of naphthoquinone derivatives was also synthesised and assayed on trypomastigote forms, including β-lapachone- and nor-β-lapachone-based 1,2,3-triazoles and 3-arylamino-nor-β-lapachones [120, 121]. 1,2,3-Triazoles are an important class of heterocyclic compounds due to their wide range of biological activities. The strategy of molecular hybridisation linking them to naphthoquinones resulted in compounds endowed with redox properties and a trypanocidal profile. The 1,2,3-triazole derivatives of nor-β-lapachone were more active than the original quinone, and the apolar phenyl-substituted derivative (2,2-dimethyl-3-(4-phenyl-1,2,3-triazol-1-yl)-2,3-dihydro-naphtho[1,2-*b*]furan-4,5-dione) was the most active compound ($IC_{50}/24\,h = 17.3 \pm 2.0\,\mu M$) [120]. Such activity could be due to its higher lipophilic character, which allows better penetration through the parasite's plasma membrane. In addition, the key intermediate azides used for the synthesis of both the β-lapachone and nor-β-lapachone series of 1,2,3-triazoles displayed higher activity than Bz ($IC_{50}/24\,h = 103.6 \pm 0.6\,\mu M$). In the case of nor-$\beta$-lapachones-3-arylamino-substituted compounds, the insertion of chlorine, bromine, nitro, and methoxy groups into the arylamino ring intensified the trypanocidal activity.

Using nor-lapachol as a starting point, substituted *ortho*-naphthofuranquinones, a nonsubstituted *para*-naphthofuranquinone, an oxyrane and an azide were prepared. Using α-lapachone as a base, a new nonsubstituted *para*-naphthofuranquinone was prepared. The most active compounds were three *ortho*-naphthofuranquinones with trypanocidal activity higher than that of Bz [122]. In another

set of experiments, three new naphthofuranquinones were synthesised and assayed on *T. cruzi*. Two of them were obtained by the addition of iodine to C-allyl-lawsone (2-hydroxy-3-allyl-naphthoquinone) followed by cyclisation, generating a furan ring; the third was obtained through an acid-catalysed reaction by dissolution of the original quinone in sulphuric acid. These compounds were active on bloodstream trypomastigote and epimastigote forms with IC_{50} values between 165–640 and 2.5–25 μM, respectively [123]. The treatment of infected murine macrophages caused a dose-dependent decrease in the percentage of infection with low toxicity to host mammalian cells (over 100 μM), with IC_{50}/24–96 h values for intracellular amastigotes between 1.2 and 3.5 μM [124]. An ultrastructural analysis of treated epimastigotes and trypomastigotes indicated a potent effect of the three naphthofuranquinones on parasitic mitochondria, which appeared drastically swollen and with a washed-out matrix profile. Fluorescence-activated cell sorting analysis of rhodamine-123-stained *T. cruzi* showed that these naphthofuranquinones caused a potent dose-dependent collapse of the mitochondrial membrane potential ($\Delta\Psi_m$), especially in epimastigote forms. Such a collapse represented a 30–60% decrease in the parasitic $\Delta\Psi_m$. These compounds also specifically decreased mitochondrial complex I-III activities parallel to a reduction in succinate-induced oxygen consumption between 64–75% in epimastigotes and 72–92% in trypomastigotes. Mitochondrial hydrogen peroxide formation was also increased 1.3–4.5-fold in epimastigotes after treatment with naphthofuranquinones. Our results indicated that the trypanocidal action of these quinones was associated with mitochondrial dysfunction, leading to increased reactive oxygen species generation and parasitic death [124]. The easy synthetic route of these compounds in the laboratory opens the possibility of large-scale production with high yields for assays in experimental mouse models.

2.3. Naphthoimidazoles and Putative Targets. Among the different classes of naphthoquinone derivatives screened against *T. cruzi* (see Section 2.2) , the most active derivatives against bloodstream trypomastigotes were three naphthoimidazoles derived from β-lapachone with the aromatic moieties phenyl (N1), 3-indolyl (N2), and methyl-*p*-phenyl (N3), which were selected for further studies [84, 85]. They were also active against intracellular amastigotes and epimastigotes (Table 1) and showed toxicity to the host cell in concentrations greater than 100 μM. The most susceptible form of the parasite was the intracellular amastigotes, with an IC_{50}/24 h between 6.5 and 9.0 μM [125, 126]. The highest activity against bloodstream forms was observed for N2, with IC_{50}/24 h values of 12.3 ± 1.2 and 61.6 ± 3.6 μM for 0% and 100% blood, respectively. In epimastigotes, N3 was the most effective, with IC_{50}/24 h values of 30.7 ± 3.6 μM. All three compounds also blocked the cell cycle (up to 96% inhibition of DNA duplication), inhibited succinate cytochrome c reductase (16–42%) and metacyclogenesis (IC_{50}/96 h values between 0.35–0.66 μM) and induced extensive morphological damage to the mitochondria, Golgi complex and reservosomes. In treated trypomastigotes, an altered kinetoplast network, mitochondrial swelling, plasma membrane blebbing and DNA fragmentation were found [125, 126]. DNA fragmentation was also evaluated using total DNA electrophoresis and flow cytometry techniques and showed a maximum of 75% TUNEL+ trypomastigotes after treatment with the naphthoimidazoles.

An investigation into their mode of action led to the characterisation of mitochondria, reservosomes, and DNA as their main targets and stimulated further studies about death pathways. Ultrastructural analysis revealed both autophagic (autophagosomes) and apoptotic-like (membrane blebbing) phenotypes. In epimastigotes and trypomastigotes, the naphthoimidazoles induced the formation of concentric membranes, autophagosomes with a loss of matrix electron density, and endoplasmic reticulum profiles surrounding different structures [127]. Apoptosis-like features, such as the release of mitochondrial cytochrome c to the cytosol, has also been detected in N3-treated parasites. Flow cytometry analysis showed a small increase in phosphatidylserine exposure in N2-treated trypomastigotes and a large increase in the percentage of necrosis caused by N1 and N2. These death phenotypes were not detected in treated epimastigotes. A strong increase in the labelling of monodansyl cadaverine (a well-known autophagic marker) was observed up to 45 and 71% in treated trypomastigotes and epimastigotes, respectively. The inhibition of the trypanocidal action of the three naphthoimidazoles by wortmannin or 3-methyladenine together with the overexpression of *ATG3, ATG4, ATG7,* and *ATG8* genes in treated epimastigotes and the ultrastructural evidence pointed to autophagy as the predominant phenotype induced by these compounds [127, 128].

To assess the mechanism of action of the naphthoimidazoles N1, N2, and N3, treated epimastigotes were submitted to two-dimensional gel electrophoresis (2-DE) and mass spectrometry to monitor changes in the protein patterns of different important pathways in *T. cruzi*. Results showed that 9 of the 30 proteins altered were mitochondrial, reinforcing previous morphological and biochemical studies that showed this organelle as the main target of these drugs [125, 126]. Treatment with the compounds led to an upregulation of three proteins: heat-shock protein 85, enolase 1 and trypanothione synthetase (Table 2). The over-modulation of the trypanothione pathway strongly suggests that increased scavenging is necessary due to the increased thiol content in treated parasites, as previously described for trypanosomatids [129].

The downregulation of 26 proteins was observed: 7 in N1, 14 in N2, and 15 in N3. Table 2 summarises all modulated proteins after treatment. A strong decrease (20–80%) in protein levels was detected after treatment with the three naphthoimidazoles. Energy metabolism is an important target of these compounds. The expression of enzymes, such as enolase, pyruvate dehydrogenase, and cytochrome c oxidase, were altered in treated epimastigotes. Interference with ATP generation occurred in different stages of the pathway, including glycolysis, the citric acid cycle, and the mitochondrial electron transport chain. The main structural target of naphthoimidazoles was microtubules. A remarkable

Table 1: IC_{50} values/1 d (μM) for the effect of N1, N2, and N3 against *T. cruzi* [a].

	Trypomastigotes		Epimastigotes	Amastigotes	
	0% blood	100% blood		Extracellular[b]	Intracellular[c]
N1	35.8 ± 1.2[d]	62.1 ± 3.0	82.8 ± 7.4	13.4 ± 1.1	9.0 ± 2.9
N2	12.3 ± 1.2	61.6 ± 3.6	36.0 ± 1.9	12.4 ± 1.9	6.5 ± 1.0
N3	28.2 ± 0.9	68.3 ± 7.3	30.7 ± 3.6	9.7 ± 0.2	7.2 ± 0.2

[a] References [123, 124].
[b] Tissue-cultured derived amastigotes.
[c] Number of amastigotes/100 peritoneal macrophages.
[d] Mean ± SD of at least 3 independent experiments.

Table 2: Modulated proteins in naphthoimidazoles-treated epimastigotes[a].

Protein description	Expression status	Treatment
Trypanothione synthetase	upregulated	N1, N2, N3
Mitochondrial heat shock	downregulated	N1
Heat shock protein 60	downregulated	N2
Elogation factor 1-α	downregulated	N1
Enolase 1	upregulated	N3
Glutamamyl carboxypeptidase	downregulated	N1
Heat shock protein 85	upregulated/downregulated	N1, N2, N3
Tyrosine aminotransferase	downregulated	N3
Cytochrome C oxidase subunit IV	downregulated	N2
Activated PKC receptor	downregulated	N3
Hypothetical protein	downregulated	N2
β-tubulin	downregulated	N1, N2
Pyruvate dehydrogenase E1 β subunit	downregulated	N3
Sterol 24-C-methyltransferases	downregulated	N2, N3
Elongation factor 1-β	downregulated	N2
Proteosome α 7 subunit	downregulated	N1
Peroxiredoxin	downregulated	N2
Hypothetical protein	downregulated	N1
IgE-dependent histamine-releasing factor	downregulated	N3
Elongation factor 2	downregulated	N2
Dehydrogenase	downregulated	N3
Cystathionine β-synthase 6	downregulated	N2
α-tubulin	downregulated	N3

[a] Reference [128].

decrease in the α- and β-tubulin content was detected in epimastigotes treated with the three compounds. Interestingly, previous ultrastructural data showed no damage to subpellicular and flagellar conformations after treatment [125, 126]. ELISA showed a decrease in tyrosinated tubulin content, suggesting interference with intracellular vesicle traffic and/or mitotic spindle formation, data reinforced by the blockage of mitosis in naphthoimidazole-treated epimastigotes [126, 130]. Amino acid metabolism was one of the most important targets of the naphthoimidazoles. The levels of tyrosine aminotransferase, elongation factors and different heat-shock proteins were affected by N1, N2, and N3, leading to a decrease in the amino acid synthesis and, consequently, an imbalance in proteins important for cell survival. N2 and N3 also arrested the latter steps of sterol biosynthesis through the downregulation of sterol 24-C-methyltransferase (Table 2). The partial impairment of this pathway could lead to alterations in the lipid composition, with a loss of membrane fluidity. Proteomic and ultrastructural data showed no evidence of necrosis or apoptosis-like cell death in treated parasites [127, 130]. A further detailed study on metabolic interactions is crucial to further elucidate the mechanisms through which naphthoimidazoles act.

3. Concluding Remarks

Despite the drawbacks found during the therapy of chagasic patients with Bz and Nf, in the last decades, only a few compounds have moved to clinical trials, possibly due to the low investments allocated to this area and the lack of standardised protocols for drug screening [31]. As previously noted, "the more leads/approaches that progress to investigational drug candidates, the better chance that new treatments for this often fatal infection will be available to patients in the

near future" [131]. Thus, the recent implementation of high-throughput compound screening against *T. cruzi* will allow for the rapid evaluation of several thousands of compounds per month against intracellular amastigotes, which represent an important tool that may yield the identification of novel promising compounds that can move to clinical trials [28].

However, these promising trypanocidal candidates will need more complete pharmacological and safety test analyses to be considered for clinical trials. However, consistent care must be provided through the adoption of a biopsychosocial model, considering patient therapy in the context of biological, psychological, and social factors, and economic difficulties, which can compromise quality of life [132, 133]. Finally, another important limitation related to moving new compounds towards clinical trials is the absence of feasible markers to monitor the progression of the chronic disease and affordable, efficient, and accessible diagnostic tests. Thus, the development of new drugs for most parasitic diseases requires a multidisciplinary approach involving diverse research areas, such as molecular and cellular biology, chemistry and biochemistry, pharmacology, and toxicology, to provide new insights related to the development and discovery of more selective compounds that could be used for Chagas disease therapy.

As concluded by Abad-Franch et al. [8] , improved specific chemotherapy, including more practical formulations (e.g., paediatric) or combinations of existing drugs, and a better understanding of pathogenesis as well as the relative contribution of the parasite and host genetic makeup are clearly needed. New strategies for drug design have been improved by the recent results in *T. cruzi* biochemistry, allowing for better elucidation of the effects of trypanocidal agents.

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Review Article

Use of Antimony in the Treatment of Leishmaniasis: Current Status and Future Directions

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In the recent past the standard treatment of kala-azar involved the use of pentavalent antimonials Sb(V). Because of progressive rise in treatment failure to Sb(V) was limited its use in the treatment program in the Indian subcontinent. Until now the mechanism of action of Sb(V) is not very clear. Recent studies indicated that both parasite and hosts contribute to the antimony efflux mechanism. Interestingly, antimonials show strong immunostimulatory abilities as evident from the upregulation of transplantation antigens and enhanced T cell stimulating ability of normal antigen presenting cells when treated with Sb(V) *in vitro*. Recently, it has been shown that some of the peroxovanadium compounds have Sb(V)-resistance modifying ability in experimental infection with Sb(V) resistant *Leishmania donovani* isolates in murine model. Thus, vanadium compounds may be used in combination with Sb(V) in the treatment of Sb(V) resistance cases of kala-azar.

1. Introduction

Leishmaniasis threatens about 350 million men, women, and children in 88 countries around the world. WHO estimates the worldwide prevalence to be approximately 12 million cases, with annual mortality of about 60,000 (http://www.who.int/vaccine_research/diseases/soa_parasitic/en/index3.html#disease%20burden) and around 1-2 million estimated new cases per year (http://www.who.int/leishmaniasis/en/).

Leishmaniasis is caused by a protozoan parasite of the genus *Leishmania* which multiplies in certain vertebrates that act as reservoirs of the disease. The parasite is transmitted to humans through the bite of sandflies that have previously fed on an infected reservoir. The outcome of the disease, however, depends on the species of *Leishmania* causing the infection and the immune response raised against that infection. The cutaneous form tends to heal spontaneously leaving the scars, which may evolve into diffuse cutaneous leishmaniasis, recidivans leishmaniasis, or mucocutaneous leishmaniasis (MCL) depending on the species of *Leishmania* causing infection. Accordingly, patients suffer from disastrous aesthetic consequences. Whereas cutaneous leishmaniasis (CL) is the most common form of leishmaniasis, visceral leishmaniasis (VL) is the most severe one. In fact, VL can be fatal when left untreated and may cause epidemic outbreaks with a high mortality rate. A varying proportion of visceral cases can also evolve into a cutaneous form known as post-kala-azar dermal leishmaniasis (PKDL), which requires lengthy and costly treatment. Depending on the geographical areas, a specific form of Leishmaniasis may be caused by different *Leishmania* spp. For example, CL and MCL in Central and South America are caused by *L. mexicana* and *L. braziliensis* whereas CL in South and Central Asia and the Middle East is caused by *L. tropica* and *L. major*. Similarly, VL (commonly known "kala-azar") is caused by *L. donovani* in India, Bangladesh, China, Nepal, and Sudan, by *L. infantum* in North Africa and southern Europe, and by *L. chagasi* in Latin America (http://www.who.int/leishmaniasis/en/). The majority of MCL cases occur in Bolivia, Brazil, and Peru. 90% of CL cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia, and Syria. Under immunosuppressive conditions such as acquired immunodeficiency syndrome (AIDS),

dermotropic species of *Leishmania* parasite has also been reported to visceralize to give rise VL. Because human immunodeficiency virus (HIV)-1 is a frequent cause of immunosuppression, an increasing number of cases of HIV-*Leishmania* coinfection are being reported in areas where both infections overlap (geographical distribution of leishmaniasis. Geneva: WHO.Available at: http://www.who.int/emc/diseases/leish/leisgeo.html). In addition, HIV modifies the clinical presentation of all forms of leishmaniasis in the coinfected patients.

As noted above, some forms of leishmaniasis, for example, VL might be fatal for patients if left untreated. In the absence of an effective vaccine, the control of leishmaniasis is solely dependent on chemotherapy. The organoantimonial compounds have remained as the first line of treatment for all forms of leishmaniasis for more than 60 years. However, until recently, little is known about the chemical structure of these compounds and the methods used in the industry for their preparation [1]. Furthermore, molecular and cellular mechanisms of their action are not well defined. In recent years, a large-scale increase in clinical resistance to pentavalent antimonials has been reported [2, 3]. In India, 65% of previously untreated patients fail to respond promptly or relapse after therapy with antimonials [4].

Second-line drugs include pentamidine and amphotericin B, but severe side effects and high cost limit their use [5]. Miltefosine (hexadecylphosphocholine), originally developed as an anticancer agent, has now been approved as the first oral drug for leishmaniasis. It can be used for both antimony-responding and nonresponding patients [6]. Although it shows good efficacy, but it is very expensive and has a long half-life. Data from phase IV clinical trials in India involving domiciliary treatment with miltefosine along with weekly supervision suggest a doubling in the relapse rate against miltefosine [7]. Beside miltefosine is found to be a potential teratogen in animals. Since there are very few affordable drugs in hand, resistance to first-line drug(s) has a very big impact on the treatment of leishmaniasis. This demands an understanding of the molecular and biochemical mechanisms of clinical resistance, which has become a World Health Organization priority (http://www.who.int/tdr/diseases/leish/strategy.htm).

2. Treatment of Leishmaniasis and Antimonials

2.1. Historical Perspective of the Disease and Therapy. Historically, the cutaneous form of leishmaniasis is a disease of antiquity and was recognized in the Old World with various names such as oriental sore, Delhi boil, Baghdad sore, and so forth. This is an ancient disease. Descriptions of conspicuous lesions have been found on tablets in the library of King Ashurbanipal from the 7th century BC, some of which are thought to have been derived from earlier texts dating from 1500 to 2500 BC. In addition, in the 10th century Arab physicians have described the oriental sore [8, 9]. Similarly, the visceral form of leishmaniasis in the Old World had been known with various other names like Jessore fever, Kala-dukh, Sarkari Beemari, Dumdum fever, Burdwan fever, Fatal-fever and kala-azar (kala-black; azar- fever). The earliest kala-azar epidemic occurred in 1824 in Jessore district of India (now in Bangladesh), which had initially been confused with malaria and named as Jessore fever [10]. This epidemic killed several thousands of patients because no treatment was known until then. The cutaneous leishmaniasis was used to be treated by local therapy in the endemic areas. However, by the end of 19th century in Tashkent, pure lactic acid was applied to the lesions to cauterize it [11]. Relapses were treated by removal "scraping" of the lesion with a sharp spoon. Other cauterizing agents included copper sulfate, old battery acid, plant extracts and heating of the lesions for 20 hours with water in circulating water baths [11]. The visceral form of the disease was often diagnosed by enlargement of abdomen and was anecdotally treated in India by burning the abdominal skin over the spleen.

Antimony has been used as therapeutics in several centuries. Some authors have suggested its earliest use in ancient Egypt for cosmetic purposes. However, it has been shown that this statement was based on a misreading of the ancient texts [12]. The importance of antimony in the early medicine is well documented, due to the debate created around their utilization in this period [13]. Paracelsus introduced antimony, as a general panacea in the 16th century (as published in Leipzig in 1604), and it was acclaimed as one of the seven wonders of the world. The modern era of usage of antimony began in 1905 when Plimmer and Thompson showed the activities of sodium and potassium tartrate against trypanosomes; subsequently these were used for the treatment of human trypanosomiasis in Africa. Use of the trivalent antimonial, tarteremetic was first reported for the treatment of CL by Vianna in 1913 [14], the efficacy was confirmed against VL by Di Cristina and Caronia in Sicily [15] and Rogers in India in 1915 [16], but later this drug was found to be highly toxic as well as very unstable in tropical climate [17]. Shortt from India was not impressed with the outcome and wrote that antimony tartrate, is an advance over no treatment at all, rather suboptimal in terms of clinical resistance and relapses [18]. In another report Cole [11] also concluded that tartar emetic was "not much better than no treatment at all." Tartar emetic was considered as an irritating drug, since it exhibited side effects such as cough, chest pain and great depression. This led to the discovery of pentavalent antimonials. Thereafter, the pentavalent antimony compound urea stibamine synthesized by Brahmachari, emerged as an effective chemotherapeutic agent against Indian kala-azar (KA) in 1920 [19, 20]. This discovery saved millions of lives of poor Indians, for which Professor Brahmchari was nominated for the Nobel Prize in 1929 (Nobel Prize official website) [10]. The development of the less toxic pentavalent antimonials by Brahmachari, Schmidt, Kikuth, and others led to the synthesis of antimony gluconate (Solustibosan) in 1937 [21] and sodium stibogluconate (Pentostam) in 1945 [22]. Now a days the most commonly used organic compounds of antimony (Sb) are sodium antimony gluconate (SAG; manufactured by Albert-David, Kolkata, India) and meglumine antimoniate (manufactured by Rhone-Poulence, Paris).

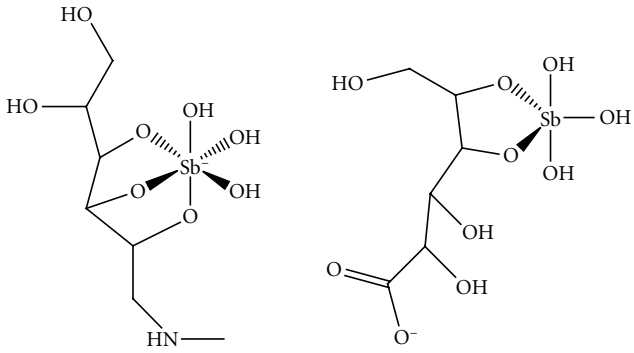

Figure 1: Proposed structural formula for 364 Da and 365 Da ions identified by ESI (−)-MS in aqueous solutions of meglumine antimoniate and stibogluconate, respectively. Adapted from [25].

2.2. Structure. Structures of two complexes of Sb(V) with *N*-methyl-D-glucamine (meglumine antimoniate or Glucantime) or sodium gluconate (sodium stibogluconate or Pentostam) remained unknown for decades due to their amorphous state. Recently, NMR and mass spectrometric approaches have allowed significant progress in this arena [1].

Fast-atom bombardment mass spectrometric (FAB-MS) data of the commercially available meglumine antimoniate suggests that two molecules of meglumine (NMG) coordinate with a single Sb atom in a symmetrical geometry [23]. On the other hand, positive ion electrospray ionization mass spectrometric (ESI(+)-MS) analyses indicate the existence of a mixture of polymeric structures with the general formula (NMG-Sb)*n*-NMG [24]. Further analyses of meglumine antimoniate by ESI-MS, in both the positive and negative modes, show negatively charged 1 : 1 (*m/z* 364) and 2 : 2 (*m/z* 765) Sb(V)-meglumine complexes and support the predominance of zwitterionic species in solution (Figure 1) [25]. ESI-MS measurements of sodium stibogluconate also showed that it consists of a mixture of oligomeric structures [25] that confirmed the earlier results obtained by molecular sieve chromatography [26], and is consistent with the general formula for meglumine antimoniate ((GLU-Sb)*n*-GLU and (GLU-Sb)*n* (GLU: gluconate). Osmolarity measurement suggested the predominance of 1 : 1 Sb-NMG and Sb-SSG complexes in diluted samples [25]. This interpretation was further in agreement with the HPLC-inductively coupled plasma-MS and ESI-MS analyses [27].

2.3. Entry of Drug. Pentavalent arsenate (As(V)), a metal related to (Sb(V)), is known to enter via a phosphate transporter [28]. Antimony transport was first studied in both stages of *Leishmania mexicana* and *Leishmania donovani* parasites using (^{125}Sb) Pentostam (Sb(V)) [29]. More recently, mass spectroscopic approaches reveal the accumulation of two forms of antimony (Sb(V) and Sb(III)) in both stages of the parasite. However, accumulation of Sb(V) has been found to be higher in axenic amastigotes than in promastigotes in a number of species [30, 31]. Because gluconate competitively inhibits uptake of Sb(V) in axenic amastigotes, Sb(V) is speculated to enter into the parasites via a protein that recognizes a sugar moiety-like structure shared with gluconate [32]. Interestingly, neither As(V) nor phosphate can compete with the uptake of Pentostam in this scenario. This ruled out the possibility that Sb(V) uses an As(V) transporter. However, the accumulation of Sb(III) is competitively inhibited by the related metal As(III) [32], suggesting that Sb(III) and As(III) enter the cell via the same route.

2.4. Mechanisms of Action. Pentavalent antimonials are in use against leishmaniases for more than six decades. However, their molecular and cellular mechanisms of action are not yet well understood. It is not even clear whether the final active form is Sb(V) or Sb(III). Three main models could be proposed regarding the mechanism of action of pentavalent antimonials.

2.4.1. Prodrug Model. According to this model, pentavalent antimony (Sb(V)) behaves as a prodrug, which undergoes biological reduction to much more active/toxic trivalent form of antimony (Sb(III)) that exhibits antileishmanial activity. However, the site of (amastigote or macrophage) and mechanism of reduction (enzymatic or nonenzymatic) remain controversial. Furthermore, the ability of Leishmania parasites to reduce Sb(V) to Sb(III) is stage-specific. For instance, amastigotes but not promastogotes can reduce Sb(V) to Sb(III). This explains why amastigotes are more susceptible to Sb(V) but promastigotes are not [33–37]. Other studies have suggested that reduction of Sb(V) to Sb(III) may also take place within macrophages, but level of reduction of Sb(V) to Sb(III) in macrophage can not be that significant since Sb(III) even at a dose of 25 μg/mL can kill 50% of the THP1 macrophages [38, 39]. Thus, conversion of Sb(V) to Sb(III) may occur at both sites, that is, macrophage and parasite, and the parasite plays a major role in the generation of higher, lethal concentrations of Sb(III) within the parasite. It has been shown that, a proportion of Sb(V) may be converted to Sb(III) in human [36, 40] and animals models [41, 42].

The reduction of Sb(V) to Sb(III) requires an active participation of thiol compounds of both mammalian host and parasite origin [43–45]. Mammalian thiols, which play important role in this process, include glutathione (GSH), cysteine (Cys) and cysteinyl-glycine (Cys-Gly). The first one is the main thiol present in the cytosol, while the second and the third are the predominant thiols within lysosomes of mammalian cells [46, 47]. The parasite-specific thiol compund, trypanothione (T(SH)2), is a complex consisting of glutathione and spermidine, has been shown to be involved in reduction of Sb(V) to Sb(III) [48]. Compared to GSH, however, the initial rate of reduction of Sb(V) is much higher in the presence of Cys-Gly, Cys, and T(SH)2 [43]. Generally, acidic pH and slightly elevated temperature favor reduction of Sb(V) to Sb(III). *In vivo* this process is mediated by T(SH)2 within *Leishmania* parasites and Cys or Cys-Gly within the acidic compartments of mammalian cells. But the stoichiometry of GSH and Sb(V) required for the reduction of antimony is equal to or more than 5 : 1. As

the rate of reduction is very low, the physiological relevance of this conversion is still open to question [49].

Interestingly, promastigotes contain higher intracellular concentrations of T(SH)2 and GSH than amastigotes [50, 51], and both stages maintain an intracellular pH value close to neutral [52]. Therefore, nonenzymic reduction of Sb(V) to Sb(III) fails to account for the insensitivity of promastigotes to Sb(V). On the other hand, recent studies have suggested the participation of an parasite-specific enzyme, thiol-dependent reductase (TDR1), in the process of reduction of Sb(V) to Sb(III) [53]. The enzyme TDR1 is a tetramer protein, containing domains of the omega class of the glutathione S transferases (GSTs), and using GSH as the reductant. Although TDR1 has been found to be highly abundant in the amastigote stage of the parasite, the enzyme activity and antimony sensitivity in Leishmania amastigotes could not be directly correlated.

An arsenate reductase homologue in *Leishmania* parasite (LmACR2) has also been shown to catalyse the reduction of Sb(V) in *L. major* in presence of GSH. LmACR2 requires glutaredoxin as cofactor for its enzyme activity and is inhibited by As(III), Sb(III) and phenylarsine oxide [54]. In contrast to TDR1, LmACR2 is a monomer. Transfection of LmACR2 in *Leishmania infantum* promastigotes augments pentostam sensitivity in intracellular amastigotes, confirming its physiological significance. It is also possible that more than one mechanism is responsible for the reduction of Sb(V) to Sb(III).

Mechanism of Killing by Reduced Sb(III). Trypanothione reductase (TR) and zinc-finger protein are the potential molecular targets of Sb(III). Such interaction is consistent with the modality of Cys binding of thiophilic metals such as As(III), Sb(III), and Bi(III). Metal-bound Cys systems are fully deprotonated thiolate anions, the nucleophilicity of which is greatly attenuated upon formation of metal complexes with high thermodynamic stability.

Action on Trypanothione/TR System. Trypanothione/TR system keeps T(SH)2 in the reduced state and thereby maintains oxidoreductive balance in Leishmania parasite. This protects the parasites from oxidative damage and toxic heavy metals, and delivers the reducing equivalents for DNA synthesis [55]. Although TR shares structural and mechanistic similarity with glutathione reductase (GR), differences in the disulfide binding site between TR and GR account for selective inhibition. Trivalent antimonials interfere with T(SH)2 metabolism by inhibiting TR and inducing rapid efflux of intracellular T(SH)2 and GSH into intact *Leishmania* cells [51, 56]. Recently, it has been shown that Sb(III) can bind to a CCHC zinc-finger peptide model and promote the ejection of Zn(II) [57]. The zinc-finger domain is characterized by the coordination of a zinc atom by several amino acid residues, which are usually cysteines and histidines. These structural elements are associated with protein-nucleic acid and protein-protein interactions [58]. The CCHC motif bearing Zn finger proteins binds to the hexanucleotide repeat sequence found in the intervening region of the GP63 (most abundant surface glycoprotein) gene cluster of Trypanosomatids. Zn finger proteins are likely to be involved in DNA replication, structure and repair [59]. Treatment of *Leishmania* amastigotes with Sb(III) has been found to induce apoptosis via induction of the oxidative-stress and increase in intracellular (Ca^{2+}) [60, 61].

2.4.2. Intrinsic Antileishmanial Activity Model. According to this model, Sb(V) has intrinsic antileishmanial activity. Initial studies suggested that sodium stibogluconate [Sb(V)] inhibits macromolecular biosynthesis in amastigotes [62], possibly via perturbation of energy metabolism due to inhibition of glycolysis and fatty acid betaoxidation [63]. However, the specific targets in these pathways have not been identified. Sodium stibogluconate, but not Sb(III), specifically inhibits type I DNA topoisomerase, thus inhibiting of unwinding and cleavage. Sb(III) mediated inhibition seems to be specific for *Leishmania donovani* topoisomerase, since Sb(III) fails to inhibit calf-thymus topoisomerase I and *Escherichia coli* DNA gyrase [64, 65]. Interestingly, *in vivo* sensitivity and resistance of *Leishmania* towards antimonial drugs have been shown to correlate with the effect of such a complex [66].

Demicheli and coworkers have reported the formation of a complex between adenine nucleosides and Sb(V) [67]. Formation of Sb(V)-ribonucleoside complexes, both in the ratio of 1 : 1 and 1 : 2 was evidenced [68, 69]. The large changes for H2 NMR resonance suggested that –OH groups in the ribose are the binding sites for Sb(V) probably via ring chelation at C2 and C3 . Complex formation between ribonucleosides and Sb(V) was found to be faster at acidic pH, indicating that it is kinetically favored in acidic biological compartments. The rate of dissociation is slow in aqueous solutions at neutral pH. Moreover, the stability constant determined for 1 : 1 Sb(V)-GMP complex is consistent with the formation of such a complex in the vertebrate host following treatment with pentavalent antimonial drugs, especially if the high accumulation and prolonged retention of antimony in macrophages is considered [70, 71]. Regarding the possible pharmacological role of Sb(V)-ribonucleoside complexes, two hypotheses may be raised: (a) formation of Sb(V)-adenine nucleotide complex might act as an inhibitor of the *Leishmania* purine transporters, or (b) these complexes might penetrate inside the parasite, encountering a neutral pH-environment where dissociation gets retarded and the complex as such behaves like the purine analog (as allopurinol), thus interfere with the purine salvage pathway [72, 73].

2.4.3. Host Immune Activation Model. According to this model antimonials clear intracellular Leishmania parasites via activation of host immune system. Action of sodium antimony gluconate (SAG) is multifaceted. SAG can activate both innate as well as adaptive immune compartments, thereby inducing effective antileishmanial immune response. This not only ameliorates existing infection but also protect from relapse.

Effect on Innate Immunity. Croft and Yardley 2002 [74] mentioned a moderate role for antimonial action in the paradigm

"the reticuloendothelial system (i.e., its stimulation by drugs, etc.) is of importance for the cure." Murrayand Nathan [75] demonstrated that MΦ activation had a significant effect on intracellular parasite killing. Treatment with SAG has been reported to induce ROS generation in peripheral blood cells of *L. infantum* infected mice on stimulation with phorbol ester (PMA) or zymosan [76], and to induce NO in canine leishmaniasis [77]. Recently, it has been reported by us [78] that SAG alone can induce both ROS and NO production in murine MΦ and promote two waves of killing of *L. donovani* amastigotes. The first phase of killing (i.e., at early time point, around 6 h post treatment) is due to induction of ROS and the second wave of killing (i.e., at a later time point, 24 h and 48 h) is mediated by NO generation. Both ROS and NO are known to be involved in parasite killing in the early stage of leishmanial infection in mice, whereas NO alone is involved in the late phase [79, 80]. The role of NO in final elimination of leishmanial parasites is further strengthened by the studies which demonstrated that treatment of *L. major* infected mice with L-NMMA drastically increases the lesion size and *L. major* is visceralized in a late phase of experimental infection in iNOS knockout mice [81].

SAG Mediating Activation of Signaling Pathways. We further deciphered the signaling mechanisms responsible for SAG-induced ROS and NO production and consequent killing of intracellular leishmania parasites within infected MΦ. SAG-induced ROS generation in MΦ requires phosphorylation of ERK via the PI3K-PKC-Ras/Raf pathway. On the other hand, activation of the PI3K/Akt pathway and downstream p38MAPK is essential for induction of NO production and subsequent parasite killing in *L. donovani*-infected MΦ following SAG treatment. It was further shown that p38MAPK mediated generation of NO by SAG treatment is an indirect mechanism. Actually p38MAPK induces TNFα production, which in turn induces iNOS2 expression and subsequent NO generation since SAG-mediated NO generation and parasite killing could be abrogated by treatment with antiTNFα neutralizing antibody [78].

Leishmania infection has been reported to increase PTPase activity mainly that of SHP1 type [82–84], which might contribute to dysregulation of PTK dependent signaling events and MΦ deactivation. SAG inhibits SHP1 and SHP2 classes of PTPases but not MKP1 type [85] by the gluconic acid backbone bound in various specific stoichiometric ratios inhibit purified SHP1 with specific efficacies. SHP1 might directly dephosphorylate ERKs [86] mechanisms by which *Leishmania* parasites can escape and regulate activation of other important signaling molecules like PI3K. Thus, inhibition of SHP1 by SAG might indirectly favor tyrosine phosphorylation of PI3K and thereby help in activating both PI3K-PKC-Ras/Raf-ERK1/2 pathway for ROS generation and the PI3K-Akt-p38 MAPK pathway for NO generation. In addition, SAG upregulates IFN-γ receptors both in uninfected and *L. donovani* infected THP1 cells, as well as in monocytes derived from kala-azar patients treated with SAG [87]. Thus it is quite possible that SAG influences the host's antileishmanial defense by altering IFN-gamma responsiveness. Indeed, SAG fails to act in IFN-γ knockout mice [88]. We have also observed that SAG and IFN-γ synergize to produce high levels of NO in MΦs. A combination of SAG and IFN-γ is also known to be therapeutically much more effective than SAG alone in the treatment of visceral leishmaniasis [89]. We have further observed that SAG triggers production of IL12 in both uninfected as well as infected MΦ. IL12 is known to induce Th cells to produce IFN-γ, which in turn activates MΦs to produce TNF-alpha and, subsequently, NO.

2.5. Effect of Antimony on Cell Mediated Immunity

2.5.1. Action on T Cell. Studies of murine VL infections (BALB/c-*L. donovani*) have established that an intact T-cell population, more specifically Th1, is required for Sb(V) to produce a curative antileishmanial effect [90, 91]. The drug itself is leishmanicidal *in vitro* and *in vivo*, however complete cure, *in vivo*, is not achieved without Th1 input. Patients coinfected with VL-HIV respond poorly to antimony treatment [92]. After an initial response, these patients frequently relapse and require alternative treatment [93]. Dermotropic infections in man usually self-cure. This can take from 3 months to 3 years depending on the species of *Leishmania* involved. In such cases antimonial treatment augments the host's immune response to rapidly resolve the infection. Exceptional cases include DCL where, in the absence of a cell mediated response, antimonials prove to be ineffective [94]. Several studies have shown that endogenous IL-2 [95], IL-4 [96, 97] and IL-12 [98] influence the effectiveness of chemotherapy with pentavalent antimony. These findings indicate the requirement of somewhat functional T cell compartment for SAG action.

Our study indicates that effect of SAG on T cell compartment is corollary to its action on antigen presenting cells like MΦ. We observed that SAG treatment enhances expression of specifically MHC I molecule on the MΦ surface and enhanced class I mediated antigen presentation, but not the presentation mediated by MHC class II (Figure 2). This may be a mechanism by which SAG can enhance antileishmanial cytotoxic T lymphocyte (CTL) response. There is a report that CTLs can kill intracellular parasites [99].

Interestingly stimulation of spleen cells, derived from either *Leishmania* infected or uninfected mice, induced IFN-γ generation (Mookerjee Basu, unpublished data). Carter et al. showed that SAG treatment of infected mice imparted resistance to reinfection while SAG treatment prior to infection imparted partial resistance to *Leishmania* infection.

SAG-induces proliferation of T-cells but not of B cells (Figure 3) even in absence of antigen presenting cells (Mookerjee-Basu, unpublished observation). Interestingly SAG-mediated proliferation of T cells does not require IL-2 (Figure 3).

Thus on the one hand SAG could activate T cell compartment (in both MHC-independent and -dependent manner), and on the other could directly activate MΦs to induce generation of microbicidal effector molecules (ROS and NO) which in concert help to potentiate both innate and cellular arms of immune system to eliminate LD parasites.

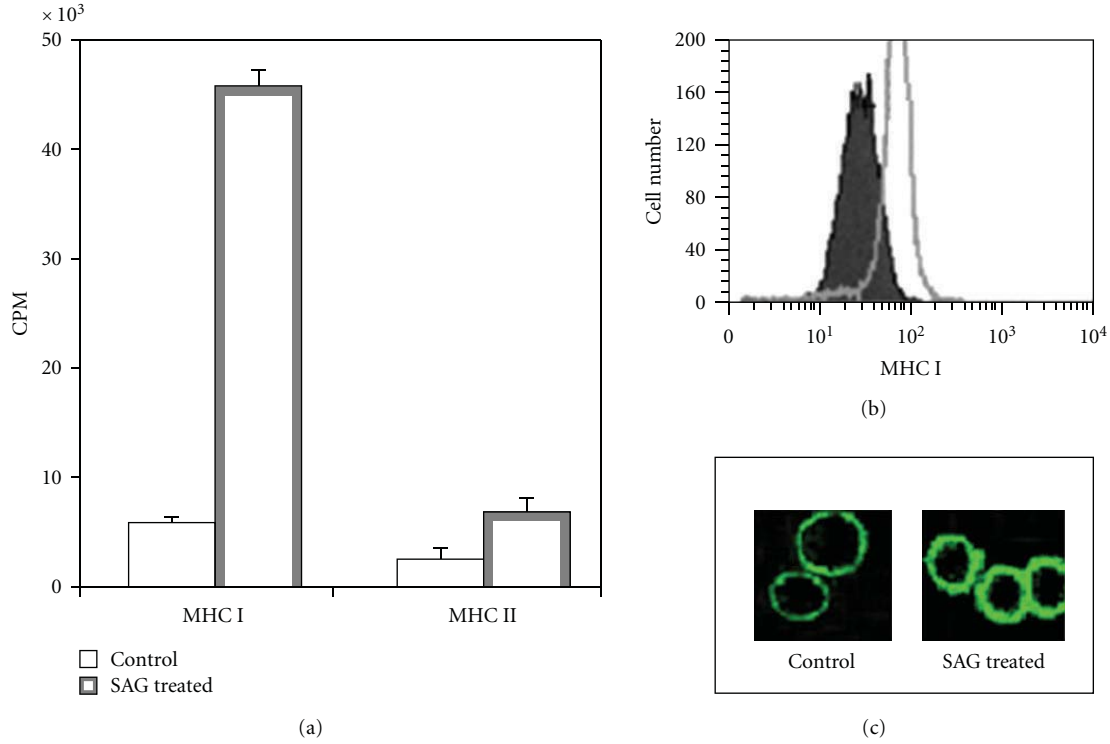

Figure 2: SAG increases MHC class I mediated antigen presentation and upregulates expression of MHC class I. MΦs isolated from BALB/c and C57BL/6 mice, cultured in presence or absence of SAG for 24 h. (a) To study the antigen presenting function, peritoneal MΦs from BALB/c and C57BL/6 mice either kept untreated or treated with SAG for 24 h, were used as antigen presenting cells to drive the T-cell hybridoma in presence of appropriate peptide and IL-2 secretion was tested on IL-2-dependent cell line (HT-2). The growth of HT-2 was studied using ^{3}H-Thymidine incorporation. The studies showed that class I but not class II restricted presentation was significantly ($P < .001$) enhanced upon SAG treatment both in normal and infected MΦ. (b) To study the expression of MHC I molecules, untreated (filled histogram) and SAG-treated (open histogram) MΦs from BALB/c mice were stained with FITC labeled anti-D^d (BD Pharmingen) according to manufacturer's instruction and either analyzed on flow cytometer or examined under a confocal laser scanning microscope. The studies showed that class I expression was significantly ($P < .001$) enhanced upon SAG treatment. Antigen presentation assay was performed at least thrice and the results are presented as mean ± SD. For flow cytometry and confocal microscopy, representative data of 3 similar experiments is presented here.

3. Resistance to Antimonials

3.1. Clinical Resistance. Pentavalent antimonial drugs were used worldwide for the treatment of VL and CL for over six decades with little evidence of resistance. There is a regional variation in response to antileishmanial drugs and thus recommendations for treatment of VL vary in different regions. Although the selection of resistant *Leishmania* has long been a part of laboratory studies, it is only in the past 15 years that acquired resistance has become a clinical threat. Pentavalent antimonials remain the treatment of choice in Africa, South America, Bangladesh, Nepal, and India (except North Bihar) at the dose of 20 mg/kg/day parenterally for 28–30 days. In the Mediterranean basin liposomal amphotericin B (L-AmB) is the treatment of choice for immunocompetent patients [100]. The drug of choice for the treatment of HIV/VL coinfection is an extended course of L-AmB [101]. However, the region endemic for VL in North Bihar, India, has the unique distinction of being the only region in the world where widespread primary failure to Sb(V) has been reported [102]. Even in this geographical region a variation in Sb(V) sensitivity occurs with significant drug resistance at the epicenter of the epidemic and a high level of sensitivity only 200 miles away [103]. This resistance is so far unique to *L. donovani*; all isolates from a large number of refractory as well as responding patients in India were identified as this species [4].

3.2. History of Antimony Resistance. Until the late 1970s, a small daily dose (10 mg/kg; 600 mg maximum of Sb(V)) for short duration (6 to 10 day) was considered adequate. In an earlier resurgence of Indian VL, which assumed epidemic proportions by 1977, an estimated 250,000 patients were affected in Bihar, when unconfirmed reports suggested a 30% treatment failure with this regimen from the four districts most severely affected, viz Muzaffarpur, Samastipur, Vaishali, and Sitamarhi [104]. Following this, an expert committee revised recommendations to use Sb(V) in two 10-day courses with an interval of 10 days and a significant improvement in cure rates (99%) was observed [105]. However, only a few years later, another study noted 86% cure rates with 20 days of continuous treatment with this regimen [106]. In 1984, a WHO expert committee recommended that Sb(V) should be used in doses of 20 mg/kg/day up to a maximum of 850 mg

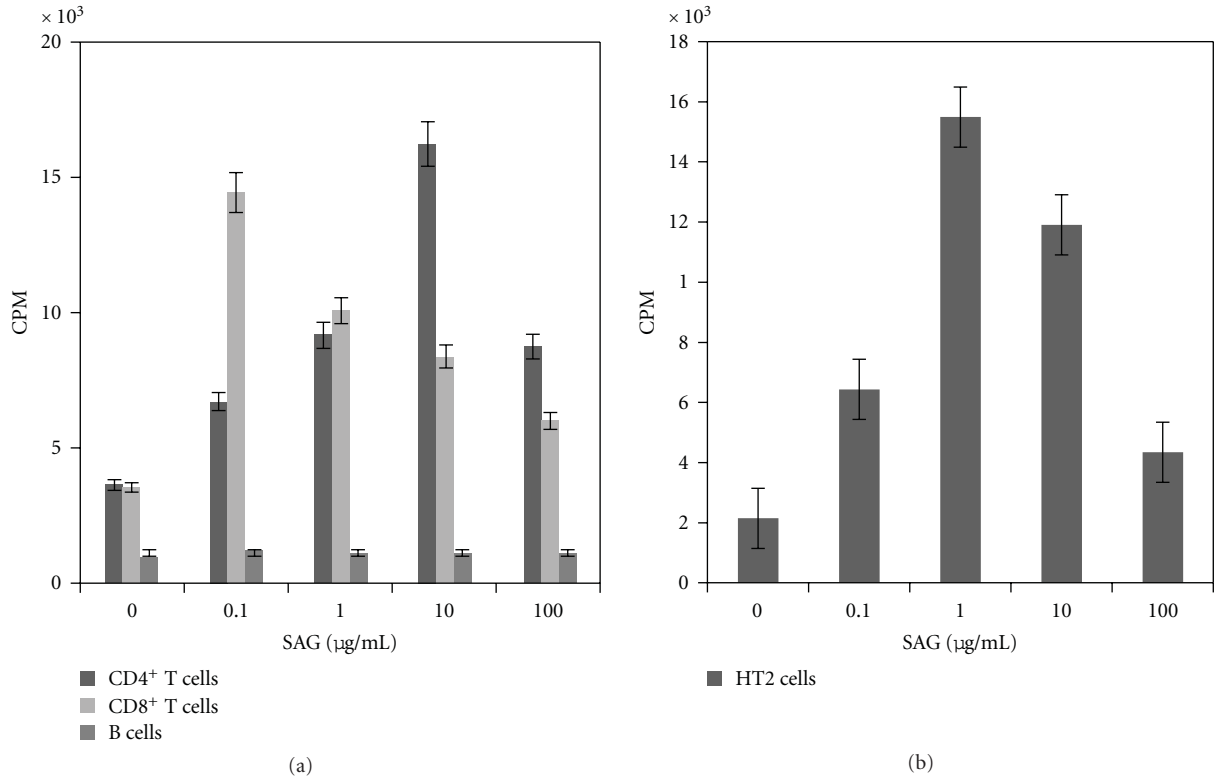

Figure 3: SAG directly stimulates proliferation of T cells. 10^5 lymphocytes, from normal BALB/c mice (a) and 5×10^4 IL-2-dependent $CD8^+$ cytotoxic T cell line (CTLL-2) were plated in each well and were kept either untreated or treated *in vitro* with various concentrations of SAG. Proliferation of each type of cells was monitored by ^3H thymidine incorporation. Each experiment was performed at least thrice and results are presented as mean ± SD.

for 20 days, with a repeat of the same regimen for 20 days in cases of treatment failure. Four years later, Thakur et al. evaluated the WHO recommendations and reported that 20 days of treatment with 20 mg/kg/day (maximum 850 mg) cured only 81% of patients, although with an extension of the treatment for 40 days 97% of patients could be cured (Table 1) [107].

Three years later, the same group noted a further decline in cure rate to 71% after 20 days of treatment, and recommended extended duration of treatment in nonresponders. Mishra et al. [5] found that extending the therapy, to 30 days could cure only 64% of patients in a hyperendemic district of Bihar, while 100 percent resistance cases of kala-azar was observed in two villages of Darbhanga and Sitamarhi districts (182 and 59 cases, resp.). From these findings it became clear that Sb(V) refractoriness was increasing although the reports came from studies that were not strictly controlled. In two following studies carried out under strictly supervised treatment schedules it was observed that only about one-third of all VL patients could be cured with the currently prevailing regimen. The incidence of primary unresponsiveness was 52%, whereas 8% of patients relapsed. During the same period, the treatment failed with only 2% of patients from the neighboring state of (Eastern) Uttar Pradesh [108]. These studies confirmed that a high level of Sb(V) unresponsiveness exists in Bihar, though the drug continues to be effective in surrounding areas. There are reports of antimony resistance spreading to the Terai regions of Nepal, especially from the district adjoining hyperendemic areas of Bihar, where up to 30% of patients seem to be unresponsive, though in eastern Nepal a 90% cure rate has been reported [109].

3.3. Reason of Antimony Treatment Failure. The reason for the emergence of resistance is widespread misuse of the drug. Sb(V) is freely available in India. Both qualified medical practitioners and unqualified quacks used the drug and this unrestricted availability of the drug led to rampant misuse. Most patients (73%) first consult unqualified medical practitioners, who might not use the drug appropriately [110]. It has been a common practice to start with a small dose and gradually increase the dose over a week. Drug-free intervals are given with the belief that they will prevent renal toxicity. On many occasions the daily dose of drug is split into two injections, to be given twice daily. These practices presumably expose the parasites to drug pressure, leading to progressive tolerance of the parasite to Sb(V). It has been observed that only a minority of patients (26%) were treated according to prescribed guidelines: irregular use and incomplete treatments were a common occurrence. These facts point to the mishandling of antileishmanial drugs in Bihar as a significant contributor to the development of drug resistance [103].

The growing resistance to Sb(V) in India while it still remained sensitive all over the world could be due to the fact that leishmaniasis usually has zoonotic transmission

Table 1: Changing therapeutic response to pentavalent antimonials (Adapted from T. K. Jha, 2006 [219]).

Study	Dose (mg/kg/day)	Duration (days)	No. of courses	No. of cases	Unresponsiveness (%)
Jha, (1980) [220]	10	10	1	200	17
Thakur et al., (1984) [221]	20	20	1	64	8
		>20	1	62	0
Jha, (1986) [222]	Child-20	Fresh-30	1	Fresh-73	1.1
	Adult-10	Relapse-60	1	Relapse-17	
		Slow	1		
		response-42			
Thakur et al., (1988) [223]	10	40	1	371	26
	15		1		14
	20		1		3
Jha, (1992) [224]	20	30	1	252	27.1
Jha, (1995) [225]	20	30	1	32	25
Jha, (1998) [226]	20	30	1	30	37
Thakur et al., (1998) [227]	20	30	1	80	54
Sundar et al., (2001) [102]	20	30	1	184	60

except in the Indian subcontinent and East Africa where the transmission is largely anthroponotic. In an anthroponotic cycle, once Sb(V) resistance gets established, it spreads exponentially and organisms sensitive to the drug get eliminated quickly, whereas the drug-resistant parasites continue to circulate in the community [111].

In CL the response is not as predictable, because there is considerable variation in sensitivity to Sb(V) among primary isolates from untreated patients with cutaneous leishmaniasis, which correlates with patients' response to treatment [112]. Except Bihar, primary resistance is quite uncommon, but resistance develops in patients with VL, CL, and MCL who have relapsed. Chances of response to further courses of antimonials diminish once there is a relapse after the initial Sb(V) treatment [113]. In *L. infantum* isolates taken from VL patients in France, drug-sensitive strains ($ED_{50} < 40\,\mu g/mL$) were isolated from patients who responded quickly to meglumine treatment, whereas all the strains which were resistant under *in vitro* conditions ($ED_{50} > 70\,\mu g/mL$) corresponded to clinical failures. *In vitro* sensitivity of strains decreased progressively in relapsing patients treated with meglumine [2].

3.4. Cellular and Molecular Mechanism of Antimony Resistance. It is evident from the above discussion that the response towards antimony treatments depends on several factors some are parasite related and some are host dependent.

3.4.1. Resistance at the Level of Parasite

Species Variation. Variation in clinical response to the pentavalent antimonials sodium stibogluconate, and meglumine antimonate (Glucantime) in VL, CL, and MCL has been a persistent problem in the treatment of leishmaniasis over the past 50 years. One explanation for this phenomenon is the intrinsic difference in species sensitivity to these drugs. In studies using the amastigote-macrophage model, *L. donovani* and *L. brasiliensis* were found to be three- to fivefold more sensitive to sodium stibogluconate than *L. major*, *L. tropica*, and *L. mexicana* [114]. This was also shown in earlier studies by Berman et al. using another amastigote macrophage model, which also demonstrated a wide variation in the sensitivity of isolates from cutaneous leishmaniasis cases to pentavalent antimonials [112]. In one controlled clinical trial in Guatemala that compared the cure rate to antimonials of CL caused by different species [115], sodium stibogluconate produced a significantly higher cure rate in patients with *L. braziliensis* (96%) lesions than those with *L. mexicana* lesions (57%).

Role of parasites in antimony treatment failure was established using *in vitro* amastigote-macrophage assay using *L. donovani* isolates from responders and nonresponders. Isolates from patients who did respond to sodium stibogluconate treatment were found be threefold more sensitive, with 50% effective doses (ED_{50}) around $2.5\,\mu g$ Sb/mL compared to isolates from patients who did not respond (ED_{50} around $7.5\,\mu g$ Sb/mL) [3]. The significant differences in amastigote sensitivity supported the concept of acquired antimony resistance in Bihar.

Other reports on VL isolates from Sudan have also shown that the clinical response to sodium stibogluconate was reflected in isolates in the amastigote-macrophage model (but not in promastigotes) [116]. Other observations support the notion that Sb resistance can be acquired. Of *L. infantum* isolates taken from immunodeficient and immunocompetent VL patients in France both before and after meglumine antimoniate treatment, those from 13 of 14 patients post-treatment had decreased sensitivity in an amastigote-macrophage assay [2]. A similar decreased sensitivity was observed in *L. infantum* isolates taken from dogs before and after meglumine antimoniate treatment [117].

In the laboratory, antimonial resistant *L. donovani* is easily generated in culture, most recently in axenic amastigotes of *L. donovani* and *L. infantum*, but *in vitro* unresponsiveness does not necessarily translate to clinical resistance [118]. Reduction of drug concentration within the parasite, either by decreasing drug uptake or by increasing efflux/sequestration of the drug, constitutes the primary mechanism of antimonial resistance. Other potential resistance mechanisms include inhibition of drug reduction, inactivation of active drug, and gene amplification [119–124].

Role of Thiol-Metabolism. Thiol metabolism has a central role in the maintenance of an intracellular reducing environment so that the cell can defend itself against the damage caused by oxidative stress inside the macrophage, oxidants, certain heavy metals and, possibly, xenobiotics [125]. As antimony causes oxidative stress [60, 126], a reducing environment within the cell and the presence of thiols become important for antimony resistance. Arsenite- or antimony-resistant laboratory mutants of all Leishmania species exhibit significantly increased levels of intracellular thiols, namely cysteine, GSH, and trypanothione (TSH), suggesting a role for thiols in resistance [127–129]. The synthesis of two precursors GSH and spermidine determines the level of TSH. The γ-GCS gene encoding γ-glutamylcysteine synthetase, which catalyses the rate-limiting step in GSH biosynthesis, has been found to be amplified in arsenite-resistant *L. tarentolae* [130], while the gene ODC which encodes ornithine decarboxylase, an enzyme involved in the regulation of spermidine biosynthesis, is also overexpressed [131, 132]. This suggests that a lowering of intracellular thiol concentration may result in the attenuation of the resistant phenotype. This proposed hypothesis is confirmed by inhibition studies. The inhibition of the γ-GCS and ODC genes by their specific inhibitors, L-buthionine-(SR)-sulphoximine (BSO) and DL-a-difluoromethylornithine (DFMO), respectively, results in the reversal of arsenite or antimony resistance in laboratory mutants [130, 133]. Although the combination of BSO and DFMO sensitizes the resistant cells, the residual level of resistance is still higher than that in wild-type cells, suggesting that GSH or TSH alone is not sufficient to confer metal resistance. Overexpression of either ODC or γ-GCS in *L. tarentolae* wild-type cells results in increased thiol levels, almost equivalent to those of resistant mutants, but the transfectants do not exhibit arsenite resistance [130]. While cotransfection of ODC or γ-GCS with MRPA in wild-type cells results in arsenite resistance [129, 132], this acquired resistance in transfectants is also reversed by the thiol depletor BSO [134]. This therefore establishes that MRPA and increased TSH concentrations act synergistically, and that TSH availability is the limiting factor in both the transport of drug conjugates and resistance to arsenite and/or antimony [135]. The tryparedoxin peroxidase family considered to be principally responsible for detoxification of peroxides [136]. The decameric type I tryparedoxin peroxidase (TryP) [137, 138], is a 2-Cys peroxidase, obtaining its reducing equivalents from T(SH)2 via the dithiol protein tryparedoxin (TryX). Studies have associated overexpression of TryP with resistance to both arsenite [139], and antimony [140] in laboratory generated *Leishmania* resistant lines and in-field isolates [141] implying that enhanced antioxidant defences, through overexpression of TryP, may well be a key feature of antimonials resistance. In *Leishmania tropica* and *Leishmania mexicana* cell lines, an increase in TSH is not associated with either the amplification of γ-GCS or overexpression of ODC [128]. Interestingly, resistance to Sb(V) in *L. donovani* clinical isolates (India) is also reversed in animal models by treatment with BSO [142, 143]. It is also noteworthy that the expression of γ-GCS in these resistant isolates is also increased significantly. Interestingly, in another study on *L. donovani* isolates from Nepal, expression of γ-GCS and ODC was significantly decreased in resistant isolates [121]. Therefore, there is a need to study the level of thiols in clinical isolates and determine their role in natural antimony resistance. It was also shown that antimony-resistant isolates downregulate the expression of γ-GCS of macrophages [144], probably by downregulating host NFκB, which is known to regulate γ-GCS expression [145]. This would result in the reduction of intramacrophage GSH levels and promote an intracellular oxidative environment, thereby minimizing the intramacrophage reduction of Sb(V) to its toxic form Sb(III) [39]. These results clearly indicate that SAG resistance in *L. donovani* is associated with manipulation of both host and parasite thiol levels. Spontaneous formation of Sb(III), complexed with GSH or TSH or both, has already been demonstrated by proton nuclear magnetic resonance spectroscopy [45, 146] and by MS [127]. Since GST is elevated in mammalian cells selected for resistance to arsenite [147], it has been proposed that GST mediates the formation of metalloid thiol pump substrates in Leishmania species also. However, in *Leishmania*, GST is not detectable; rather, a related trypanothione S-transferase activity is observed [148]. Thus, the thiols have a dual role in antimony resistance, that is, sensitization of the parasite by the reduction of pentavalent to trivalent antimony, and promotion resistance by forming conjugates with trivalent antimony for efflux and/or sequestration.

Efflux of the Drug. The efflux of a drug or its active derivative is a very common mechanism of drug resistance in bacteria, yeasts and fungi, and various pathogenic protozoa, for example, *Plasmodium falciparum, Entamoeba histolytica, Giardia lamblia, Trypanosoma cruzi, and Trichomonas vaginalis.* This may be the case in Leishmania too. Two types of ABC transporters are known to be responsible for multidrug resistance (MDR) in cancer cells: P-glycoprotein (P-gp) and multidrug resistance-related protein (MRP). P-gp is encoded by the mdr1 gene, which confers resistance to many hydrophobic drugs (MDR), and is characterized by reversion with verapamil and cyclosporine A. In Leishmania, MRP also confers MDR, although this cannot be reversed by conventional MDR modulators; the protein responsible is known as MRP1.

In Leishmania, both classes of ABC transporters have also been reported to be amplified in various species in response to different drugs under laboratory conditions [149].

Analysis of the complete Leishmania genome (http://www.genedb.org/) has revealed eight putative protein

homologues belonging to the MRP1 family, known to be involved in thiol-associated efflux and metal resistance in mammalian cells [150]. Two of them appear to be involved in antimony resistance in the parasite. The first one is PGPA (renamed as MRPA). However, Leishmania MRPA is functionally distinct from mammalian MRP, as resistance is not conferred to pentavalent antimonials, zinc and cadmium, or the typical multidrug-resistant P-gp substrates vinblastine and puromycin [151]. The gene has been found to be amplified in a number of laboratory mutants of Leishmania species selected for resistance to Sb(III), Sb(V), and As(III) [152]. Its role in antimony resistance has been confirmed by transfection studies [128]. However, this transporter is not responsible for the drug efflux across the plasma membrane. Rather, it confers resistance by sequestration of metal-thiol conjugates, a mode of metal detoxification in yeast cells [28]. MRPA is localized in membrane vesicles that are close to the flagellar pocket, the site of endo- and exocytosis in the parasite [153]. Overexpression of MRPA has been reported to decrease influx of antimony rather than increase efflux [33], and this may be due to a dominant negative effect through interaction with other membrane proteins. Thus, MRPA is an intracellular rather than an efflux transporter, and may play a major role in antimony resistance [154]. Recently, it has been shown by DNA microarray assay that MRPA is overexpressed in the axenic amastigote stage of Sb(III)-resistant *L. infantum* [134]. Transfection of MRPA confers Sb(III) resistance in promastigotes and Sb(V) resistance in the intracellular stage of *L. infantum*. However, MRPA has not been found to be upregulated in a comparative transcriptomic study of antimony-resistant *L. donovani* field isolates [121].

Further, no reports are available regarding the amplification of ABC transporter gene(s) in-field isolates. Thus, it is still of great interest to determine whether or not drug-resistant field isolates adopt the same strategies to resist antimony as the laboratory mutants. A second ABC transporter protein (PRP1), involved in antimony resistance, has been isolated by functional cloning selecting for pentamidine resistance [155]. This protein has been shown to confer cross-resistance to antimony. The localization of this protein and the mechanism by which it confers resistance remain to be determined. Another transporter that confers antimony resistance by an active extrusion system independent of MRPA is also present in *L. tarentolae* laboratory mutants [156]. Using everted vesicles enriched in plasma membrane, it has been shown that a metal efflux pump is present in the Leishmania plasma membrane. Like MRPA, this efflux pump also recognizes the metal conjugated to thiols such as GSH and TSH [127] and requires ATP. The identity of this efflux pump is still unknown even 10 years after its discovery. Further, it also appears that this efflux system does not play a significant role in antimony resistance, as the transport kinetics of the vesicles prepared from sensitive and resistant isolates are similar [157].

Dfferential gene expression study showed that expression of aquaglyceroporins AQP1, responsible for Sb(III) uptake, was downregulated at both the promastigote and the intracellular amastigote stages in antimony-resistant *L. donovani* isolates from Nepal [121]. The mRNA of AQP1 has also been shown to be decreased in antimony-resistant mutants of several Leishmania species.

3.5. Changes in the Cytoskeleton. Microtubules are dynamic cytoskeleton polymers consisting of repeating α-/β-tubulin heterodimers along with α-tubulin, and are vital for cell shape, growth and differentiation of Leishmania [158]. Altered expression, polymerisation and cellular distribution of α-/β-tubulin and apoptosis-like cell death in arsenite resistant Leishmania donovani promastigotes. Expression of α-tubulin is similar in both wild-type promastigotes and arsenite-resistant mutants. A twofold increased sensitivity of a mutant resistant to Paclitaxel (known to promote tubulin assembly) is found to decrease the expression of α-tubulin in arsenite-resistant mutant promastigotes [159]. On the other hand, the expression level of β-tubulin is higher in both stages of an arsenite-resistant mutant than in the wild-type [160], while α-tubulin expression is upregulated in the amastigote stage only and is unaltered in the promastigote stage. Although Paclitaxel treatment significantly increases the expression of b-tubulin in resistant promastigotes, it has no effect on c-tubulin expression in either strain, either before or after differentiation [160]. Further, arsenite treatment has been shown to decrease the expression of alpha- and betatubulin in wild-type promastigotes, while expression remains unaltered in an arsenite-resistant mutant [161]. Since tubulin synthesis is regulated by the unpolymerized tubulins, and arsenite has been shown to inhibit microtubule polymerization in the parasite, arsenite may decrease the synthesis of tubulins by inhibiting polymerization. It is noteworthy that phosphorylation of α- and β-tubulin is highly increased in the arsenite-resistant mutant [162]. Phosphorylation of tubulins could directly affect the dynamics of tubulin assembly and regulate and affect several signal-transduction pathways [163]. Since As and Sb are both are metalloid and mutual cross resistance has been seen in some Leishmania mutants, it could be speculated that tubulin may play an important role in Sb resistance.

3.6. Resistance at the Level of Host. The immune status of Leishmania infected patients has long been known to affect drug efficacy. This has proven to be of particular importance in relation to pentavalent antimonial treatment of DCL [164] and coinfections with HIV in the visceral form [165, 166], where there is both an absence of a specific T-cell mediated immune response and mutual exacerbation of infection. The basis for this lack of activity of pentavalent antimonials has been explored in immunodeficient mouse models for which the effects are probably due to deficiencies of both Th1-cell-mediated and macrophage responses [90, 167]. The introduction of highly active antiretroviral therapy [168] again suggesting an important role for CD4 lymphocytes in preventing relapses and controlling the infection.

It was further shown by our group that antimonials activate important signaling pathways of host immune cells like macrophage to induce ROS and NO that ultimately leads killing of intracellular parasites [78]. Interestingly, SAG

can also induce the generation of gamma interferon from splenic lymphocytes and the proliferation of splenocytes [169]. Therefore, it was necessary to decipher the role played by the host cell, if any, in Sb unresponsiveness. Further endeavor in this direction by our group revealed that resistant parasites strongly increase expression of host's P-gp and MRP1 transporters on the surface of infected macrophages resulting in Sb clearance from the host cells in the course of *in vitro* as well as *in vivo* experimental infection. Moreover, studies performed on patient samples from Sb-resistant infection areas unequivocally indicate that a similar phenomenon occurs during natural human infection. In contrast to infection with Sb-sensitive *L. donovani* isolates, infection with Sb-resistant *L. donovani* isolates upregulates the multidrug resistance-associated protein 1 (MRP1) and the permeability glycoprotein (P-gp) in host cells, thus inhibiting intracellular drug accumulation [170]. Indeed, it is well established that monocytes do not harbor parasites at the active stage of the disease. In spite of this, peripheral blood monocytes from Sb(V) resistant VL patients upregulate P-gp and MRP1. Therefore, it is likely that soluble and circulating parasite antigens can cause upregulation of expression of these transporters. This is supported by our findings that formalin-fixed Sb resistant *L. donovani* or even extracts from Sb resistant *L. donovani* strains can induce upregulation of MRP1 and P-gp in uninfected murine macrophages and reduce Sb accumulation following SAG treatment. Thus the resistance mechanism may operate in different cells of parasite reservoirs even in the absence of parasite replication *in situ*. Our results also show that inhibitors of P-gp and MRP1 could restore sensitivity toward Sb not only *in vitro* but also *in vivo*. In animal models, inhibition of the proteins MRP1 and P-gp by lovastatin reverses their action on drug accumulation and allows them to escape a fatal outcome. These results indicate that lovastatin, which can inhibit P-gp and MRP1, might be beneficial for reverting Sb resistance in VL.

A recent study [171] by our group has shown that antimony sensitive and resistant clinical isolates of *L. donovani* differentially regulate activation of dendritic cells (DCs). SAG-induced signaling pathway associated with DC activation/maturation is selectively targeted by antimony resistant *L. donovani* infection. In contrast to antimony sensitive *L. donovani*, antimony resistant *L. donovani* infection inhibits SAG-induced proinflammatory cytokine secretion as well as upregulation of costimulatory molecule and MHC expression in DCs. Antimony resistant *L. donovani* mediates these inhibitory effects in DCs by blocking SAG-induced activation of the PI3K/AKT and downstream NF-κB pathway. In addition, the suppression of NF-κB activation by antimony resistant *L. donovani* results in inhibition of SAG-induced γGCS heavy-chain (γGCS_{hc}) gene expression in DCs. Regulation of host γ GCS_{hc} expression and, therefore, of host GSH level by antimony resistant *L. donovani* is important in the view of antimony resistance in LD infection. This study establishes a key role for NF-κB in antimony resistant *L. donovani* -mediated suppression of DCs. Notably, antimony resistant but not antimony sensitive *L. donovani* induces increased IL-10 secretion by DCs. IL-10, a potent suppressor of antileishmanial immunity, is known to minimize responsiveness to SAG. Therefore, increased IL-10 production may play a critical role in disease pathogenesis in the host infected with antimony resistant *L. donovani*. Studies are underway to confirm whether the inhibition of SAG-induced signaling pathways observed in antimony resistant *L. donovani* infected DCs is due to lack of accumulation of the drug itself (as observed previously in case of macrophage system) or due to the effect of antimony resistant *L. donovani* infection.

3.7. Antimonials for Cancer. The immune system performs meticulously balanced and harmonious functions and thus protects the host from any undesirable foreign insult. Despite the existence of a multifunctional immunosurveillance process, immunocompetent individuals develop cancer. Cancer induces immense local immunosuppression and global immunosuppression in late stage. Antimonials possess immunomodulatory activity, can activate multiple signaling pathways including NFκB [78], and are also able to modulate intracellular redox balace [39]. Antimonial has been shown to activate T cells, and ameliorate renal cell carcinoma in combination with IL-2 [172]. SAG as well as antimony trioxide have also been shown to possess antileukemic activity [85, 173–175]. Since antimony is cheap and shows both direct action as well as indirect action on both immune cells and tumor cells, therefore antimony compounds are being tried clinically for cancer therapy mainly against leukemia.

At present novel cost-effective delivery systems for antimonials using liposome and cyclodextrin are being developed by Frezard's group and are showing enhanced efficacy. Interestingly cyclodextrin-based [176] antimony delivery has been found to be orally active. These formulations will not only improve therapeutic use of antimony for leishmaniasis but also for other diseases.

3.8. Other Available Drugs

3.8.1. Amphotericin B. Conventional by, amphotericin B has been used as a second-line treatment for VL since the 1960s. This drug exhibits an excellent antileishmanial activity with >90%–95% cure rates in Indian VL cases. The routine scheme of conventional amhotericin B is 1 mg/kg administered on alternate days for a total of 30 days. However, a recent study in India showed 96% cure rates with a dose of 0.75 mg/kg/day for 15 days [176]. Major disadvantages of conventional amphotericin B are its prolonged administration and the frequent adverse effects, such as infusion-related fever and chills, nephrotoxicity, and hypokalemia, which necessitate administration in hospital [176]. Conventional amphotericin B is used extensively in India for cases unresponsive to antimonials or even as a first line drug. However, outside India this drug does not offer any advantage over pentavalent antimonials.

Unresponsiveness and relapses occur rarely, except among HIV-infected patients. In this population, secondary episodes of VL are common and are attributed mainly to relapse but also to reinfection [177]. A recent study failed to disclose decreased susceptibility among *Leishmania* parasites

collected from HIV-infected patients during repeated VL episodes (mean follow-up period: 35.6 months; range: 3–137 months), despite repeated courses of amphotericin B.

3.8.2. Miltefosine. Miltefosine (hexadecylphosphocholine) is the first orally administered drug for VL and the latest to enter the market. This agent is associated with high efficacy rates, including cases unresponsive to antimonials [178, 179]. In a phase IV multicenter trial in India of 1132 adults and children with VL treated with miltefosine, cure rates were 82% per intention-to-treat analysis and 95% per protocol analysis [180]. In this study, 3% of patients developed adverse effects, mainly gastrointenstinal toxicity, and elevated hepatic transaminases and creatinine [180]. Data from phase IV clinical trials in India involving domiciliary treatment with miltefosine along with weekly supervision suggested a doubling in the relapse rate against miltefosine [180]. So far, miltefosine is licenced in India, Germany, and Colombia. The scheme of miltefosine treatment is 100 mg/kg/day for 28 days in adults weighing 50 kg, 50 mg/kg/day in adults <50 kg, and 2.5 mg/kg/day in children (maximum dose: 100 mg/day). Major concerns for the wide use of miltefosine include its teratogenic potential and its long half-life (approximately 150 hours) which may facilitate the emergence of resistance. Miltefosine is strictly forbidden in women of child-bearing age who may become pregnant up to two months following drug discontinuation. In India miltefosine is available over the counter, a fact that may expose this drug to misuse and emergence of resistance. Once generated, resistant parasites could spread rapidly, endangering the life span of miltefosine in a country where it is needed most [7].

The exact antileishmanial mechanism of miltefosine remains largely unknown. The intracellular accumulation of the drug appears to be the critical step for its action. The intracellular accumulation of miltefosine includes the following steps: binding to plasma membrane, internalization in the parasite cell (two proteins, the miltefosine transporter LdMT and its beta subunit LdRos3, are the most significant), and intracellular targeting and metabolism [181]. It has been found that miltefosine induces an apoptosis-like cell death in *L. donovani* by producing numerous defects [181]. Miltefosine also induces several immunologic and inflammatory effects on macrophages. In animal models, miltefosine does not require T-cell-dependent immune mechanisms in order to act, indicating that this agent can be used in T-cell-deficient patients [182]. Recently, it was found that miltefosine enhanced IFN-γ receptors and thus IFN-γ responsiveness in *L. donovani*-infected macrophages; in the same model, miltefosine induced an IL-12-dependent Th1 response and reversed the Th2 response to Th1 response [183].

Resistance to miltefosine may emerge easily during treatment due to single point mutations. Decrease in drug accumulation is the common denominator in all miltefosine resistant Leishmania lines studied to date, and this could be achieved through decreased uptake, increased efflux, faster metabolism, or altered plasma membrane permeability; the first two mechanisms have been already described in models of experimental miltefosine resistance [184]. Two proteins, miltefosine transporter LdMT and its specific beta subunit LdRos3, form part of the miltefosine translocation machinery at the parasite plasma membrane, and are required for miltefosine uptake [181]. Experimental mutations at LdMT or LdRos3 rendered the parasites remarkably less sensitive to miltefosine, and this resistance persisted *in vivo*; cross-resistance with other antileishmanials was not detected [185]. The overexpression of ABC transporters is another mechanism for acquisition of miltefosine resistance, through reduction of the drug intracellular accumulation [185]. Recently, a novel flavonoid derivative was designed and it was shown that the use of suboptimal doses in order to overcome the overexpression of LtrMDR1 (a P-glucoprotein-like transporter belonging to the ATP-binding cassette superfamily) was associated with a fourfold increase of intracellular miltefosine accumulation in the resistant Leishmania lines [186]. Furthermore, modifications in lipid compositions of membranes and sterol biosynthesis have been detected in miltefosine-resistant L. donovani promastigotes [187]. Since membrane fluidity and permeability are influenced by lipid composition, their modification may affect drug-membrane interactions [187]. A case of a healthy patient with VL, who relapsed 10 months after successful treatment with miltefosine for 28 days, was reported recently [188].

3.8.3. Paromomycin. Paromomycin (aminosidine) is an aminoglycoside with antileishmanial activity. In a phase III study of VL in India, this drug was associated with 94.6% cure rates, similar to amphotericin B [189]. Adverse effects were more frequent in the paromomycin-treated group compared with the amphotericin B-treated group (6% versus 2%, resp.); included increased hepatic transaminases, ototoxicity, and pain at injection-site [189]. Currently, paromomycin is under phase IV clinical trials. Paromomycin is inexpensive but requires daily intramuscular injections for 21 days [176].

Paromomycin inhibits protein synthesis and modifies membrane fluidity and permeability. An *in vitro* study showed that following a 72-hour exposure of *L. donovani* promastigotes and amastigotes to paromomycin, the mitochondrial potential was decreased, which indicates that mitochondria are the targets of the drug [190]. In laboratory-derived resistant parasites developed through serial-passage increasing-drug concentrations, paramomycin uptake was decreased compared to the wild-type parasite, in association with inhibition of protein synthesis; no cross-resistance with other antimonial agents was detected [190]. Since paromomycin is an aminoglycoside, it is possible that resistance will emerge rapidly if used as monotherapy.

3.8.4. Combination Regimens. The rational for using combination regimens with different resistance mechanisms over monotherapy relies on the expected enhanced efficacy (through synergy or additive activity without drug interaction), shorter treatment duration, less toxicity, improved compliance, reduced likelihood of emergence of resistance, and reduced costs. A combination policy for VL is supported by the fact that antileishmanial drugs belong to different chemical classes [195]. Recent studies have investigated this

option. In a retrospective study conducted among Sudanese patients with VL, it was found that combination of sodium stibogluconate and paromomycin administered for 17 days was associated with higher cure and survival rates compared to sodium stibogluconate monotherapy administered for 30 days (44%–86% lower odds of death in the combination group) [191]. Combinations of miltefosine with amphotericin B, paromomycin or pentavalent antimonials have been evaluated in an *in vivo* model and this revealed that the combinations of miltefosine with amphotericin B or paromomycin were efficacious [192]. These preliminary data justified a recent study in Bihar, India, comparing 5 mg/kg of liposomal amphotericin B administered once (group A; 45 patients), 5 mg/kg of liposomal amphotericin B administered once plus miltefosine for either 10 days (group B; 46 patients) or 14 days (group C; 45 patients), 3.75 mg/kg of liposomal amphotericin B administered once plus miltefosine for 14 days (group D; 45 patients), and 5 mg/kg of liposomal amphotericin B administered once followed by miltefosine for 7 days (group E; 45 patients); in this study, similar final cure rates (91%–98%) were noted in all treatment groups. These data indicate that a single dose of liposomal amphotericin B followed by 7–14 days of miltefosine is active against Indian VL [193]. In this study, all patients were treated in an outpatient setting. Large, randomized-controlled trials are required before adaptation of combination regimens.

Several combination regimens with investigational agents have been tested *in vitro* and in animal models [194]. The plant-derived immunostimulant agent picroliv has no antileishmanial activity; however, when administered with half-dose miltefosine, it increases significantly the activity of the latter [195].

3.9. Peroxovanadium Compounds towards the Reversal of Antimony Resistance. There are reports that peroxo- and diperoxo-vanadate compounds are potential antileishmanial agents in a number of *in vitro* and *in vivo* assays [196, 197]. The peroxide of vanadium (PV, a mixture of vanadate and H_2O_2) is an insulinomimetic agent and a potent inhibitor of protein tyrosine phosphatase (PTP) [198–202]. Inhibition of PTP by peroxovanadate can modulate the leishmanicidal response by inducing microbicidal effector molecules (like NO, ROS) along with IFN-γ [196, 197]. The peroxovanadate compounds that are used against experimental infection contain 1,10-phenanthroline, pyridine-2-carboxyl or bipyridine as ancillary ligands [197, 198, 202]. A number of chemically defined PV derivatives, each containing an oxo ligand, one or two peroxo anions in the inner coordination sphere of vanadium, and an ancillary ligand, are equally potent PTP inhibitors stable in aqueous solution [202]. These can activate the insulin receptor kinase, mimic insulin biological action *in vivo* [198], and also activate the response of immune cells [203]. Both in human and mice, the severity of visceral leishmaniasis have been most closely associated with increased levels IL-10, where the ratio of IFN-γ : IL-10 is the important denominator for the protection [204–206]. Thus peroxovanadate complexes appear to possess the potential to become antileishmanial agents.

We tested a number of vanadium compounds, which are different from those used against experimental infection, with respect to their ancillary ligands in the coordination sphere of the compounds (Figure 4), to get the potent variety that may be of therapeutic application against leishmaniasis. Another compelling reason to test vanadium compounds is that vanadate is an inhibitor of P-gp [207–209], which is well related to Sb-resistace in leishmaniasis [210]. We have studied six peroxovanadate compounds ((three dinuclear triperoxovanadate (TPV, (a)–(C) in Figure 4) complexes and three mononuclear diperoxovanadate (DPV, (d)–(f) in Figure 4) complexes). Our study showed that one of the mononuclear diperoxovanadate compounds (designated as PV6) is highly effective in killing intracellular *Leishmania* parasites. When PV6 was injected together with SAG, the combination showed enhanced antileishmanial activity *in vivo* in terms of reduction in organ parasite burden in BALB/c mice infected either with SAG sensitive or SAG unresponsive strain. Our study also showed that immune parameters like antileishmanial T cell response as also ROS and NO production were enhanced in response to the combination treatment. Most importantly, such therapy allowed increased IFN-γ production with concomitant decrease in IL-10 generation, an indicator for favorable antileishmanial immune.

4. Strategies Available to Combat Drug Resistance

4.1. Drug Resistance Monitoring. Improved methods to monitor drug resistance are essential that determine either the (i) phenotypic sensitivity of parasite isolates or (ii) molecular changes that indicate alterations in either the drug target or mechanisms that alter the intraparasite level of active drug. There are problems with both approaches. First, the determination of drug sensitivity of clinical isolates is open to the criticism that pathogen adaptation from host to culture media immediately selects for a subpopulation of pathogens best suited for growth in that medium. The drug sensitivity of parasites must therefore be tested as soon as possible after isolation from the patient using defined agreed protocols. Although promastigote assays are easiest and quickest, this assay is not predictive for pentavalent antimonials, and possibly not for other antileishmanials also, for example, paromomycin, pentamidine, and miltefosine. The amastigote-macrophage assay is currently the only model able to correlate clinical response to the sensitivity of the isolate, as demonstrated in relation to pentavalent antimonials [3]. Axenic amastigotes are sensitive to antimonials but adaptation of isolates is both too selective and too lengthy a process to be used in this type of assay [211]. Second, the ability to develop molecular probes or PCR-based diagnostics to monitor the development and spread of drug resistance is severely limited by a lack of knowledge of the molecular and biochemical mechanisms of action and resistance of most antileishmanial drugs, especially in clinical isolates [114].

4.2. Monitoring Therapy. The introduction of an oral drug for leishmaniasis offers advantages of improved compliance, self administration, and reduced costs. In the phase IV trial

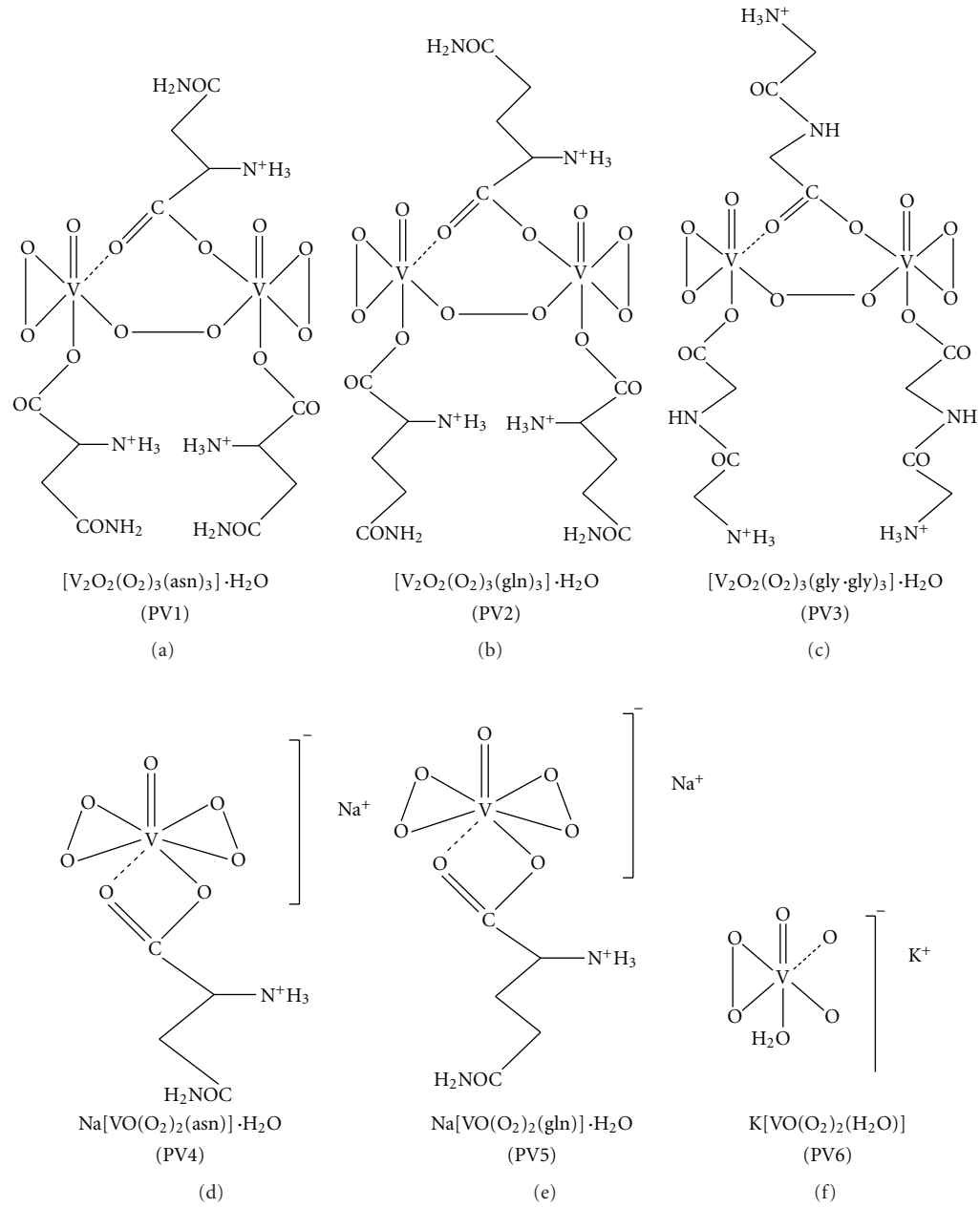

Figure 4: Structures and formulae of the PV compounds [218].

for miltefosine, a 7-day supply is issued to patients who have to return to the clinic each week for examination and resupply. For drugs like miltefosine which have a long half-life and a propensity for selection of resistant forms, the monitoring of daily dosing and the completion of a course of treatment are essential. The directly observed treatment strategy for tuberculosis chemotherapy has been successfully introduced in India by the Revised National TB Control Programme in 1997 (http://www.who.int/gtb/publications/globerep/index.html). The potential for use of a parallel system for the control of leishmaniasis, for miltefosine at present possibly also for sitamaquine in future, should be considered [114].

4.3. Cost and Distribution of Drugs. The approximate cost of treatment of a patient with VL in India is given in Progressive failure of antimonial drug treatment, which is the only available drug treatment in the public health program in India, has driven most of the VL patients in India towards the private sector. The drugs, including antimonials, amphotericin B, and now miltefosine, can be bought over the counter without restriction on quantity. The cash-starved population buys antileishmanial drugs in instalments, and most do not complete treatment [102] as disease symptoms are alleviated quickly. Considering the cost of drugs, antimonials have been the only drugs that are barely affordable. Miltefosine, which is being used extensively in

the private sector, is 6 times more expensive and it is not mandatory to buy the full course. This is likely to result in widespread underdosing, sharing of doses among patients, and ultimately emergence of resistance to this important and only oral antileishmanial compound. Considering the inability of the majority of the population to purchase and complete a full course of the drug, and the chaotic system of drug marketing, it has been suggested that, miltefosine should be withdrawn from the private sector and made available free through public and/or private health care providers to prolong the effective life of this important drug [7].

4.4. Diagnostic Methods. The improvement in noninvasive serological diagnostic methods with high sensitivity and specificity, for example, DAT, K39, and Katex (urine dipstick), is a major advance in the control of leishmaniasis [212, 213]. In the context of chemotherapy what is required is a noninvasive diagnostic kit that can be used to monitor drug response and determine cure in patients. Antibody levels do not always indicate active infection, vary between individuals, and are of no use in HIV/VL coinfection cases. Antigen detection is far more important for monitoring drug response; further improvement of methods such as the Katex kit [214] might be of particular interest in this case. The variation in species sensitivity has greatest clinical significance in Central and South America, where the distribution of *L. mexicana*, *L. amazonensis*, *L. panamensis*, *L. braziliensis*, and other members of these groups overlap. The distinctive amastigote and macrophage interaction of mexicana group parasites makes some level of diagnosis by microscopy feasible for trained staff. Molecular tools that have been developed need to be implemented to distinguish the braziliensis group species.

4.5. Combination Therapies. Drug combinations have proven to be essential features of antimicrobial treatment through design or use to (i) increase activity through use of compounds with synergistic or additive activity, (ii) prevent the emergence of drug resistance, (iii) lower required doses, reducing chances of toxic side effects and cost, or (iv) increase the spectrum of activity, for example, the use of an antileishmanial with either an antiinflammatory or immunodulator in cutaneous leishmaniasis. Previous studies on drug combinations for VL, for example allopurinol plus sodium stibogluconate and paromomycin plus sodium stibogluconate [215]. The use of combinations to combat resistance has been well rehearsed in antimalarials; studies to identify such combinations are new for leishmaniasis; limited studies are under way to examine interactions of miltefosine with other antileishmanials to identify suitable combinations. Bryceson [216] advocated the examination of combinations of strong antileishmanials with "weak" drugs (e.g., azoles); this is an approach also used in malaria treatment, for example, the inclusion of clindamycin or azithromycin in combinations. A combination therapy also needs to be evaluated for safety and optimized for either concomitant or sequential administration of component drugs.

4.6. Resistance Reversal Agents. The strategy to reverse resistance has long been discussed in relation to chloroquine resistance in *Plasmodium falciparum* and produced interesting experimental results without any clinical impact. In laboratory studies on *Leishmania,* a series of sesquiterpenes have been shown to reverse drug resistance due to P-glycoproteins in an *L. tropica* clone. Another study suggested a strategy of inhibition of thiol levels by coadministration of antimony with an inhibitor of glutathione biosynthesis.

4.7. New Targets, New Drugs. There are few better ways to avoid drug resistance than to have an adequate armory of drugs with different targets and no cross-resistance. Although miltefosine has been approved for use in the treatment of VL in India, paromomycin is moving through phase III trials in India and Africa, and sitamaquine remains in phase II development for leishmaniasis [217], all these drugs have clear limitations of toxicity, long courses of treatment, or parenteral administration. More clearly defined criteria of the needs and target profiles for new drugs and new treatments are required.

5. Conclusions

The control of VL globally is challenged by the widespread emergence of antimonial resistance in India. In the last decade new formulations of conventional antileishmanial drugs as well as new agents became available. The wide use of the oral agent miltefosine was hampered by the potential for teratogenicity and emergence of resistance. Combination regimens should be evaluated in large trials. During last few years several mechanisms of in-field antileishmanial resistance were identified. Understanding their molecular and biochemical characteristics will lead to the design of new drugs and also the molecular surveillance of resistance. In order not to jeopardize the life span of available antileishmanials, their delivery, clinical response, and resistance should be monitored. Overall the development of antileishmanials has been generally slow; new drugs are needed.

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Review Article

Identification and Characterization of Genes Involved in *Leishmania* Pathogenesis: The Potential for Drug Target Selection

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Identifying and characterizing *Leishmania donovani* genes and the proteins they encode for their role in pathogenesis can reveal the value of this approach for finding new drug targets. Effective drug targets are likely to be proteins differentially expressed or required in the amastigote life cycle stage found in the patient. Several examples and their potential for chemotherapeutic disruption are presented. A pathway nearly ubiquitous in living cells targeted by anticancer drugs, the ubiquitin system, is examined. New findings in ubiquitin and ubiquitin-like modifiers in *Leishmania* show how disruption of those pathways could point to additional drug targets. The programmed cell death pathway, now recognized among protozoan parasites, is reviewed for some of its components and evidence that suggests they could be targeted for antiparasitic drug therapy. Finally, the endoplasmic reticulum quality control system is involved in secretion of many virulence factors. How disruptions in this pathway reduce virulence as evidence for potential drug targets is presented.

1. Introduction

Leishmania is the causative agent of leishmaniasis, a spectrum of diseases affecting more than 12 million people worldwide. The two major clinical forms of leishmaniasis, cutaneous and visceral, are the result of infection by different species of the parasite. Visceral leishmaniasis (VL), which causes splenomegaly and hepatomegaly, is fatal if not treated and is caused by *L. donovani* and *L. infantum* (also designated *L. chagasi* in the new world). More than 90% of the visceral cases in the world are reported from Bangladesh, India, Nepal, Sudan, and Brazil [1]. Cutaneous leishmaniasis (CL) causes lesions which are mostly self-healing and are caused by *L. major*, *L. tropica* or *L. aethiopica*, in the old world and by *L. mexicana* or the *L. braziliensis* complex in the new world [2]. Both environmental risk factors such as massive displacement of populations, urbanization, deforestation, and new irrigation plans and individual risk factors such as HIV, malnutrition, and genetic susceptibility make leishmaniasis an important public health problem [1]. Though the most significant public health effects of leishmaniasis are concentrated in developing countries, occasional cases occur in developed countries as well. In the European countries around the Mediterranean basin and throughout the Middle East, as well as Latin America, there are large populations that must still consider the risk of leishmaniasis. In some of these countries, dogs represent an important reservoir for the parasite. In the USA, even though leishmaniasis is not endemic, infections can be found in pockets of the country especially in the southwest [3]. In addition, *Leishmania* infection was found in dogs in the northeastern part of the USA [4]. Increasing immigration, tourism, and military activity in *Leishmania* endemic areas has led to leishmaniasis becoming an increasing threat in nonendemic areas of the world. This was underscored by the recent US military deployments to *Leishmania* endemic areas such as Iraq and Afghanistan, which have resulted in infected US soldiers [5]. In addition, there have been several documented cases of parasite transmission by blood transfusion worldwide forcing the deferral of exposed individuals from blood

donation [6]. Studies in animal models, such as hamsters and dogs, show that *Leishmania* not only survives blood-banking storage conditions, but also retains its infectivity [7, 8]. Therefore, *Leishmania* has a potential to impact blood safety in developed as well as developing countries.

In the *Leishmania* life cycle, the motile promastigote form that resides in the alimentary canal of the sandfly vector is transmitted to a mammalian host during a blood meal. Host macrophages ingest the parasites, which must differentiate into the nonmotile, amastigote, form to persist in the macrophage's lysosomal compartment [9]. These two life stages have been adapted to *in vitro* culture for many *Leishmania* species [10, 11] allowing manipulation of the genome and assessment of the altered phenotypes *in vitro* [12, 13].

The only available cure for visceral leishmaniasis is drug treatment. Though most cutaneous leishmaniases are self-healing, drug treatment is employed to relieve the painful sores, avoid scarring and other complications. However, currently available drugs for leishmaniasis are far from satisfactory because they are toxic, expensive or lose effectiveness due to the development of drug resistance after prolonged use [14–16]. Vaccination is not a viable option either, because there are as yet no effective vaccines for leishmaniasis. Recent technological advances in the understanding of the pathogenesis of leishmaniasis beg the question how these advances could be translated into either development of better drug or vaccination strategies that could eradicate this disease.

Many investigators in the field have pointed to the importance of the publically available DNA sequence for *Trypanosomatid* genomes as a pathway to new drug discovery [17, 18]. However, for the visceral genome sequenced, *L. infantum*, there are 8387 genes annotated, of which 5,342 are "hypothetical" and only 3,288 have been assigned gene ontology terms. Thus the majority of the building blocks of this parasite are uncharacterized. A similar situation exists for the cutaneous species, *L. major*, with 5,396 hypothetical genes out of 9,388 annotated. Clearly to make advances in the development of new drugs, parasite components that are required for survival need to be identified and characterized to the point where rational drug design can target inactivation of these molecules or their activities. The annotated genome information is essential in the process of identifying and characterizing parasite proteins and the genes that encode them. Therefore, further characterization of such genes is needed to focus on the following important questions, for example: (a) how essential is a protein encoded by such genes for survival of the parasite, (b) what functional role does it play in the parasite's physiology, (c) how does it fit into biochemical pathways that are crucial for parasite pathogenesis, (d) are there life cycle stage-specific expression patterns, in particular, is the protein required in the amastigote stage that will be subject to the drug impact in treated patients, (e) how divergent is the parasite protein or activity from similar human proteins to avoid toxicity of any proposed drug, and (f) have the activities of similar proteins been inhibited with compounds that suggest drug treatment is feasible?

This paper focuses on our efforts to identify and characterize *Leishmania donovani* genes and the proteins they encode for their role in pathogenesis. A brief survey of those proteins and their novel attributes can reveal the value of this approach for finding new drug targets and illustrate specific characteristics that could suggest a target is "druggable." We are indeed cognizant of the efforts by other investigators in this field, but have not attempted to cover those studies because of the limited scope of the paper. The search for such proteins and activities in these human pathogens requires a broad perspective on the physiology of the parasite. We present below a survey that spans diverse pathways with potential for therapeutic disruption. Any pathway that is to be targeted by drugs given to the mammalian host must be essential in the amastigote life cycle stage found in the patient. We review some examples of newly described proteins and their pathways that are differentially expressed or required in this intracellular stage in the first section. A pathway nearly ubiquitous in living cells already has been targeted by anticancer drugs, the ubiquitin system. Section two reviews new findings in ubiquitin and ubiquitin-like modifiers in *Leishmania* and how disruption of those pathways could reduce the viability of the parasite. The existence of a programmed cell death pathway has been well documented in protozoan parasites. We review some of the components of this pathway and evidence that suggests they could be targeted for drug therapy in Section three. At the very inception of synthesis of many secreted virulence factors is the endoplasmic reticulum quality control system. How disruptions in this pathway reduce virulence as evidence for a potential drug target is presented in Section four.

2. Targeting Proteins Uniquely Required for Survival in the Mammalian-Infecting, Amastigote, Life Cycle Stage

In search of functions that may be unique to amastigotes, we noted that the shift of metabolism from promastigotes to amastigotes leads to the expression of a spectrum of genes that could be targets to control *Leishmania* pathogenesis. Whereas promastigotes utilize glucose as their primary energy source, intracellular amastigotes depend primarily on amino acids and fatty acids as their carbon source [19, 20]. Increased mitochondrial activity may play a crucial role in the survival of amastigotes inside host cells [20, 21]. The mitochondrion harnesses the energy from numerous substrates through the electron transport chain. Electron transport depends on multiprotein complexes I, II, III, and IV embedded in the inner mitochondrial membrane ultimately passing the electron to oxygen. This oxygen consumption is referred to as respiration. The proton gradient produced by electron transport drives the F_1/F_0 ATPase (complex V) in a coupled process termed oxidative phosphorylation. Active respiration is required for survival of both promastigote and amastigote forms of *Leishmania* [22, 23]. Investigations of the individual complexes of the respiratory chain suggest NADH dehydrogenase (complex I) is not found in its classical form in trypanosomatids [24]. However, evidence for succinate dehydrogenase (complex II), cytochrome c reductase (complex III), and cytochrome c

oxidase (complex IV) has been demonstrated for both *Leishmania* and *Trypanosoma* [24, 25]. Recent studies suggest that *Leishmania* cytochrome c oxidase is a potential target for the oral drug, Miltefosine [26, 27]. The trypanosomatid cytochrome c oxidase (COX) complex (complex IV) is a multicomponent complex composed of more than 14 subunits [28, 29]. It has three mitochondrially encoded subunits, and all the others are nuclear encoded subunits. Most of the nuclear encoded components have no apparent homologue outside the *Trypanosomatids* [28, 30] thus fulfilling one of the criteria of a drug target. Some of the nuclear encoded subunits are essential for proper function of complex IV [31] including the recently described MIX protein [32, 33].

Recently, we characterized a gene encoding a 27 kDa mitochondrial membrane protein (Ldp27), a subunit of the active COX complex, specific to amastigotes and metacyclics, the infectious stages in *Leishmania* [34]. We also demonstrated that Ldp27 is necessary for the high level of COX activity in amastigotes and that Ldp27 gene deleted parasites ($Ldp27^{-/-}$) show significantly less COX activity and reduced ATP synthesis in intracellular amastigotes compared to wild type. Moreover, the $Ldp27^{-/-}$ parasites are less virulent both in human macrophages and in BALB/c mice.

A functional role for Ldp27 is also suggested by the lower level of COX activity in the wild-type procyclic promastigote stage that does not express Ldp27. It has been established that the respiratory chain is active in *Leishmania* promastigotes [24], and the inhibition of promastigote proliferation by cyanide indicates the requirement for an active COX in this stage [23]. In our recent study, COX activity was also detected in the promastigote form, although significantly less than in the amastigote form. Thus Ldp27 may play a role in increasing the enzymatic activity of the COX complex, but not in the abundance or assembly of at least some of its components.

The utility of the electron transport chain as a target of antiparasitic drugs is illustrated by the ability of atovaquone to block growth of *Plasmodium* [35], and inhibition of the cytochrome c oxidase complex in particular is the mode of action of the antimalarials artesunate [36] and artemisinin [37]. Further study will be required to determine what specific function allows Ldp27 to substantially increase COX activity potentially through evaluation of the effect of mutating key amino acid residues. However, from the investigation so far, this protein is essential in the amastigote stage, is demonstrated to be in a critical biochemical pathway that is already known to be an effective drug target, and is a unique parasite protein suggesting specific inhibitors will not affect mammalian COX activity. These features illustrate how careful characterization of parasite proteins can set the stage for rational drug design.

In our efforts to identify genes that are differentially expressed in the virulent amastigote stage of the parasite, we identified a *Leishmania* homologue of the mammalian argininosuccinate synthase (ASS) gene first identified in a screen for genes altered in expression when amastigote cells undergo mitotic arrest. The ASS gene was also shown to be more abundantly expressed in the amastigotes than in the promastigote forms by Northern and Western blot analyses [38]. Thus this protein presents as an available target in the human infection for drug intervention.

Mammalian ASS, 59.6% similar to *Leishmania* ASS, is the limiting enzyme of the urea cycle that catalyses the ATP-dependent condensation of citrulline and aspartate to form argininosuccinate, immediate precursor of arginine, thus leading to the production of urea in the liver and Nitric Oxide (NO) in many other cells [39]. Though the high level of similarity raises early concerns about drugs having a toxic effect on the human cells as well as *Leishmania*, the subcellular compartmentalization of the protein may lead to differential sensitivity. The intracellular ASS location in mammals may depend on its physiological function, and its gene regulation differs greatly depending on the tissue [40]. Unlike the mammalian homologue, the *Leishmania* ASS is isolated to a glycosome-like vesicle, which might suggest a drug effect that differs between *Leishmania* and humans. The glycosomal localization is suggested by the glycosomal targeting signal (amino acids Serine-Serine-Leucine) encoded at the C-terminal of the amino acid sequence [41]. Further evidence comes from IFA studies using parasites overexpressing ASS with a native C terminus or ASS for which the SSL at the C-terminus was blocked with an epitope tag. The native ASS is localized in small punctate spots distributed throughout the cell, but the protein with the C terminal tag remained in the cytosol in the parasite and did not target to any cytoplasmic vesicle [42]. The unique compartmentalization in glycosomes has been suggested as a means to develop *Leishmania*-specific inhibitors of other metabolic enzymes as well [43]. An added advantage of characterization of ASS as drug target is the availability of inhibitors already used in other species that could be evaluated for anti-*Leishmania* activity. Fumonisin B1, a fungal mycotoxin altering sphingolipid metabolism through interruption of de novo ceramide synthesis, inhibits *in vitro* argininosuccinate synthase [44]. Saccharopine, another ASS inhibitor, is a potent inhibitor of crude and purified preparations of argininosuccinate synthase [45]. Though these inhibitors may be toxic or nondiscriminating in their current form, they could be a starting point to screen chemical derivatives with improved properties. The criterion that ASS should be an essential activity has not been fully demonstrated. More characterization such as the demonstration that ASS is essential for survival as an amastigote and the description of subsequent steps in its metabolic pathway since the parasite does not have the enzymes to convert argininosuccinate to arginine will be needed; however the increased expression in the amastigote stage, the important biochemical pathway, the existence of specific inhibitors, and the divergence in subcellular localization between the mammalian enzyme and the *Leishmania* enzyme indicate a potential for ASS as a target of therapeutic drugs to treat leishmaniasis.

3. The Ubiquitin Conjugation System as Target for Chemotherapy

Covalent attachment of ubiquitin (Ub) to protein targets has been recognized as an important step in the specific

destruction of proteins in the proteasome [46]. On the other hand, a broad range of physiological processes are regulated by an expanding array of Ub-like modifiers (NEDD, SUMO, Ufm1). The Ub/Ubl modifiers share a structural fold and are probably evolved from prokaryotic sulphurtransferase systems [47]. Ubiquitin, a 76-amino-acid protein, is covalently linked to lysine residues of substrate proteins in a multistep process. Such ubiquitination is common in normal, as well as pathological, cellular processes. The concept that ubiquitination is solely the process that targets proteins for degradation by the proteasome has been rendered over simplistic by the discovery of expanding functions regulated by ubiquitination such as protein trafficking, the assembly of protein signaling complexes, cellular remodeling through autophagy, and the activation or inactivation of enzymes [47]. The attachment of Ub to a substrate requires the consecutive action of three enzymes. The first step involves the activation of Ub by the formation of a thioester bond with the ubiquitin-activating enzyme, E1. In the second step, E1 delivers the activated Ub to the E2 ubiquitin-conjugating enzyme. Finally, E3 ligases catalyse the transfer of Ub from E2 to a lysine residue in the substrate protein. Ubiquitin contains seven acceptor lysines that can be conjugated with ubiquitin, giving rise to ubiquitin chains of different topologies, lengths, and functional consequences [48].

Significant progress has been made not only in understanding the function and important regulatory roles of the Ubl network but also the alterations of ubiquitination in cellular processes pertinent in the development of various human diseases including cancer [49]. This has led to the development of chemical and/or peptide molecules that inhibit components of the ubiquitination system [48], Bortezomib, the proteasome inhibitor, being the well known example [50]. Notably, E3 ligases that confer specificity of conjugation to substrate proteins and the deubiquitinating enzymes also have been extensively investigated as potential drug targets [51, 52]. In comparison, studies on the ubiquitin conjugation system as a source of potential drug targets in parasitic protozoa are very limited [53].

Studies on Ub in trypanosomatid parasites such as *T. brucei* and *T. cruzi* focused on revealing the Ub gene structure, Ub-dependent protein degradation, and its role in differentiation from the trypomastigote into an amastigote [54, 55]. Studies in *Plasmodium* identified deubiquitinating/deNeddylating activities and sumoylation of telomere associated protein PfSir2, a novel substrate protein for SUMO [56, 57]. Recent studies have demonstrated the role of ubiquitination in the degradation of transmembrane surface proteins in trypanosomes, cell cycle regulation by the single SUMO homologue in *T. brucei*, and interactions with several nuclear proteins in the host cell by a protein that possesses a ubiquitin ligase activity secreted by *T. cruzi*. [58, 59]. Further studies elucidating structural mechanisms of UCHL3, a hydrolase with uniquely dual specificities to Ub and NEDD in *Plasmodium*, further emphasize the increasing interest in parasitic Ub conjugation/deconjugation pathways as potential drug targets [60].

Studies in our laboratory with *Leishmania* Ufm1, a mitochondrial associated Ubl, revealed ways in which Ubl conjugation in these human parasites could represent novel protein drug targets [61]. The description of a Ubl (Ufm1), E1 enzyme (Uba5), and E2 enzyme (Ufc1) shows remarkable similarity of the *Leishmania* conjugation system to mammalian systems. This similarity suggests that anticancer drugs, for example, that target the ubiquitin pathway, may provide a starting point for development of effective antiparasitics. Yet, the sequence divergence of the *Leishmania* components from their mammalian homologues and the lack of similarity of Ufm1-conjugated target proteins to mammalian conjugates suggest that drugs can be developed avoiding toxic side effects. The antiparasitic effect of chemical disruption of this pathway is indicated by the reduced survival of intracellular amastigotes in which Ufm-1 function has been disrupted by overexpression of dominant negative mutant forms of Ufm1 or the E1 enzyme, Uba5 [61]. Identification of Ufm1-mediated protein modification pathways in *Leishmania*, with its distinct subset of substrate proteins associated with mitochondrial activities, may provide specific targets for novel drug therapies against this human pathogen.

The diversity of functions regulated by the Ubls in eukaryotic organisms in general and the fact that inhibitors of the ubiquitin-proteasome pathway are either in clinical use or are being studied for their potential as anticancer drugs indicate the importance of this pathway as a drug target. The ubiquitin-dependent proteolysis system (UPS) is increasingly recognized as a viable therapeutic pathway in the treatment of cancer after the successful treatment of hematological malignancies with proteasome inhibitors [62]. Deubiquitinases, the key effectors of UPS and intracellular signaling cascades, and Ub ligases because of their narrow substrate specificity are emerging as important targets for potential anticancer therapies. This effectiveness at stopping uncontrolled cancer cell growth suggests that targeting the ubiquitin pathways in human parasitic organisms may be successful as well. Importantly, the finding that protozoan parasites such as *Leishmania* interfere with the host protein degradation system to promote their intracellular survival [63] supports the concept that chemotherapy to reverse this interference could help clear the infection. Therefore, systematic studies of Ubl pathways in the human trypanosomatid parasites such as *Leishmania* could yield better understanding of the pathogenesis and lead to novel therapeutic reagents.

4. The Programmed Cell Death Pathway Presents Many Potential Targets for Antileishmanial Drug Therapy

Programmed cell death, commonly manifested as apoptosis, plays crucial roles in a multitude of physiological processes starting from embryogenesis to maintenance of the immune system. Evolutionarily, apoptosis emerged along with multicellular organisms, primarily as a defense against viral infections. However, increasing experimental evidence is showing that mechanistically similar processes also appear in many single-celled organisms including trypanosomatid parasites.

In trypanosomatids, features suggesting apoptosis have been reported in response to a wide range of stimuli such as heat shock, reactive oxygen species, antiparasitic drugs, prostaglandins, and antimicrobial peptides. Many biochemical events that accompany mammalian apoptosis such as generation of reactive oxygen species, increase in cytosolic Ca^{2+} levels, alterations in mitochondrial outer membrane potential, exposure of phosphatidylserine in the outer leaflet of the plasma membrane, release of cytochrome c and nucleases that cleave genomic DNA have also been widely documented in trypanosomatid parasites [64, 65].

In comparison to *C. elegans* and yeast, studies elucidating molecular mechanisms of PCD in trypanosomatid parasites are limited primarily because of the apparent absence of homologues to key regulatory or effector molecules of apoptosis in the trypanosomatid genomes that have been described in mammalian or nematode apoptosis such as Bcl-2 family members and caspases [66]. However, progress is being made with regard to systematic identification and characterization of proteases and/or nucleases with pro-apoptotic activities in these organisms [67]. We provided evidence that metacaspases (protease belonging to the caspase family) could be involved in *Leishmania* PCD [67]. Metacaspases have also been shown to be associated with cell cycle progression in *Leishmania* [68] and associated with RAB11-positive endosomes in *Trypanosoma brucei* [69] indicating additional roles not related to the cell death pathway. Several mammalian cell death regulators have additional functions in healthy cells and are not simply "latent" death factors waiting to kill cells [70]. A series of metacaspase inhibitors have been evaluated as potential antiparasitic drugs [71]. Recently, we and others have shown the involvement of mitochondrial nuclease endonuclease G in trypanosomatid PCD [72, 73]. The absence of homologues of regulatory or effector molecules of mammalian apoptosis indicates that the apoptotic pathways in these parasitic organisms are probably more austere/less complicated than in mammalian cells.

Although the impact of PCD pathways in regulating host-pathogen interaction in terms of parasite cell densities on the one hand and modulating host immune responses that favor the parasite on the other continues to be unraveled, the existence of conserved apoptotic cell death pathways in trypanosomatid parasites can provide targets for identifying novel chemotherapies [74]. Recent pharmacological studies elicited interest in several molecules with activities that trigger apoptotic death in cancerous cells as potential antiparasitic agents [75]. This is partly because of the common biochemical pathways used by the cancer cells and the parasites such as protein kinase pathways, DNA, and polyamine metabolism and also immune evasion strategies that underlie successful survival in the host.

Apoptotic death was observed in *Leishmania* treated with known antileishmanial drugs such as antimonial compounds [76] and antifungal compounds [64]. Antivirals, such as HIV-1 protease inhibitor Nelfinavir, induced oxidant stress-mediated apoptosis in *Leishmania* [77]. Cysteine cathepsin inhibitors have been shown to induce cell death in *Leishmania* [78]. Importantly, recent studies that characterized the action of novel drugs in *Leishmania* indicated that these drugs interfere and/or impair mitochondrial activities including an imbalance of antioxidant homeostasis [79–81]. There is indication that plant products such as yangambin and diospyrin induce apoptosis like death in *Leishmania* [82, 83]. Tafenoquines, an antimalarial compound, also induces apoptotic cell death in *Leishmania* by inhibiting mitochondrial cytochrome c reductase [84]. Fungal peptides with antitumoral activities kill *Leishmania* through apoptosis-like processes [85] involving depletion of ATP pools indicating impaired mitochondrial functions. Interestingly, overexpression of ascorbate peroxidase, a mitochondrial enzyme that scavenges reactive oxygen species in *Leishmania*, resulted in reduced cell death induced either by chemical agents or by reduced ATP generation [86].

Systematic characterization of programmed cell death pathways in trypanosomatid parasites could lead to identification of novel drug targets as it is evident that the human parasites utilize these pathways in unique ways for promoting infection [87]. In addition, such studies will be useful in defining the mechanism of action of novel drugs that induce apoptosis in these parasites. Several studies referenced above have shown apoptosis-like death in the parasites when treated with pharmacological compounds even though at present molecular mechanisms regulating such apoptotic death in trypanosomatid parasites are far from complete.

5. *Leishmania* Endoplasmic Reticulum Quality Control Molecules Involved in Secretion of Virulence Factors as Potential Targets for Novel Antileishmanial Drugs

Leishmania secrete a significant number of proteins into their environment that traffic through the secretory pathway (e.g., secretory acid phosphatase, chitinase, or thiol-specific antioxidant) [88–91]. Some of these secreted molecules have been shown to be important virulence factors involved in *Leishmania* pathogenesis. Although poorly studied, it is believed that secreted proteins traffic in *Leishmania* via a typical eukaryotic secretion pathway in which proteins are first folded in the ER and then transported via a Golgi apparatus to the flagellar reservoir for secretion outside the cell [92]. Therefore, the processing of putative virulence factors in the ER and their proper transport via the Golgi is essential for the survival of *Leishmania* parasites in their hosts.

A number of homologues of proteins involved in the quality control of glycoprotein folding of higher eukaryotes have been described in trypanosomatid parasites. These include calreticulin (CR), BiP, and protein disulfide isomerase (PDI) [93–95]. Our studies have focused on the characterization of CR and PDI and their possible involvement in the control of protein secretion in *L. donovani*. *L. donovani* calreticulin (LdCR) possesses the hallmarks of calreticulins, including its presence in the ER and conservation of protein structure suggesting conservation of function as a chaperone molecule [12]. The role as chaperone is indicated when altering the function of calreticulin affected the secretion of secretory acid phosphatases and resulted in

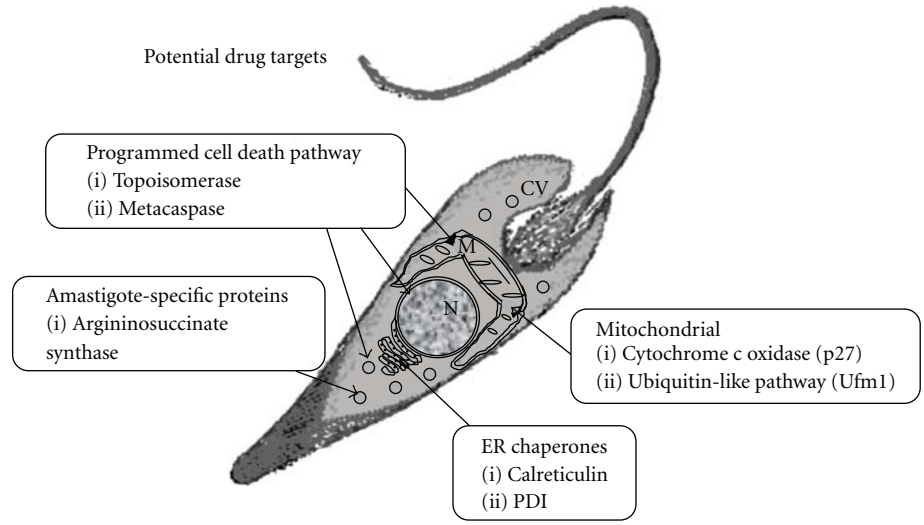

Figure 1: The potential drug targets discussed in this paper are listed, grouped according to the pathways and sites of action. N: nucleus, M: mitochondrion, CV: cytoplasmic vesicle, ER: endoplasmic reticulum, and PDI: protein disulfide isomerase.

significant decrease in survivability of *L. donovani* in human macrophages [12]. In addition, attempts to delete LdCR, a single-copy gene, in *L. donovani* were unsuccessful, only resulting in gene rearrangements [96]. Failure to generate a null mutant in *Leishmania* coupled with the absence of calnexin, a functional homolog of calreticulin, further suggests that LdCR plays an essential function in this organism.

We have also shown that the *L. donovani* PDI (LdPDI) is a 12 kDa protein with a single domain containing the-CGHC-PDI signature [97]. That LdPDI has both oxidase and isomerase activities and is localized in the ER of *Leishmania* strongly suggests its role as an ER quality control enzyme responsible for disulfide bond formation in nascent polypeptides as described in higher eukaryotes [97]. The essential nature of PDIs was reported recently in mammalian cells by knocking down PDI in human breast cancer cells using small interfering RNAs [98]. PDI transcript depletion had a strong cytotoxic effect and triggered apoptosis in these cells.

Evidence that LdPDI could be involved in the control of protein secretion in the ER came from the analysis of mutant *Leishmania* parasites overexpressing mutated versions of this protein. Results showed that the secretion of the *Leishmania* secretory acid phosphatases was significantly reduced [12, 97].

The exact molecular mechanisms involved in altered trafficking and secretion of SAcP proteins in the two *Leishmania* mutants remain unclear. The proposed hypothesis for this effect is that the expression of either mutated/inactive chaperone has a dominant negative effect on the interaction of nascent glycoproteins with the native LdCR and LdPDI and with other folding molecules in the ER.

As a drug target, disruption of LdCR or LdPDI function using a small molecule inhibition approach could result in a similar disruption of secretion. In that regard, a complete inhibition of parasite growth was observed when *Leishmania major* was incubated in vitro with 2 mM zinc bacitracin, a known PDI inhibitor, and disease progression was attenuated when zinc bacitracin was locally applied as an ointment on the parasite inoculation site in BALB/c mice [99].

The findings that disruption of CR and PDI alter the function of the secretory pathway, *Leishmania* parasites with disrupted CR showed reduced survival in macrophages, and the antiparasitic activity of a PDI inhibitor suggest that this pathway is well worth further exploration as a source of drug targets.

6. Conclusion

The crucial need to develop new affordable drugs to cure leishmaniasis that can be delivered in a way that assures patient compliance and avoids rapid evolution of resistance on the part of this disfiguring and deadly parasite demands a multifaceted approach. Research to identify and characterize genes and the proteins they encode that are only known by untested homology or merely as hypothetical takes its place among others. High-throughput screening of off-the-shelf drugs and combinatorial libraries, repurposing of drugs with mechanisms that could suggest antiparasitic activity such as anticancer drugs and *in silico* approaches taking advantage of the annotated databases are all effective strategies in this multifaceted approach. In this paper, we have highlighted the important role that can be played by systematic molecular and cell biological studies of previously unknown genes and the proteins they encode to identify new drug targets and lay the bases for rational drug design (Figure 1).

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Review Article

Role of cAMP Signaling in the Survival and Infectivity of the Protozoan Parasite, *Leishmania donovani*

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Leishmania donovani, while invading macrophages, encounters striking shift in temperature and pH (from 22°C and pH 7.2 to 37°C and pH 5.5), which act as the key environmental trigger for differentiation, and increases cAMP level and cAMP-mediated responses. For comprehensive understanding of cAMP signaling, we studied the enzymes related to cAMP metabolism. A stage-specific and developmentally regulated isoform of receptor adenylate cyclase (LdRACA) showed to regulate differentiation-coupled induction of cAMP. The soluble acidocalcisomal pyrophosphatase, Ldvsp1, was the major isoform regulating cAMP level in association with LdRACA. A differentially expressed soluble cytosolic cAMP phosphodiesterase (LdPDEA) might be related to infection establishment by shifting trypanothione pool utilization bias toward antioxidant defense. We identified and cloned a functional cAMP-binding effector molecule from *L. donovani* (a regulatory subunit of cAMP-dependent protein kinase, LdPKAR) that may modulate metacyclogenesis through induction of autophagy. This study reveals the significance of cAMP signaling in parasite survival and infectivity.

1. Introduction

Infection by protozoan parasites of the genus *Leishmania* results in a spectrum of clinical manifestations referred to collectively as leishmaniases. The clinical manifestations range in severity from spontaneously healing cutaneous ulcers by *L. major* infection to potentially fatal visceral disease by *L. donovani* infection. The parasite is a digenic one and in its infective cycle, the parasite is transmitted as promastigote from the gut of insect vector female phlebotomine flies to mammalian hosts. The procyclic promastigotes get converted to metacyclic ones and are phagocytosed by mammalian macrophages where they convert into amastigote form, which is able to survive, and replicate within the phagolysosome. Along with a substantial alteration of nutrient availability, the parasite must adapt to new conditions of temperature and pH (37°C and pH 5.5) which acts as an initial environmental stress to the parasite. This physical conditioning has proved indispensable for *Leishmania* differentiation and *in vitro* transformation protocols are already in use mimicking the physical condition encountered in mammalian host [1, 2]. After their phagocytosis by macrophages at the initial stages of infection the parasites suffer another stress caused by the respiratory burst of macrophages, its first line of defense, producing reactive oxygen and reactive nitrogen species [3, 4]. Macrophages also produce different cytokines and chemokines that regulate their activity as well as regulate the recruitment and activation of other inflammatory cells. Cell-mediated immunity, which depends on the differentiation of Th0 cells to Th1 cells is also regulated by macrophage functions. IL-12 and IFN-γ secreted by activated macrophages play important role in differentiation of naïve T-helper cells into proinflammatory Th1 subset. However, there are some intracellular parasites like *Leishmania* that are able to impair these activities by taking advantage of the host anti-inflammatory response to avoid self-damage by

modulating its own biology and host environment to persist successfully inside the host. Even in the face of exposure to toxic prooxidants a subset of the *Leishmania* parasites that invades the host macrophages survives and subsequently converts into intracellular amastigotes, finally leading to disease manifestation [5]. But the molecular mechanism by which the parasite circumvents the toxic effects of these reactive oxygen and nitrogen species is yet to be deciphered.

Few previous studies suggested that in *Leishmania,* genes like superoxide dismutase, peroxidoxin, and trypanothione reductase are implicated in antioxidant defense against reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) [6–8]. Disruption of these genes or transfection with transdominant inactive counterpart renders parasites more susceptible to intracellular killing in macrophages capable of generating reactive oxygen intermediates [6–10]. But, in *Leishmania*, in the absence of any known transcription factors, it is really unknown what triggers the expressions of the genes speculated to be associated with its antioxidant system. Interestingly, like in many lower organisms, environmental cues seemed to play some important roles in controlling biology of the parasite. Preexposure to environmental stress (pH 5.5 and temperature 37°C) has been shown to induce resistance against oxidative damage in this organism [3, 11]. The ability of *Leishmania* parasites to resist oxidative damage was observed to be coupled with their transformation to amastigote stage and there may be more than one mechanism of environmental sensing along with stress exposure, which finally trigger differentiation of the parasite. cAMP response has been implicated as one of the major environmental sensing machineries associated with stress response in many unicellular eukaryotes like *Plasmodium*, *Trypanosoma,* and others. cAMP in malarial parasite, *Plasmodium falciparum* triggers the conversion of asexual erythrocytic ring-stage parasites to sexual precursors, gametocytes [12]. *P. falciparum* could synthesize its own cAMP by adenylate cyclase (AC) which is uniquely not stimulated by mammalian AC activator Forskolin or heteromeric G-protein activators AIF_4. Moreover, cAMP signaling effector molecule Protein Kinase A (PKA) plays an important role in conductance of anions across the host cell membrane of *Plasmodium*-infected RBC [13]. It is now known that PKAR (PKA regulatory subunit) may be involved in activation of anion conductance channel in *P. falciparum*-infected RBC [14]. Activation of PKC or cAMP-dependent signaling pathways in *Entamoeba histolytica* triggers the phosphorylation of proteins involved in actin rear-arrangements necessary for adhesion and locomotion. Moreover, cAMP-response elements could play an important role in regulating actin expression and organization in signaling processes activated during tissue invasion. cAMP also plays an important role in Trypanosome differentiation from long slender form to short stumpy form, the form in which the cAMP level declines [15]. Moreover, adenylate cyclase activity is stimulated by Ca^{2+}, which seemed to have a receptor located in the membrane or as a part of AC. Also in *Plasmodium*, evidences identified intracellular Ca^{2+} store utilized by both melatonin and cAMP pathways. Also another component of cAMP signaling, the phosphodiesterases (PDEs) has transmembrane domains suggesting that they are integral membrane proteins. Hence, it was indeed necessary to study whether cAMP has similar importance in the parasite survival and infectivity. The Ras-cAMP pathway serves as a negative regulator of stress response in *Saccharomyces cerevisae* [16, 17].

This review will focus on developments in the field of *Leishmania* cAMP signaling and its control. We have tried to assess the functions of all the enzymes that are intimately associated with cAMP metabolism in the parasite (Figure 1). The multitudinous functions of cAMP require precise spatial and temporal control of its production, degradation, and detection. Though novel proteins have recently been identified that critically modulate cAMP signal in several organisms, not much is known about cAMP signaling in *Leishmania* (Figure 1). In this review, we sought to focus on the molecular mechanisms whereby *Leishmania* parasites can subvert host surveillance by activating its own antioxidant machineries by cAMP-mediated signaling. We would also like to shed some light on the mechanism of action of the leishmanial adenylyl cyclases towards the positive modulation of cAMP in the absence of canonical heteromeric G proteins and genes for G-protein-coupled receptors [18]. We focus on the action of cAMP on *Leishmania* lifecycle that helps its survival inside macrophages and sought to discuss the role of cAMP-dependent phosphodiesterases in modulating the cAMP signaling in the parasite.

2. Role of cAMP in *Leishmania* Survival and Infectivity

Leishmania thrives inside the gut of sand fly at a temperature of 22°C and pH 7.4 and encounters a huge shift in temperature and pH to 37°C and pH 5.5 when it invades mammalian macrophages where a subset of parasite survives the oxidative stress of the macrophages to get converted into amastigotes. The initial environmental stress in the macrophage environment induces differentiation of the parasites from promastigotes to amastigotes and it was deciphered by us that the differentiation condition (37°C and pH 5.5) increases the resistivity of the parasites against induced oxidative stress by H_2O_2 and peroxynitrite [19]. Moreover, it was also shown that such parasites could infect IFN-γ-activated macrophages with more efficiency than the parasites not exposed to differentiation condition [19]. Since differentiation condition is nothing but an environmental cue for the parasite, it was exigent to look for a molecule which could sense such cue to trigger a signaling cascade leading to parasite infectivity and survival within the macrophages. As cyclic nucleotides were known to be important modulators of environmental conditions and speculations were there regarding its role in kinetoplastidae differentiation, cAMP level in the parasite was checked after exposing them to differentiation condition. Results indicated striking elevation of cAMP level in such parasites within 1 hourour of stress exposure. Not only that, cAMP-dependent protein kinase activity (PKA) also increased simultaneously and the substrate level phosphorylation of the same was also elevated [19]. This indicated that cAMP might have an interesting role to play in leishmanial survival and infectivity.

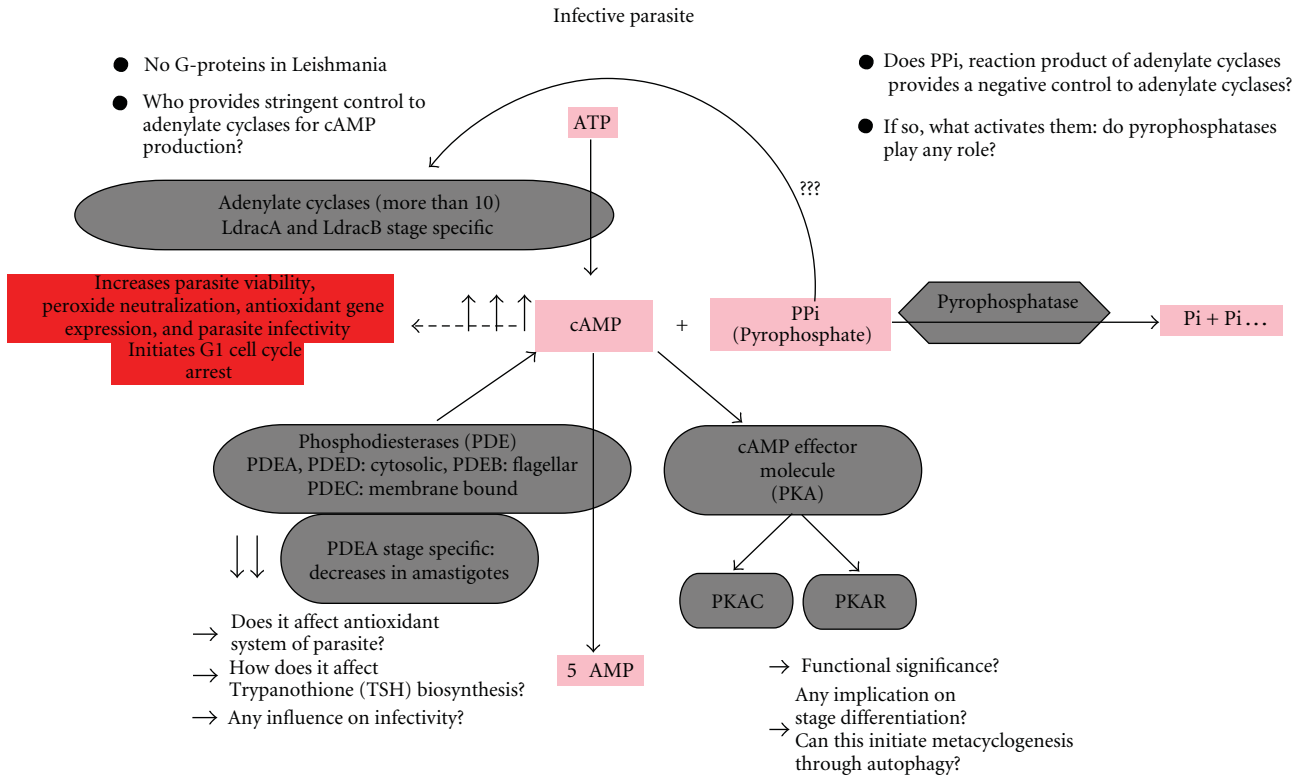

Figure 1: Enzymes intimately associated with cAMP metabolism in *Leishmania*. Cyclic adenosine monophosphate (cAMP) is formed from adenosine triphosphate (ATP) by adenylate cyclases where pyrophosphate (PPi) is also produced as one of reaction products which is hydrolyzed by pyrophosphatases to inorganic phosphate (Pi). Downstream to cAMP, leishmanial phosphodiesterases (PDE) hydrolyzes cAMP to 5′ adenosine monophosphate (5′AMP). There are 5 different PDEs in the parasite (PDEA, PDEB1, PDEB2, PDEC, and PDED). cAMP-dependent protein kinase A (PKA) exists as an inactive tetramer consisting of two catalytic subunits (PKAC) and two regulatory subunits (PKAR). Binding of cAMP to regulatory subunit releases catalytic subunit.

2.1. cAMP Is an Environmental Sensor and Cytoprotector in Leishmania. Since cAMP plays a pivotal role in the differentiation, cell movement, and stress response in several organisms like *Dictyostellium* and *Trypanosoma*, more understanding was required regarding the role of cAMP in parasite survival in macrophage's hostile environment. Increasing intracellular cAMP level by cell permeable cAMP analog, pCPT-cAMP resulted in increased resistance against H_2O_2 and peroxynitrite. On the other hand, enhanced resistance by exposure to differentiation condition could be reversed by adenylate cyclase inhibitor, DDA (dideoxyadenosine), and PKA inhibitor, H89. To further ascertain the ability of cAMP in cytoprotection of the parasites against H_2O_2, three parameters were checked: DNA degradation, protein carbonylation, and ultrastructural analysis. The extent of DNA degradation and protein carbonylation by H_2O_2 was reduced in pCPT-cAMP-treated and differentiation condition-exposed cells, which got reversed by treatment with DDA, and H89 [19]. Similarly, ultrastructural integrity was retained more in pCPT-CMP-treated cells than in normal macrophages. These observations suggest that differentiation condition triggers cAMP response, which enhances resistance against oxidants.

2.2. Role of cAMP in Cell Cycle Blockage. Morphological transformation of promastigotes to amastigotes by exposure to 37°C and pH 5.5 occurs during cell cycle arrest at G1 phase [20]. As resistance against oxidative damage and transformation are coupled and because cell cycle arrest initiates differentiation, we studied the effects of cAMP modulation on cell cycle of *Leishmania*. Cell cycle was studied after intracellular cAMP concentration was modulated by treating the cells with pCPTcAMP, DDA and H89. pCPTcAMP caused a significant G1 phase arrest whereas treatment with DDA, and H89 decreased such arrested condition [19]. This data indicated the involvement of cAMP in G1 arrest of the parasite during transformation. But why such G1 arrest was required to drive the transformation in the parasite could not be answered. Later, we tried to address this question while probing the downstream signaling of cAMP by phosphodiesterases (PDEs).

2.3. cAMP: A Major Upregulator of Antioxidant Genes of the Parasite. Normally, cytoprotection in eukaryotes depends on a number of molecular machineries, the most important of which are antioxidant enzymes. *Leishmania* has unique antioxidant machinery devoid of catalase and glutathione

peroxidase. In most eukaryotic systems four enzymes have been implicated in antioxidant defense, namely, catalase, glutathione peroxidase, superoxide dismutase (SOD), and peroxidoxins (PXN). In *Leishmania*, instead of glutathione, trypanothione, a unique redox cycling glutathione-spermidine conjugate, is present, which in concert with trypanothione reductase (TR) maintains the intracellular reducing environment and resistance to reactive oxygen species (ROS). From genome analysis we know that at least 2 Sods and 3 different Pxns are present in *Leishmania,* of which Pxn1 is found to be differentially expressed and active against both ROS and reactive nitrogen intermediates (RNIs). A direct correlation between these antioxidant gene expression and intracellular cAMP response could be suggested from observations at both mRNA and protein levels of the genes, namely LdPxn1, LdSodA, and LdTr. They were all elevated by positive modulation of cAMP as well as on exposure to differential condition. Such upregulation of antioxidant genes of the parasite appears to be essential for induction of stress-resistance response of the parasite [19].

2.4. The Regulation of Leishmanial Adenylate Cyclases towards Positive Modulation of cAMP. Only very few publications have addressed adenylate cyclases in *Leishmania* for the last 20 years. Reports suggested that there are more than 10 adenylyl cyclases in this parasite. This surprisingly high number of different adenylyl cyclases might be related to any other peculiarity of the parasite. Interestingly, there is no report of any G proteins in the parasite, and therefore, possible functions of adenylate cyclases are yet to be deciphered. Previously, two receptor adenylate cyclases from *L. donovani* (LdracA and LdracB) were analyzed which form part of a cluster of five similar genes. They were observed to be developmentally regulated with their expression in promastigote stage and not in amastigote stage [21]. Since cAMP level was observed to be modulated during transformation from promastigote to amastigote stage, experiments were carried with these two isoforms. Interestingly, LdRACA knocked-down cells showed significantly decreased intracellular cAMP levels after exposure to differentiation condition starting from 30 minutes which decreased maximally after 2 hours of stress compared to uninduced set. Stress-unexposed parasites also showed decrease in cAMP levels in tetracycline-induced LdRACA knocked-down cells. LdRACB knocked-down cells showed little decrease in intracellular cAMP levels by tetracycline induction in both normal and 1 hourour stress exposure. This indicates towards the fact that LdRACA might be primarily responsible for modulation of cAMP level during stress (personal communications).

2.5. Receptor Adenylyl Cyclase Control in Leishmania: Probable Role of Pyrophosphate Pool and Pyrophosphatases. Since *Leishmania* lacks G-proteins, it was important to seek what provides a stringent control to the receptor adenylyl cyclases so that the strict local confinement of a cAMP signal, crucial for allowing local effect to occur, could be maintained. Many lower organisms were observed to have the total inorganic pyrophosphate pool (PPi) and polyphosphate pool (polyP) as environmental sensors. In *Leishmania*, control of adenylyl cyclases might be brought about by a further peculiarity of the parasite, that is, their high concentration of cytoplasmic PPi. This high concentration might effectively block cAMP synthesis via product inhibition of the adenylate cyclase reaction, the products being cAMP and PPi. Experiments were, therefore, designed to observe whether such PPi pool generates a negative feedback to receptor adenylyl cyclases towards formation of cAMP. The total PPi pool in log phase promastigote was found to be quite high (milimolar range), but it was interesting to note that differentiation condition exposure decreased the total PPi pool significantly by 1 hour. Modulation of PPi level might be largely due to the hydrolyzing enzyme, pyrophosphatase. *Leishmania* genome showed the existence of 3 different pyrophosphatases, namely, putative vacuolar type proton translocating pyrophosphatase ($V\text{-}H^+$ ppase), soluble acidocalcisomal pyrophosphatase (LdVSP1), and putative inorganic pyrophosphatase (Ioppase). $V\text{-}H^+$ ppase is known to be associated with acidocalcisomal membrane whereas vsp1 is soluble acidocalcisomal form and ioppase is of cytosolic localization. Expressions of all these pyrophosphatases were observed in both cytoplasmic and membrane fractions of *L. donovani* promastigotes by Western blot with antibodies raised against each of them by the administration of custom peptide in rabbit. $V\text{-}H^+$ ppase was found to be predominantly present in membrane fraction of both normal and stress- (37°C and pH 5.5) exposed parasites. Its expression was not altered by the duration of stress exposure. Ldvsp1, on the other hand, was found to be present mainly in the cytoplasmic fraction of normal promastigotes. However, upon stress exposure, its expression was gradually enhanced in the membrane fraction with a maximum expression at 2 h after stress exposure with a plateau after 4 h. Further exposure did not alter LdVSP1 expression level (personal communications). The putatively cytosolic inorganic pyrophosphatase (ioppase) was mainly detected in the cytoplasmic fraction with its expression unaltered after stress exposure. Expression pattern of LdVSP1 could provide some clue of its control on PPi pool.

2.5.1. Soluble Acidocalcisomal Pyrophosphatase: The Controller of Pyrophosphate Level in the Parasite during Stress. Relative expression and localization of the different pyrophosphatase proteins were then assessed in *L. donovani* promastigotes after stress exposure for various time periods by indirect immune fluorescence using antibodies raised against respective pyrophosphatases. As evidenced from immunofluoroscence, inorganic pyrophosphatase (ioppase) was cytosolic in both normal and stress-exposed cells. Neither did it colocalize with the acidocalcisome marker, nor it was found to be associated with any kind of membrane structure after stress exposure. The acidocalcisomal enzyme was found to be diminished significantly with time. LdVSP1 is primarily localized in vesicle-like structures of various sizes in normal unexposed promastigotes. Such cellular organization is typical of acidocalcisome distribution [22] and LdVSP1 seemed to be associated with parasite acidocalcisome in normal circumstances. Ldvsp1 was found to

be colocalized with acidocalcisome marker (DND lysotraker green) in normal promastigotes, but after stress exposure for various time periods, the relative expression of LdVSP1 increased significantly. Moreover, after stress exposure, most of the LdVSP1 were localized near membrane structures. Acidocalcisomes was observed to move near membrane vicinity after 30 min of stress exposure and they could not be traced by its marker after 1 hour of stress. On the other hand, the V-H^+ ppase was found to be colocalized with acidocalcisomal marker in both normal and stressed conditions and V-H^+ ppase being a membrane bound acidocalcisomal pyrophosphatase could not be visualized after 1 hour of stress. These observations point towards the fact that the soluble acidocalcisomal LdVSP1 moves towards membrane vicinity by change in acidocalcisome biogenesis and function during stress (personal communications).

2.5.2. Possible Role of Pyrophosphate in Leishmanial Adenylyl Cyclase Function. During stress, PPi pool was observed to get modulated, and interestingly, Ldvsp1 was found to be located near membrane vicinity after stress exposure, where leishmanial adenylyl cyclases (LdRACA and LdRACB) reside. To fully understand the regulation of leishmanial receptor adenylyl cyclases, experiment was carried to observe whether leishmanial adenylyl cyclases interact with LdVSP1 during stress. Observation indicated that LdVSP1 could interact with LdRACA but not with LdRACB during stress exposure (personal communications). This indicated that one part of cAMP regulation might have been contributed by pyrophosphatase enzymes along with the total inorganic pyrophosphate pool modulating the function of leishmanial adenylyl cyclases. The high concentration of PPi might effectively block cAMP synthesis via product inhibition of adenylyl cyclase reaction, the products of which are cAMP and PPi. These observations agree well with the previous speculation that LdRAC enzymes might have their catalytic domains stuck in a soup of PPi, being so strongly downregulated that they need activation which our study showed to be the action of soluble acidocalcisomal pyrophosphatase LdVSP1 (personal communication).

2.6. Downregulation of Intracellular cAMP by Cytosolic Phosphodiesterase (PDE). Since it could be speculated that intracellular cAMP pool is regulated by adenylate cyclase (AC) with the help of enzymes like pyrophosphatases, it was exigent to observe another part of cAMP regulation by phosphodiesterases (PDE) that hydrolyze cAMP to 5′-AMP or cGMP to 5′-GMP. Since PDE activity is contributed by several families of PDE of which some might be located in the immediate vicinity of LdRAC, we would discuss the concrete information availed by us studying the leishmanial phosphodiesterases. Depending on the catalytic properties, PDEs are classified into 3 different categories, namely, class I, class II, and class III. 21 genes for PDE have been identified in mammals and several in *Drosophila* and *Dictyostellium*. Though, several class I isoforms have been identified in *T. bruci* and *T. cruzi* and PDE activity was previously reported in *Leishmania*, only very recently 2 PDEs have been cloned from *L. major* [23]. Since there is a large variety of PDEs in this lower eukaryote, some precise regulatory mechanism of intracellular cAMP must be maintained by them during the differentiation of the parasites. Among 4 different leishmanial phosphodiesterases (PDEA, PDEB1 and PDEB2, PDEC and PDED), PDEB and PDEC are predominantly membrane bound whereas PDEA and PDED are cytosolic. These PDEs might be a controlling factor for the differentiation of the parasites as the cytosolic PDE activity decreased during stage differentiation whereas the membrane bound PDE activity remained unaltered [24]. We extensively studied different PDEs of *L. donovani* and inferred that LdPDEA is differentially expressed and decrease of cytosolic activity is due to PDEA downregulation. Kinetic analysis showed detectable reduction of PDE expression 6 hours after exposure to differentiation condition and this was supported by immunofluorescence analysis [24]. We then tried to characterize PDEA by cloning the ORF in PET16b vector and expressed it bacterially. Enzyme kinetics showed a Km of 166.66 μM for cAMP with no activity against cGMP. It was found to be a typical class 1 metal-dependent PDE (Ca^{2+}-calmodulin independent and Mg^{2+}-dependent). The mammalian PDE inhibitors could cause inhibition of this leishmanial PDE at very high concentrations barring 2 inhibitors (dipyridamole and trequinsin) proving it to be somewhat different from the mammalian counterpart [24].

2.6.1. cAMP-Dependent PDEA: A Possible Target for Controlling Anti-Oxidant Machinery of the Parasite. In order to look into the functional significance of LdPDEA we then silenced the gene using tetracycline-inducible knock-down system [24]. When we used inhibitors of PDE, parasites showed enhanced viability against peroxide and peroxynitrite. Further, inhibition by pharmacologic inhibitors or knocking down PDEA caused enhanced peroxide degradation in the parasite. Peroxide neutralization in *Leishmania* is done by peroxidase as it lacks functional catalase. Since glutathione (GSH) is absent in *Leishmania,* peroxide action is mainly based on trypanothione (TSH), a glutathione-spermidine conjugate. Trypanothione is biosynthesized from arginine by arginase, ornithine decarboxylase (ODC), and other enzymes, which convert it to spermidine. It then conjugates with GSH. First we checked the availability of precursors like arginine and ornithine and found that the expression of arginine and ornithine transporter was not affected by PDEA inhibiton. Functional arginine and ornithine transport was also not affected [24]. But when we checked the expression of the enzymes for biosynthesis like arginase and ODC, we found that the expression of both these enzymes was increased under PDEA-inhibited condition suggesting thereby that PDEA inhibition might have caused increased TSH synthesis. But when we analyzed total thiol or intracellular TSH content, there was not much alteration [24]. We, therefore, wanted to check whether utilization of TSH pool was affected by PDEA inhibition. Normally, TSH pool is utilized in the parasite either by ribonucleotide reductase for DNA replication or by peroxidoxin and ascorbate peroxidase for peroxide degradation.

In PDEA-inhibited parasites, expressions of all the enzymes which drive towards peroxide degradation like peroxidoxin and ascorbate peroxidase were elevated. Even the expression of intermediate electron shuttler like tryparedoxin was observed to get upregulated, which points to the fact that downregulation of PDEA may be needed for shifting the bias of TSH pool utilization toward antioxidant defense [24].

2.7. Downstream Effector of cAMP in Leishmania: Protein Kinase A. Though cAMP-dependent protein Kinase (PKA) is well characterized in eukaryote world, very little information is available on this particular downstream effector of cAMP signaling in the parasite. Our observations clearly indicated that temperature and pH stress which is responsible for transformation of promastigote to amastigote can also induce the PKA activity along with increasing cAMP levels. Moreover, substrate level phosphorylation on serine and threonine residues also increased during temperature and pH stress and in the case of positive modulation of cAMP by cell permeable cAMP analogs. PKA exists as inactive R_2C_2 heterotetramer consisting of two catalytic and two cAMP-binding regulatory subunits. Binding of cAMP to the regulatory subunits releases the active catalytic subunits, which are then free to phosphorylate a broad range of substrates. Recently, two PKA catalytic subunits (PKAC) from *Leishmania* have been cloned, characterized, and found to be sensitive to mammalian PKA inhibitors [25, 26] suggesting that PKA exists in *Leishmania* and perhaps plays a regulatory role in the parasite. In spite of the failure of previous attempts to identify PKA holoenzyme from kinetoplastidae parasites [27], functional PKAC-PKAR holoenzyme has recently been identified in *T. cruzi* [28]. To decipher the intricate role of PKA, it was indeed of utmost importance to study not only the catalytic subunit of PKA but also the regulatory counterpart. We for the first time have identified and characterized a functional PKA regulatory subunit (PKAR) from *L. donovani* [29]. Moreover, this report further extends the knowledge of cAMP-mediated responses in *Leishmania* as LdPKAR from *L. donovani* seemed to modulate metacyclogenesis, the process where the promastigotes get converted to infective form through induction of autophagy. Since regulatory subunits of PKA (PKAR) spatially and functionally interact with m-TOR during autophagosome maturation and deletion of PKAR results in activation of m-TOR leading to autophagic deficiency in mammalian cells and tissues [30, 31], our observation suggesting the role of LdPKAR in autophagy was really in line with the previous observations in the mammalian cells.

2.7.1. Cloning and Characterization of a Regulatory Subunit of Protein Kinase A. The LdPKAR gene-encoding sequence was successfully cloned into the bacterial expression vector pET16b and expressed in *E. coli* BL21 (DE3) *pLysS* host. The fusion protein with an N- hexahistidine-tag was purified under nondenaturing conditions using Ni-NTA columns. It was found to be a single copy gene. The ORF of LdPKAR encodes a 502 amino acid polypeptide of molecular weight of 58.5 kDa. Comparison of protein sequences with *Trypanosoma* showed extensive identity with two of them, *T. brucei* (60%) and *T. cruzi* (66.1%), and showed 32.1% homology with bovine PKAR1-α. The N-terminal moiety of LdPKAR1 is longer than that of mammalian or *S. cerevisiae* PKARs and bears no identifiable functional domain. In analogy to other type 1 regulatory subunits, residues 133–137 and 203–207 probably represent the candidate pseudosubstrate sequences required for interaction and inhibition of PKA catalytic domain. Apart from this, residues 244–355 and 364–476 form the cyclic nucleotide binding domains A and B, respectively, which are composed of a number of conserved residues fitting the structural mode of bovine regulatory subunit PKAR1-α [32]. LdPKAR1 was regulated throughout the growth and differentiation cycle of the parasite as it is elevated in late stationary stage significantly compared to log phase promastigotes. Moreover, indirect immunofluorescence analysis in stationary phase promastigotes using polyclonal antibody raised against LdPKAR1 suggested it to be a predominantly cytosolic protein. The functional activation of PKA depends on the extent of dissociation of the catalytic and regulatory subunits. *Leishmania* reportedly encodes two functional PKACs [26], and therefore, each of these PKACs was tested for interaction with LdPKAR. LdPKAR interacted normally with LdPKAC1 and LdPKAC2 although LdPKAC2 interaction seemed to be weaker than LdPKAC1.

2.7.2. Role of LdPKAR on Metacyclogenesis. LdPKAR expression was found to be increased in late stationary phase promastigotes, a condition metabolically similar to metacyclic promastigotes. In line with this observation, we found that the expression of LdPKAR significantly increases under starvation condition, a trigger for metacyclogenesis. Moreover, its overexpression could induce the onset of metacyclogenesis in the cells. Several properties like morphology, agglutination to PNA, increased expression of soluble acidocalcisomal pyrophosphatase (VSP1), sensitivity to human serum, and macrophage infectivity which distinguish metacyclic promastigotes from procyclic ones were all assessed to observe the role of LdPKAR on metacyclogenesis. Observations confirmed a definite role of LdPKAR on metacyclogenesis. Moreover, LdPKAR-over expressing cells were also found to be more efficient in surviving within IFN-γ-activated macrophages compared to wild-type parasites indicating greater infectivity of LdPKAR-over-expressing cells. LdPKAR appeared to have a role in the metacyclogenesis of *L. donovani* [29].

2.7.3. Association of LdPKAR with Induction of Autophagy. PKA activity serves as a regulator of autophagy in a number of mammalian cell lines and such regulation seems to be evolutionarily conserved as autophagy is negatively regulated by Ras/PKA pathway in *S. cerevisiae* [33]. LdPKAR over-expression could also induce autophagy. Monodansyl cadaverine (MDC), an autoflurosescent marker that specifically labels autophagic vacuoles, was found to increase in LdPKAR-over-expressing starved cells. Possibly there was induction of autophagy in over expressed cells

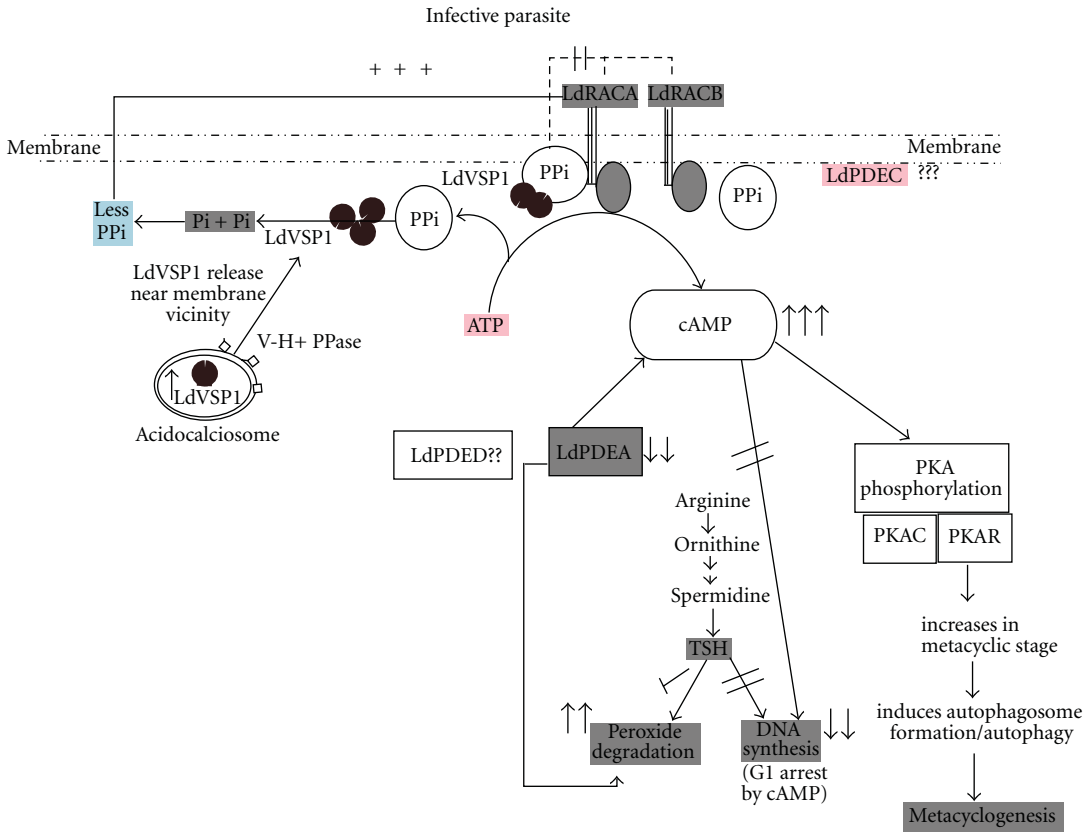

Figure 2: Model for comprehensive cAMP signaling in *Leishmania* parasites. Receptor adenylate cyclase A (LdRACA) and receptor adenylate cyclase B (LdRACB) are G-protein independent membranae bound adenylate cyclases (AC). LdRACA primarily converts ATP to cAMP along with the formation of PPi. This PPi pool provides an inhibition to AC towards the formation of cAMP. During stress, the PPi pool is hydrolyzed by vacuolar acidocalcisomal soluble pyrophosphatase (LdVSP1) which is released by membrane disintegration of acidocalcisomes releasing the inhibition on LdRACA to produce more cAMP. The increased level of cAMP stalls the cell cycle of the parasite at G1 stage and also elevates the expression of antioxidant genes like peroxidoxin, superoxide dismutase and tryparedoxin peroxidase. cAMP also downregulates a stage specific cytosolic PDE, LdPDEA leading to peroxide degradation due to trypanothione (TSH) pool utilization bias towards peroxide degradation instead of DNA synthesis by ribonucleotide reductase which helps in the survival of the parasite in macrophages. Moreover, LdPKAR might have a role in the infective metacyclic stage of the parasite as it induces the formation of autophagosome and process of autophagy initiating metacyclogenesis.

in starvation condition. *L. major* encodes a single copy ATG8 gene (LmjATG8) and fluorescent tracking of ATG8 entails autophagy monitoring in *Leishmania* parasites as efficiently as in mammalian and yeast cells [34]. Percentage of cells with ATG8-positive structures enumerated by using anti-LmjATG8 antibody was significantly higher in LdPKAR-over expressed cells compared to empty vector-bearing cells at 2 hours after starvation. ATG8-positive autophagosome formation in LdPKAR-over expressing cells could be prevented by addition of 3-methyl adenine and wortmanin, inhibitors of autopghagosome formation [35]. Ultrastructural analysis also showed more structures like autophagosomes and autophagolysomes in LdPKAR-over-expressing cells compared to cells bearing empty vectors at 2 h after starvation. These observations suggested that induction of metacyclogenesis by LdPKAR over expression might be due to induction of autophagy in *L. donovani* promastigotes. The significance of these observations with respect to cAMP signaling of the parasite for establishment of its infectivity seemed quite clear but further studies are required to decipher the intricate roles of all the components of leishmanial PKA [33].

3. Synopsis

We first showed that differentiation-coupled induction of resistance of *Leishmania* parasites to macrophage oxidative damage is associated with increased intracellular cAMP and cAMP-mediated response. Parasites having increased cAMP-response elements were more cytoprotective, having higher levels of antioxidant enzymes and having more free radical scavenging capacity. For comprehensive understanding of cAMP signaling, we then studied the cAMP synthesizing enzyme, adenylate cyclase, the degrading enzyme phosphodiesterase (PDE), the regulatory enzyme pyrophosphatase (PPase) and the functional enzyme,

cAMP-dependent protein kinase (PKA). Of 10 different leishmanial receptor adenylate cyclases (LdRACs), two (LdRACA and LdRACB) are stage-specific and developmentally regulated. Silencing and other biochemical parameters showed that differentiation-coupled induction of cAMP is regulated by LdRACA. We are the first to clone and characterize all five isoforms of PDE from *Leishmania* and showed that the soluble cytosolic isoform, PDEA, is heavily downregulated as the parasite is differentiated from promastigotes to amastigotes. Knockingdown the enzyme as well as by using specific inhibitors, we found that PDEA-inhibited parasites have markedly higher peroxide degradative capacity. This increased peroxide degradation is not due to increased trypanothione (TSH) biosynthesis or transport; rather it is due to the shifting of TSH pool utilization bias toward peroxide degradation, that is, antioxidant defense. Since pyrophosphate, one of the reaction product of adenylate cycase, is related with functionality of receptor adenylate cyclase, we studied the enzyme providing stringent control for it, that is, pyrophatases. Of the three different phosphatases present in *Leishmania,* the soluble acidocalcisomal form, LdVSP1 was found to be the major isoform regulating cAMP level and peroxide neutralizing capacity. The study on Ldvsp1 further suggests the significance of its association with LdRACA in regulating the cAMP pool which perhaps triggers the differentiation-associated events that ultimately affect the infectivity of the parasite (Figure 2). We then wanted to determine the downstream effector molecules of cAMP-mediated events. In mammalian cells, there are a number of intracellular effectors of cAMP, most important of which is cAMP-dependent protein kinase (PKA). PKA exists as inactive R_2C_2 heterotetramer consisting of two catalytic and two cAMP-binding regulatory subunits. Binding of cAMP to the regulatory subunits releases the active catalytic subunits, which are then free to phosphorylate a broad range of substrates. PKA catalytic (PKAC) subunits have been cloned and characterized from different *Leishmania* species, but the regulatory subunit has not yet been characterized from any *Leishmania* species. We identified a regulatory subunit of PKA from *L. donovani* (LdPKAR), which is expressed in all life cycle stages. Its expression attained maximum level in stationary phase promastigotes which are biochemically similar to infective metacyclic promastigotes. Starvation condition, the trigger for metacyclogenesis in the parasite, elevates PKAR expression, and under starvation condition, promastigotes overexpressing PKAR attained metacyclic features earlier than normal cells. Furthermore, PKAR overexpression accelerates autophagy, a starvation-induced cytological event necessary for metacyclogenesis and amastigote formation (Figure 2). Conditional silencing of PKAR delays the induction of autophagy in the parasite. The study, for the first time, reports the identification of a functional cAMP-binding effector molecule from *L. donovani* that may modulate important cytological events affecting metacyclogenesis. Since no *bona fide* cAMP-binding protein of defined function has yet been identified in *Leishmania* or in any other kinetoplastidae, the biological significance and molecular mechanism behind cAMP signaling is still an open field to be explored.

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Review Article

Evasion of Host Defence by *Leishmania donovani*: Subversion of Signaling Pathways

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Protozoan parasites of the genus *Leishmania* are responsible for causing a variety of human diseases known as leishmaniasis, which range from self-healing skin lesions to severe infection of visceral organs that are often fatal if left untreated. *Leishmania donovani* (*L. donovani*), the causative agent of visceral leishmaniasis, exemplifys a devious organism that has developed the ability to invade and replicate within host macrophage. In fact, the parasite has evolved strategies to interfere with a broad range of signaling processes in macrophage that includes Protein Kinase C, the JAK2/STAT1 cascade, and the MAP Kinase pathway. This paper focuses on how *L. donovani* modulates these signaling pathways that favour its survival and persistence in host cells.

1. Introduction

Leishmaniasis, caused by more than 20 species of *Leishmania* and transmitted by approximately 30 species of sand fly, is one of the major infectious diseases affecting 12 million people worldwide [1–3]. *Leishmania* are obligate intracellular parasites that infect the hematopoietic cells of the monocyte/macrophage lineage. They exhibit dimorphic life cycle, residing as extracellular flagellate promastigotes in the digestive tract of the sand fly vector and as intracellular flagellate amastigotes within macrophage phagolysosome in their mammalian host [4]. Depending upon the type of species, infection results in a spectrum of clinical manifestations ranging from self-healing skin ulcers to disfiguring mucosal lesions to life-threatening infections of visceral organs (liver and spleen). Among all these forms, visceral leishmaniasis (VL, also known as kala-azar), caused by *Leishmania donovani* complex (i.e., *L. donovani* and *L. infantum* in Old World and *L. chagasi* in New World), is often fatal if left untreated. An estimated annual incidence of 0.5 million new cases of VL is reported to occur in 62 countries [5].

Monocytes and macrophages are considered as sentinels of the immune system. These cells participate in innate immunity and act as the first line of defence in immune response to foreign invaders. They also participate in initiation of the acquired immune response by ingesting foreign particles and presenting them on their surface with major histocompatibility (MHC) complex. In their resting stage, macrophages are relatively quiescent, showing low levels of oxygen consumption, MHC class II gene expression, and cytokine secretion. But once activated, they exhibit maximal secretion of factors like IL-1, IL-6, TNF-α, reactive oxygen species, and nitric oxide produced by inducible nitric oxide synthase (iNOS) [6]. Production of reactive nitrogen and oxygen intermediates (RNIs and ROIs) has made these cells potentially microbicidal [7, 8]. In spite of these, a pathogen like *L. donovani* is able to replicate and survive inside these cells. This suggests that this pathogen has evolved intricate mechanisms to evade or impair macrophage antimicrobial functions [9].

The parasite has been observed to interfere with the host signal transduction in a way that the effector function of macrophage gets impaired, which in turn facilitates parasite survival. Signalling pathways inside the cell are tightly regulated by protein phosphorylation, and levels of cellular protein phosphorylation are controlled by the activities of both protein kinases and protein phosphatases [10, 11].

Therefore, it is not surprising that the parasite interferes with the protein phosphorylation process, impairing kinase-phosphatase balance, and hence distorting macrophage's antimicrobial functions. This paper, therefore, highlights the molecular mechanism by which *L. donovani* modulates macrophage's signalling machinery that promotes its intracellular survival and propagation within the host.

2. MAPK Mediated Pathway

Mitogen-activated protein kinases (MAPKs), a group of serine/threonine-specific protein kinases, constitute one of the important intracellular signalling pathways in eukaryotic cells like macrophages, regulating their accessory and effector functions including production of proinflammatory cytokines and NO [12]. MAPK family includes extracellular signal-related kinases 1 and 2 (ERK1/2), c-jun NH_2-terminal kinase (JNK), and p38 MAPK. Activation of these kinases requires dual phosphorylation of serine/threonine and tyrosine residues, located in a Thr-X-Tyr motif in their regulatory domain [12, 13], by upstream kinases like MAP/ERK Kinase (MEK), which is itself activated by MEK Kinase (MEKK) [7]. Once activated, these kinases phosphorylate a number of selected intracellular proteins including the ubiquitous transcription factors such as activating protein 1 (AP-1), NF-κB and IFN regulatory factors (IRFs), because of which a diverse signalling cascade is triggered that regulates gene expression by transcriptional and posttranscriptional mechanisms [14–16]. A number of studies implicated that *L. donovani* infection of macrophage leads to the alteration of MAP Kinase pathway, which in turn promotes parasite survival and propagation within the host cell. For example, Phorbol 12-myristate 13-acetate-(PMA-) dependent activation of MAP kinase and subsequent expression of c-Fos and elk-1 is impaired in macrophage infected with *L. donovani* [17]. Further, the observation that these effects largely negate when the macrophage is treated with sodium orthovanadate prior to infection [17] suggests that *Leishmania*-induced cellular phosphotyrosine phosphatases are responsible for resulting in such macrophage deactivation. In fact, it was found that the specific activity of Src homology 2 (SH2) domain containing PTP (SHP-1) towards MAP Kinase increases in *L. donovani*-infected macrophage. Consistent to this, there was also reported an increased activity of SHP-1 as well as that of total PTP [18], which apparently supports the finding that SHP-1-deficient macrophage unlike normal macrophage activates JAK2, ERK1/2, and the downstream transcription factors, NF-κB and AP-1 and NO production when treated with IFN-γ even in infected conditions [19]. Recently, Kar et al. demonstrated some different phosphatases that are also induced during *Leishmania* infection and promotes parasitic survival. These specific MAPK-directed phosphatases, MKP1 and PP2A, are shown to inhibit ERK1/2 MAP Kinase resulting in diminished expression of iNOS mRNA [20].

Additional possible mechanism of MAP Kinase inactivation by *Leishmania* could be explained by the elevation of endogenous ceramide in parasitised macrophage [21]. Ceramide is an intracellular lipid mediator, which plays an important role in regulating such diverse responses as cell cycle arrest, apoptosis, and cell senescence [22]. It exerts its cellular functions by means of a delicate regulation of downstream kinases and phosphatases [23]. It was found that intracellular ceramide dephosphorylates ERK by activating tyrosine phosphatase [21]. This impairment of ERK is further shown to attenuate AP-1 and NF-κB transactivation and production of NO in infected macrophage [24] (Figure 1). Moreover, these results are in agreement with previous reports that infection of naïve macrophage with promastigotes of *L. donovani* evades activation of MAPKs leading to impaired proinflammatory cytokines production [25]. However, treatment of macrophage with IFN-γ prior to infection is also shown to induce the phosphorylation of p38 MAPK and ERK1/2 and production of proinflammatory cytokines [25]. Recently, it was identified that priming of macrophage with IFN-γ lead to the expression of Toll-like Receptor 3 (TLR3) which is recognised by the parasite, leading to production of proinflammatory cytokines like tumor necrosis factor alpha (TNF-α) and NO [26]. TLR-mediated regulation of MAP Kinase in macrophage infected with *Leishmania* was also demonstrated by Chandra and Naik. They showed that *L. donovani* infection of both THP-1 cells and human monocytes downregulates Toll-like Receptor 2 (TLR2) and Toll-like Receptor 4 (TLR4), stimulated IL-12p40 production and increases IL-10 production, by suppressing MAPK P38 phosphorylation and activating ERK1/2 MAP kinase phosphorylation through a contact-dependent mechanism [27]. As previous studies have shown that TLR ligation results in phosphorylation of MAPK p38 and ERK1/2 leading to the production of IL-12 and IL-10, respectively [28, 29]. Therefore, it seems that *Leishmania* infection modulates macrophage function by counter regulating p38 and ERK1/2 phosphorylation. In addition, such differential regulation is a direct implication of parasitic infection without much influence of cytokines and is evidenced by the observation that neutralisation of macrophage with anti-IL-10 antibody prior to infection did not abrogate the suppression of IL-12 production [27]. A recent study by Rub et al. [30] also showed that *L. major* infection of macrophage inhibits CD40-induced phosphorylation of the kinases Lyn and p38 resulting in diminished production of IL-12, whereas it upregulates CD40-induced phosphorylation of the Kinases Syk and ERK1/2 and enhances the production of IL-10. Moreover, this has been found to be dependent on the assembly of distinct CD40 signalosomes which is influenced by the level of membrane cholesterol. This represents a unique strategy that the parasite has evolved to survive and needs to be investigated in case of *L. donovani* infection of host macrophage.

The synthetic *Leishmania* molecule LPG is also shown to exhibit differential regulation of MAP kinase pathway in J774 macrophage [31]. By stimulating ERK MAP kinase that inhibits macrophage IL-12 production, LPG has been shown to skew the immune response towards Th 2 type. This suggests that *Leishmania* parasite employs this molecule to evade macrophage effector function [31]. But a subsequent study by Privé and Descoteaux contradicted these results that

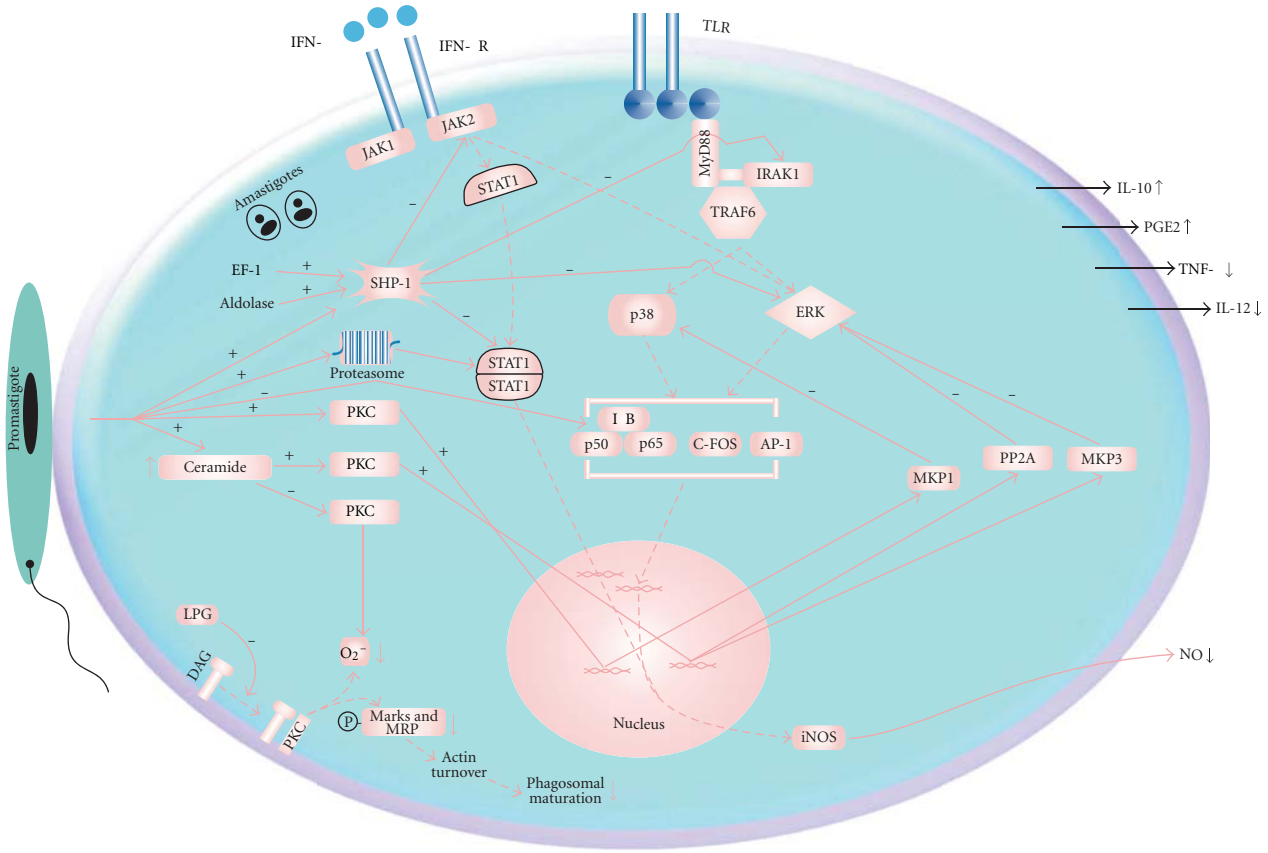

Figure 1: Manipulation of macrophage signaling pathways by *L. donovani*. *L. donovani*-derived molecules like EF-1α and Fructose 1,6 bisphosphate aldolase activate SHP-1 which negatively affects JAK2, STAT1, ERK1/2, and IRAK-1 inhibiting IFN-γ-induced NO production and TLR-mediated production of cytokines like IL-12, TNF-α. Impairment of IFN-γ-dependent pathway also includes reduced level of JAK2 expression and proteasome-mediated degradation of STAT1α. NF-κB-dependent pathway is blocked by impaired degradation of IκB. Other than SHP-1, *L. donovani* also leads to an enhanced expression of MKP3, PP2A, and MKP1 by inducing PKCζ, and PKCε respectively. These dual phosphatases or serine/threonine phsophatases inhibit p38 (MKP1) and ERK1/2 (MKP3/PP2A) resulting in upregulation of IL-10 and downregulation of NO and TNF-α production. PKC-mediated secretion of immunosuppressive molecule PGE2 is also observed. Enhanced level of endogenous ceramide inhibits PKCβ leading to an impaired oxidative burst. PKC-dependent phosphorylation of MARKS and MRP and resulting phagosomal maturation is also inhibited by the parasite. Solid lines: Interaction or positive/negative modulation; dashed lines, interrupted pathway; MyD88: myeloid differentiation primary-response gene 88; IRAK1: IL-1R-associated Kinase 1; TRAF6: TNF receptor-associated factor 6.

LPG instead of stimulating ERK exerts inhibitory effect on ERK activation in murine bone marrow-derived macrophage (BMM) [25]. In addition, the influence of LPG on ERK is specific as infection of naïve macrophage with LPG defective parasite was shown to induce ERK activation while having insignificant effect on p38 and JNK MAP kinase activation [25]. These findings are further supported by a the observation that inhibition of the ERK pathway with PD059089 (ERK inhibitor) increases the parasitic survival in infected macrophage either through increased uptake or decreased killing of the parasite [32]. Nevertheless, recently it has also been explained that the *Leishmania* surface molecule LPG stimulates the simultaneous activation of all three classes of MAP kinases, ERKs, JNK, and the p38 MAP kinase with differential kinetics in J774A.1 macrophage with production of IL-12 and NO [33]. In conclusion, these demonstrations suggest that use of different macrophages in respective studies might have contributed to these contradictory results and therefore needs additional studies to address such disparities in alteration of signal transduction pathways in response to *Leishmania* infection.

Several studies on *Leishmania*-dependent modulation of MAP Kinase pathway implicates that regulation of p38 activation in host macrophage is important in the control of *Leishmania* infection. For example, it was demonstrated that treatment of macrophage with anisomycin, which activates p38, diminishes the survival of the parasite in macrophage [32]. This is in consistency with a current finding that testosterone suppresses *L. donovani*-induced activation of p38 and enhances the persistence of the parasite in macrophage [34]. Furthermore, the observation that the specific MAPK-directed phosphatase, MKP1, induced by *L. donovani* infection downregulated p38 activation and enhanced the survival of the parasite in macrophage again emphasizes the importance of p38 MAP Kinase activation in *Leishmania* infection [20].

3. Protein Kinase C-Dependent Pathway

Protein kinase C (PKC) is a family of calcium and phospholipid-dependent serine/threonine kinases having closely related structures. Based on their intracellular distribution, cofactor requirement, and substrate specificities, these have been grouped into three subfamilies, namely, classical PKCs (cPKC; α, β, γ), novel PKCs (nPKC; δ, ε, η, θ), and atypical PKCs (aPKCs; ζ, ι, λ), [35–37]. While classical PKCs are activated by the intracellular second messengers Ca^{2+} and diacylglycerol (DAG) together with the membrane lipid phosphatidylserine (PS), novel PKCs are activated by diacylglycerol and phosphatidylserine; and atypical PKCs, whose activity is yet not clearly determined, are apparently shown to be stimulated by phosphatidylserine [38]. These kinases reside in the cytosol of the cell in their inactive conformation. Upon activation by stimuli like hormones or phorbol esters, they translocate to cell membrane or to different cell organelles. The mechanism of activation and the localization to subcellular compartments varies among the various isoforms [38]. PMA, a well-known phorbol ester, has been shown to activate [39] and deplete [40] PKC from cells depending upon the time of incubation. *L. donovani* has been shown to evade several macrophage microbicidal activities by altering PKC-mediated signaling pathways.

L. donovani promastigotes, amastigotes, and its major surface molecule LPG have been shown to inhibit PKC-mediated c-fos gene expression in murine macrophage while exhibiting little or no effect on PKA-mediated gene expression [41, 42]. This suggests that the parasite has selectively evolved PKC inhibitory mechanisms, which assist in its survival and propagation within the host macrophage. Interestingly, the observation that LPG-deficient amastigotes are also able to inhibit PKC-mediated c-fos gene expression [41], and PKC activity [43] implicates the role of additional *Leishmania* molecules in blocking PKC-mediated events. Indeed, McNeely et al. demonstrated GIPL to be responsible for PKC inactivation in vitro [44], although its role in intact macrophage still needs to be determined. *L. donovani* infection of human monocytes has also been shown to attenuate PMA-induced oxidative burst activity and protein phosphorylation, by impairing PKC activation [43]. Phosphorylation of both the PKC-specific VRKRTRLLR substrate peptide and MARCKS and endogenous PKC substrate is also shown to be inhibited by LPG treatment of macrophage [45]. Giorgione et al. further demonstrated by an assay using large unilamellar vesicles that LPG inhibits PKC-α catalyzed phosphorylation of histone proteins. This study also showed that inhibition is likely a result of alterations in the physical properties of the membrane [46] and supports a recent finding that uptake and multiplication of parasite increases in PKC-depleted macrophage having diminished membrane microviscosity [47]. The level of MARCKS-related proteins (MRP, MacMARCKS) in macrophage is found to be attenuated by all species and strains of *Leishmania* parasites, including LPG-deficient *Leishmania major* L119 [48]. Thus, this indicates that *Leishmania* parasites, in addition of impairing PKC-dependent protein phosphorylation, have developed a novel mechanism to modulate downstream PKC substrates, which interferes with PKC-mediated signalling pathways. Furthermore, the observation that depletion of PKC renders macrophages more permissive to the proliferation of *L. donovani* again reinforces the fact that inhibition of PKC-dependent events is one of the important strategies that the parasite employs, for promoting its survival within the host cell [45].

One of the studies demonstrated that mere attachment of parasite on macrophage surface leads to the activation of PKC and production of O_2 and NO, whereas internalization of the parasite inhibits these responses [49]. From such observations, it was suggested that *L. donovani* attached to the surface of host cell during initial phase of infection behaves like other organisms that are killed by macrophages. But once they are internalized, triggering of these effector molecules like O_2^- and NO is switched off in part, due to the impairment of PKC-mediated signal transduction pathways.

The finding that the activity of PKC increases after it attaches to the plasma membrane in infected macrophage appears to indicate that translocation of PKC isoforms remains unaffected during *Leishmania* infection [49, 50]. However, the affinity of these isoforms towards their activator DAG is shown to be diminished in infected macrophage correlating reduced generation of oxygen radicals [43]. Furthermore, this reduction in affinity has been suggested to be linked with direct interference of LPG in binding of the regulators like calcium and DAG to PKC [45]. LPG is also shown to inhibit phagosomal maturation, a process requiring depolymerization of periphagosomal F-actin [51]. Holm et al. demonstrated that treatment of macrophage with LPG induces the accumulation of periphagosomal F-actin, which was found to be associated with impaired recruitment of the lysosomal marker LAMP1 and PKCα to the phagosome [52]. Recently, it was demonstrated that PKC-α is involved in F-actin turnover in macrophages and PKC-α-dependent breakdown of periphagosomal F-actin is required for phagosomal maturation [53]. Therefore, there is no doubt that LPG inhibits phagosomal maturation by impairing PKC-α-dependent depolymeristion of F-actin, resulting in enhanced intracellular survival of the parasite in infected macrophage [53]. These findings further corroborate the previous observations that intracellular survival of the parasite was enhanced by 10- to 20-fold in the murine macrophage cell line RAW 264.7 overexpressing a dominant-negative (DN) mutant of PKC-α [54].

Infection of murine cells in vivo and in vitro with *Leishmania* parasite has been shown to induce an increased synthesis of prostaglandin E2 (PGE2) that favours parasite persistence and progression [55, 56]. Recently, it was demonstrated that generation of PGE2 in *L. donovani*-infected U937 human monocytes is, in part, dependent upon PKC-mediated signalling pathway [57]. This shows that *L. donovani*, in addition to downmodulating macrophage functions by affecting important signalling pathways, induces secretion of immunosuppressive molecules (e.g., PGE2) to potentially affect functions of surrounding uninfected cells, which in turn renders macrophage suitable for the survival and establishment of the parasite.

L. donovani infection of macrophage, whereas selectively attenuates both the expression and activity of calcium-dependent PKC-β, is shown to induce the expression and activity of calcium-independent PKC-ζ isoform with diminished production of O_2^- and TNF-α [58]. Attenuation of the expression and activity of calcium-dependent PKC-β has been suggested to be mediated by IL-10 overproduction, as pretreatment of infected macrophage with neutralizing anti-IL-10 restoring the activity of PKC as well as production of O_2^-, NO, and TNF-α [59]. From these findings, it can be thus speculated that *L. donovani* infection induces endogenous secretion of murine IL-10, in order to facilitate its intracellular survival via selective impairment of PKC-mediated signal transduction. One possible mechanism for this differential regulation of both the expression and activity of PKC isotypes by *Leishmania* infection was demonstrated by Ghosh et al. They elaborated that *Leishmania* infection induces elevation of intracellular ceramide in infected macrophage largely due to its denovo synthesis. The enhanced ceramide then downregulates classical calcium-dependent PKC, enhances expression of atypical PKC-ζ isoform, and diminishes MAPK activity and generation of NO [21]. Consistent with this, Dey et al. also reported ceramide-mediated upregulation of atypical PKC-ζ isoform in infected macrophage. However, they further showed that this ceramide-induced atypical PKC-ζ inhibits PKB (Akt) phosphorylation which is dependent upon PKCζ-Akt interaction, as the treatment of the cell with PKC-ζ inhibitor prior to infection showed a significant translocation of Akt from cytoplasm to the membrane [60]. Moreover, *L. donovani* infection of macrophage has been found to induce the expression of MAPK-directed phosphatases such as MKP1, MKP3, and a thronine/serine phosphatase PP2A by stimulating various PKC isoforms. While MKP3 and PP2A, activated by PKC-ζ were further found to be responsible for ERK1/2 dephosphorylation, MKP1 induced by PKC-ε is shown to inhibit p38 phosphorylation, which resulted in diminished production of NO and TNF alpha favouring enhanced survival of the parasite in macrophage [20]. In conclusion, the observation that C-C chemokines restore calcium-dependent PKC activity and inhibit calcium-independent atypical PKC activity in *L. donovani*-infected macrophages under both in vivo and in vitro conditions restricting the parasitic load again supports the fact that impairment of PKC-mediated signaling is a key to the establishment of *Leishmania* parasites in their host cells [61].

4. JAK2/STAT1-Dependent Pathway

IFN-γ is a potent cytokine that induces macrophage activation and helps resisting *Leishmania* infection [62, 63]. It mediates its biological functions via IFN-γ receptor- (IFN-γR-) mediated pathway involving receptor-associated kinases JAK1/JAK2 and STAT-1 [64, 65]. Binding of IFN-γ to its multisubunit receptor triggers its dimerization and allows transphosphorylation of the Jak1 and Jak2. These kinases in turn phosphorylate the cytoplasmic tail of the receptor itself which recruits the cytoplasmic molecule STAT1α. This transcription factor is then phosphorylated, becomes a homodimer, and then translocates to the nucleus to enhance transcription of IFN-γ-induced genes, such as FcgRI [66]. *Leishmania* induced macrophage dysfunction such as defective production of NO [67] and MHC [68] expression in response to IFN-γ may not exclude the possibility that the parasite could have impaired this pathway. In fact, a number of studies implicated *Leishmania*-mediated impairment of JAK2/STAT1 pathway, which correlates with such macrophage deactivation. For instance, one of the studies showed that *L. donovani* infection attenuates IFN-γ-induced tyrosine phosphorylation and selectively impairs IFN-γ-induced Jak1 and Jak2 activation and phosphorylation of Stat1 in both differentiated U-937 cells and human monocytes [69]. A probable mechanism for this was demonstrated by Blanchette et al. that *L. donovani* infection of macrophage rapidly induces host PTP activity simultaneously with dephosphorylation of macrophage protein tyrosyl residues and inhibition of protein tyrosine kinase [18]. They further revealed that upon infection, PTP SHP-1 is also rapidly induced, which interacts strongly with JAK2, and impairs IFN-γ signaling [18]. However, a recent observation that IFN-γ-stimulated STAT1α activity is also reduced in SHP-1-deficient macrophages following *L. donovani* infection indicates that *Leishmania* employs further mechanisms to inhibit STAT1 activity [19]. One possible mechanism could be the proteasome-mediated degradation of STAT1α in infected macrophage, as treatment of macrophage with proteasome inhibitors prior to infection is shown to rescue STAT1α nuclear translocation as well as restore its general protein level in *Leishmania*-infected macrophage [70]. Additionally, *L. donovani* infection of macrophage has been shown to attenuate IFN-γR alpha subunit expression [71] and induce the transient expression of the cytokine signaling 3 (SOCS3) [72], which also shown to negatively regulate IFN-γ signaling. More recently, *L. donovani* amastigote is found to inhibit the expression of IRF-1 while having no effect on STAT1α protein levels. This inhibition of IRF-1 expression correlates with the defective nuclear translocation of STAT1 and further revealed that the IFN-induced STAT1α association with the nuclear transport adaptor importin-5 is compromised in *L. donovani* amastigote-infected macrophage [73]. These results thus provide evidence for a novel mechanism used by *L. donovani* to interfere with IFN-γ-activated macrophage functions.

5. Implication of Phosphatases

Protein phosphatases are key regulatory components in signal transduction pathways [74, 75]. Based on their substrate specificity, these have been divided into two main groups. One of them specifically hydrolyzes serine/threonine phosphoesters (PPs) and the other is phosphotyrosine specific called protein tyrosine phosphatases (PTPs). Apart from these, a subfamily of PTPs also exists that are capable of efficiently hydrolysing both phosphotyrosine and phosphoserine/threonine residues and are therefore known as dual-specificity phosphatases. PTP-regulated protein dephosphorylation is a critical control mechanism for numerous physiological processes such as cell growth, motility, metabolism,

cell cycle regulation, and cytoskeletal integrity [76, 77]. However, for parasites like *Leishmania* these molecules have been proved fruitful in enhancing their survival within host macrophage by inhibiting several intracellular signaling cascades involved in host effector functions.

5.1. SHP-1 Protein Tyrosine Phosphatase. Protein Tyrosine Phosphatases containing Src homology 2 (SH2) domains have been identified in a wide variety of species [74, 75]. One of them is PTP SHP-1, which is also known as PTP1C, HCP, SHPTP1, and SHP [75]. This phosphatase is expressed not only in haematopoietic cells but also in smooth muscle [78] and epithelial cells [79] and is considered as an important negative regulator of numerous signaling pathways, such as those related to the actions of interferons [80, 81] and erythropoietin [82, 83].

Structural analysis of SHP-1 showed that this phosphatase contains two SH2 domains in its N-terminal portion, a phosphatase domain conserved in a central position and a C-terminal tail [84]. The SH2 domains which contain specific amino acid sequences have been found to interact with the target protein through an immunoreceptor tyrosine-based inhibitory motif (ITIMs) within the consensus sequence I/V/LxYxxL/V [85]. These specialized motifs are known to be present in many signaling molecules [86, 87], and multiple types of ITIMs exist and display specific abilities to recruit and activate SH2 containing PTPs. SHP-1 has been shown to bind to receptors and dephosphorylate them directly or associate with a receptor and dephosphorylate other members of the receptor binding complex. Moreover, it also interacts with other cytosolic proteins and was found to dephosphorylate them or their associated proteins [86]. Several studies on *Leishmania* infection have implicated a negative role of these phosphatases.

A study by Olivier et al. for the first time demonstrated a role of protein tyrosine phosphatases in *Leishmania* infection, by using PTP inhibitors such as the peroxovanadium (pv) compound bpv(phen), which restricted the progression of both visceral and cutaneous leishmaniasis in vitro as well as in vivo [88]. Consistent with this, Blanchette et al. showed that *L. donovani* infection of macrophage induces a rapid elevation of total PTP activity and SHP-1 activity, leading to a widespread dephosphorylation of high-molecular-weight proteins [18]. In addition, activated SHP-1 is observed to interact with JAK2 and impair its activation in response to IFN-γ [18]. Accordingly, it was also found that *Leishmania*-induced SHP-1 interacts strongly with MAP kinases and impairs PMA-stimulated ERK1/2 phosphorylation, Elk-1 activation, and c-fos mRNA expression resulting in attenuated expression of iNOS [17]. These results are strongly supported by a recent finding that infection of SHP-1 deficient macrophage with *L. donovani* exhibits normal JAK2 and ERK1/2 activity and increased NO production in response to IFNγ [19]. Taken together, these findings suggest that *L. donovani* exploits host PTP SHP-1 in modulating several key signalling molecules to evade macrophage effector functions.

Studies aimed at understanding the mechanism responsible for the change in activation state of SHP-1 led to the identification of *Leishmania* EF-1α and subsequently fructose-1,6 bisphosphate aldolase, which were shown to bind and activate PTP SHP-1 in vitro and in vivo, in a similar fashion [89, 90]. In both these cases, although the trafficking mechanism of the molecules is not yet clear, it appears that they are exported out of the phagosome into the cytosol, where they activate SHP-1 [89, 90]. These observations lead to the speculation that more than one *Leishmania*-derived molecule is likely to be needed for optimum activation of SHP-1 as these molecules are reported to cooperate in activating this PTP by interacting at different sites on it [90].

SHP-1 is also shown to inhibit a critical kinase (IRAK-1) involved TLR signaling. This has been linked to a rapid binding of SHP-1 with IRAK-1 through an evolutionarily conserved ITIM-like motif identified in the kinase. This motif was also present in other kinases involved in Toll signalling and therefore could represent a regulatory mechanism of relevance to many kinases. This work therefore reports a unique mechanism by which *Leishmania* can avoid harmful TLR signalling [91].

5.2. Other Phosphatases. It is apparent from several studies that SHP-1 plays an important role in pathogenesis during *Leishmania* infection. Nevertheless, the finding that SHP-1-deficient macrophage witnessed an increased PTP activity and inhibition of NF-κB and AP-1 during *L. donovani* infection points to the induction of additional PTPs that could also be involved in disease progression [19]. In fact, Olivier et al. showed that macrophage PTP-1B is rapidly induced upon *Leishmania* infection (Gomez and Olivier, unpublished data), although the underlying mechanism involved in its activation and in its enrolment in macrophage dysfunction during *L. donovani* infection remains undiscovered and needs further investigations. The elevated level of endogenous ceramide, generated during *Leishmania* infection, is shown to activate a vanadate-sensitive tyrosine phosphatase which dephosphorylates ERK1/2 resulting in a diminished production of NO [21]. Similarly, Dey et al. described another phosphatase PP2A, induced during *L. donovani* infection of macrophage, mediated through ceramide. PP2A was found to inhibit PKB (Akt), a kinase involved in respiratory burst activity in infected macrophage, and enhanced survival of the parasite in infected macrophage [60]. *L. donovani* infection of macrophage is also shown to induce a significant upregulation of a serine/threonine phosphatase PP2A and two specific MAPK-directed phosphatases such as MKP1 and MKP3. [20]. While MKP3- and PP2A- mediated dephosphorylation of ERK1/2 resulted in substantial decrease in iNOS expression in infected macrophage, MKP1 is shown to skew cytokine balance towards Th2 response that favoured persistence and propagation of the disease in in vitro as well as in vivo model of *Leishmania* infection [20].

6. Conclusion

Parasitic protozoa like *Leishmania* are a major cause of severe morbidity and mortality in several parts of the world. These pathogens have evolved with the mammalian immune system and typically produce long lasting chronic infections.

They exhibit an efficient survival in host macrophage by manipulating host signaling machinery in its favour. This paper has covered some of these mechanisms which would facilitate further studies in knowing the unidentified strategies that the parasite employs in subverting host immune system. Moreover, given that these signalling pathways could be manipulated pharmacologically, an improved understanding of the host parasite interaction would allow the development of new therapies to control such infectious agents.

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Review Article

A Perspective on the Emergence of Sialic Acids as Potent Determinants Affecting *Leishmania* Biology

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Leishmaniasis caused by *Leishmania sp*. has a wide range of manifestations from cutaneous to the deadly visceral form. They shuttle between the invertebrate and vertebrate hosts as promastigotes and amastigotes having adaptations for subverting host immune responses. Parasite-specific glycoconjugates have served as important determinants influencing parasite recognition, internalization, differentiation, multiplication, and virulence. Despite the steady progress in the field of parasite glycobiology, sialobiology has been a less traversed domain of research in leishmaniasis. The present paper focuses on identification, characterization, and differential distribution of sialoglycotope having the linkage-specific 9-*O*-acetylated sialic acid in promastigotes of different *Leishmania sp*. causing different clinical ramifications emphasizing possible role of these sialoglycotopes in infectivity, virulence, nitric oxide resistance, and host modulation in *Leishmania spp*. asserting them to be important molecules influencing parasite biology.

1. Introduction

Human beings are often infested by parasites that manipulate the milieu of the host causing several pathological manifestations. Today one of the biggest risks to man are infectious/parasitic diseases. Such parasites include obligate intracellular kinetoplastids, of which *Leishmania* comprising 21 diverse species causes a range of disease manifestations from cutaneous leishmaniasis (CL) to lethal (if untreated) visceral leishmaniasis (VL; also known as kala-azar). Less recurrent forms of the disease include mucosal leishmaniasis, diffuse CL with nodular lesions and post-kala-azar dermal (PKDL) occurring as a followup to VL. *L. infantum* or *L. donovani* causes VL, characterized by systemic infection of the reticuloendothelial system. The cutaneous form is caused by *L. major, L. tropica,* and *L. aethiopica* in the Old World; *L. mexicana, L. braziliensis,* and *L. amazonensis* in the New World [1–4]. The present scenario of leishmaniasis is severe, being endemic in 88 countries affecting approximately 12 million people worldwide. The disease has an incidence of 1.5 to 2 million new cases every year. VL has an estimated global incidence of 200,000–500,000, and CL shows a prevalence of more than 5 million cases worldwide (http://apps.who.int/tdr/). The situation is all the more alarming in India and Sudan where recent epidemics have caused 100,000 deaths [5–8]. Considering the disaster to life, knowledge about *Leishmania* biology, disease pathogenesis, and host-parasite relationship encompassing numerous molecular and biochemical interactions is the need of the hour. Already many parasite-surface glycoconjugates and other factors influencing parasite biology with or within the host cell are reported. Could we find many more? In spite of many potential roles of diverse forms of sialic acids in different cellular processes, sialoglycobiology is a less explored area in leishmaniasis [9–17]. Could elucidation of sialoglycobiology save us from this grave situation? Hence, this review has made a modest attempt to assemble the observations on the sialobiology of *Leishmania sp.* highlighting their importance in leishmaniasis.

2. Biology of *Leishmania*: Key to Different Manipulative Strategies

Leishmania is a digenetic parasite completing its life cycle within human beings and female sand-flies (e.g., *Phlebotomus* species in the Old World, *Lutzomyia* species in the New World). The parasite while shuttling between the hosts differentiates into different flagellated (promastigotes) and aflagellated forms (amastigotes). The amastigotes are taken up by female sand flies, within whose midgut they differentiate into motile procyclic forms that transform into the nectomonads [18]. After their escape they attach to the sand-fly gut-epithelium [19]. The successful multiplication, predominance and infectivity of both these stages within the respective host require several adaptive modifications [7]. The mode of attachment may be through one major glycan molecule lipophosphoglycan (LPG), as evidenced in *L. major,* or may be mediated by receptor-ligand interaction [20–25]. The infective metacyclic promastigotes are transmitted into humans. This constant differentiation all throughout the life cycle forces the parasite to acquire special adaptive mechanisms by exploiting different parasite-derived moieties. Thus knowledge of the parasite topology becomes important to understand its course within the host that could probably shed light on the identification of novel targets for diagnosis and therapy.

3. Glycoconjugates of *Leishmania* and Their Significance

Diverse range of glycoconjugates involved in recognition, attachment, internalization, and differentiation in *L. adleri* [26], *L. major* [27], *L. mexicana* [28], *L. tarentolae* [29], and *L. donovani* [30–32] have been documented. Shedding and secretion of many *Leishmania*-derived glycoconjugates like acid phosphatase, proteophosphoglycans and phosphoglycans and their role in virulence and differentiation is known [33–37]. The role of macrophage mannosyl fucosyl receptor in invasion of *L. donovani* promastigotes indicates the presence of these sugars on parasite surface [38]. LPG, glycoprotein 63 (gp63), a zinc metalloprotease increases infectivity of *L. major* and *L. amazonensis* within the host [39, 40]. Additionally, gp63 also alters host signaling causing intracellular survival of parasites by cleaving activated tyrosine phosphatases of the macrophage and controls the parasite burden within dendritic cells [41–44]. LPG plays an important role as a regulator of nitric oxide (NO) and a modulator of host responses that affects intracellular survival [37, 45, 46]. It reduces the activity of protein kinase C and protein tyrosine phosphatase accompanied with decrease in levels of IFN-γ, IL-12, NO, and reactive oxygen intermediates produced by macrophages and increased production of IL-10 and TGF-β by T cells [47]. Additionally, glycoinositolphospholipids also plays a potent role in inhibition of NO in murine macrophages [34]. Hence, these glycoconjugates are considered as important markers of virulence. Differences in the distribution of proteophosphoglycans on promastigotes and amastigotes in pentavalent antimony resistant and sensitive clinical isolates suggest the implications of surface topology in determining the clinical outcome of the disease [48]. Therefore glycoconjugates and their association with *leishmania* have always instigated the scientific community to unravel their importance.

4. Sialic Acids and Parasite

Sialic acids or *N*-acetylneuraminic acid (Neu5Ac or SA) are nine carbon sugar molecules comprising of more than 50 forms based on modifications of the amino and hydroxyl group [11, 16, 17, 49]. The predominantly occurring forms are the *O*-acetylated SA (at C-7/8/9) forming *O*-acetylated sialoglycoconjugates [12–15, 49, 50]. It is usually present at the terminal end of glycoconjugates and affects different cell-mediated physiological processes. The parasites being manipulative often utilize SA for mediating different facets necessary for establishing successful infection, like recognition, adhesion, infectivity and survival. *Trypanosoma cruzi,* causing chagas disease has a cell surface decorated with SA-bearing mucin like structures forming a negatively charged coat which prevents killing of the infective trypomastigotes by human anti-α-galactosyl antibodies [51]. The presence of tran-sialidase in trypanosomes is a landmark discovery in the domain of sialoglycobiology. This unique enzyme cleaves SA from host-derived glycans and incorporates it into mucin-like molecules on their surface without the need of metabolic energy; hence, serve as virulence factors [51, 52]. These sialylated structures also serve as ligands for siglecs (sialic acid binding immunoglobulin-like lectins) naturally present on different hematopoietic cells [53, 54]. A majority of molecules like reticulocyte binding protein homologue used by the merozoites of *Plasmodium falciparum* include terminal SA containing glycans joined to glycophorin on the host cell [55, 56]. Encystation of the infective cyst-stage in *Entamoeba histolytica* involves distinct sialoglycans indicating the association of SA [57]. *Toxoplasma gondii*, the causative agent of toxoplasmosis demonstrated uptake of fetuin (a sialoglycoprotein) by tachyzoites, signifying that sialoglycans are inevitable for the maintenance of their lifecycle [58]. Therefore the integration of SA with parasites has always suggested their probable implications as effective drug targets.

5. Sialic Acids on *L. donovani*

Over the past decade, we have demonstrated the presence of sialic acids and distinct sialoglycoproteins on promastigotes and amastigotes of *L. donovani* using *Sambucus nigra agglutinin* (SNA) and *Maackia amurensis agglutinin* (MAA) having preferential binding specificity towards α2-6 and α2-3 SA, respectively. The predominance of α2-6 linked SA on parasites suggests their stage-specific association indicating their probable implication in parasite biology [59–61]. The complex pattern of binding of *L. donovani* with different recombinant siglecs provides evidence in support of these sialoglycotopes serving as important determinants for recognition. Additionally, we have also demonstrated the presence

of 9-*O*-acetylated sialic acid (9-*O*-AcSA) and distinct 9-*O*-acetylated sialoglycoproteins (9-*O*-AcSGPs) on *L. donovani* using a snail lectin, Achatinin-H [59, 61, 62]. Interestingly, the amastigotes of *L. donovani* exhibit an unusual derivative of SA, *N*-glycolylneuraminic acid (Neu5Gc), absent on promastigotes. Neu5Gc is the evolutionary missing link present in the apes while its absence has been documented in humans due to a mutation in the gene coding CMP-*N*-acetylneuraminic acid hydroxylase for its biosynthesis [63]. However, increased presence of Neu5Gc in various forms of human cancers and its association with certain parasites like *Nippostrongylus brasiliensis* makes it a relevant target for detailed exploration [64, 65]. The occurrence of Neu5Gc on amastigotes and the evidence of a completely new set of sialoglycoprotein bands in Western blot analysis with SNA and MAA using amastigotes membrane fraction suggest that probably these parasites are capable of accommodating new molecule which could influence their biology within the host cells. Thus the intracellular pools within the host cells could serve as the source of these molecules. Since Neu5Gc is absent in healthy humans, the presence of this sugar indicates the interplay of some biochemical modification that occurs during the transformation of promastigotes to amastigotes. However, the exact mechanism remains to be investigated. Elucidation of its functional significance in stage-specific *Leishmania* biology could probably make it a novel target for therapy. Such studies are currently ongoing.

6. Acquisition of Sialic Acids by *Leishmania*

The occurrence of SA at the termini of glycoconjugates on the parasite surface obviously haunts us about the mechanism of their acquisition. SA may either be synthesized *de novo* or may be transferred with the help of transsialidase or could also be scavenged from other external sources. Whatever the mechanism may be, it employs a specialized system that demands detailed exploration and varies in different parasites. The pivotal role in biosynthesis of SA is played by UDP-GlcNAc 2-epimerase which catalyzes the first step, hence its presence or absence provides direct evidence in favor of the sialic acid synthesis machinery. The absence of the activity of UDP-GlcNAc 2-epimerase in *Leishmania* rules out the possible presence of biosynthetic machinery in it. The culture medium containing fetal calf serum (FCS) exhibits a few distinct serum α2-6- and α2-3-linked sialoglycoproteins analogous to those present on the promastigotes as visualized by binding with SNA (130, 123, 90, and 70 kDa) and MAA (123, 90, and 70 kDa) [59, 60]. These observations provide probable evidence in support of the direct transfer of SA containing glycoconjugates by polyanionic adsorption and their incorporation in the glycocalyx comprising LPG. Decrease binding of both the sialic acid binding lectins with promastigotes, grown in reduced serum concentration, corroborates the theory of borrowing of SA from environment to reimburse the deficient status. Speculations suggest that this transfer of sialoglycoconjugates could occur either wholly or partially by the aid of enzymatic fragmentation which would ultimately fit within the complex outer glycocalyx of the parasite. However, the exact mechanism of the transfer remains to be investigated. The incorporation of SA-modified glycoconjugates under different growth conditions may have a probable effect on the surface architecture of the parasite. It may be possible that the incorporated sialoglycoconjugates mask some moieties or modify them leading to newer conformations being displayed on the surface which could potentially serve as more stringent markers of virulence. Alternative possibility could be that changes in SA opsonization reflect alteration in other cell surface molecules that are required for virulence. However, whatever may be the consequences the exact interplay of these molecules on the parasite surface remains to be investigated.

Acquisition of SA in the absence of *de novo* SA biosynthesis enzyme machinery is also achieved by transsialidases that not only cleave SA from host glycoconjugates but also transfers it on itself [52, 60]. Another mode of acquisition could be the transfer of SA from CMP-SA acting as the nucleotide sugar donor by serum or ectosialyltransferases on the parasite surface glycoconjugates. However, the evidence of the occurrence of serum CMP-SA is still lacking, hence the role of ectosialyltransferases is doubtful and requires elaborate study. Under experimental conditions, we could not detect 9-*O*-AcSGPs in FCS, possibly due their minimal presence. 9-*O*-acetylation of SA occurs due to the fine balance of four key enzymes, namely, *O*-acetyl transferases, sialyltransferases, esterases, and sialidase [17]. Although, to date the occurrence of these enzymes is lacking in the *Leishmania,* but one cannot completely rule out the fact. Moreover the presence of inactive enzymes cannot be ruled out and is a domain of elaborate future research. Recently reported genome sequences of *L. major*, *L. braziliensis,* and *L. infantum* show conserved gene sequences [66]. *N*-acetyl transferase producing *N*1-acetylspermidine in *L. amazonensis* indicates the presence of enzymes for acetylation [67]. However, any such claim for the presence of *O*-acetyltransferase requires the identification of the respective genes which at present is lacking. Direct analogies correlating the transfer of sialoglycoproteins from the serum demands elaborate proteomic characterization of surface proteins on promastigotes and are a subject of future research.

7. Differential Distribution of Sialic Acids on Promastigotes of Virulent and Avirulent *Leishmania sp.*

Interestingly, in our study four virulent strains of *L. donovani (*AG83, GE1, NS1, and NS2) and six other virulent strains K27 (*L. tropica*), JISH118 (*L. major*), LV4 (*L. mexicana*), LV81 (*L. amazonensis*), L280 (*L. braziliensis*), MON29 (*L. infantum*) demonstrate a differential distribution of SA [68, 69]. All virulent strains reveal higher presence of total SA content as compared to avirulent UR6 strain ([69–72], Figure 1(a)). The strain UR6 (MHOM/IN/78/UR6) failed to induce visceral infection by intracardiac inoculation and was cultured accordingly. Therefore, UR6 has been considered as

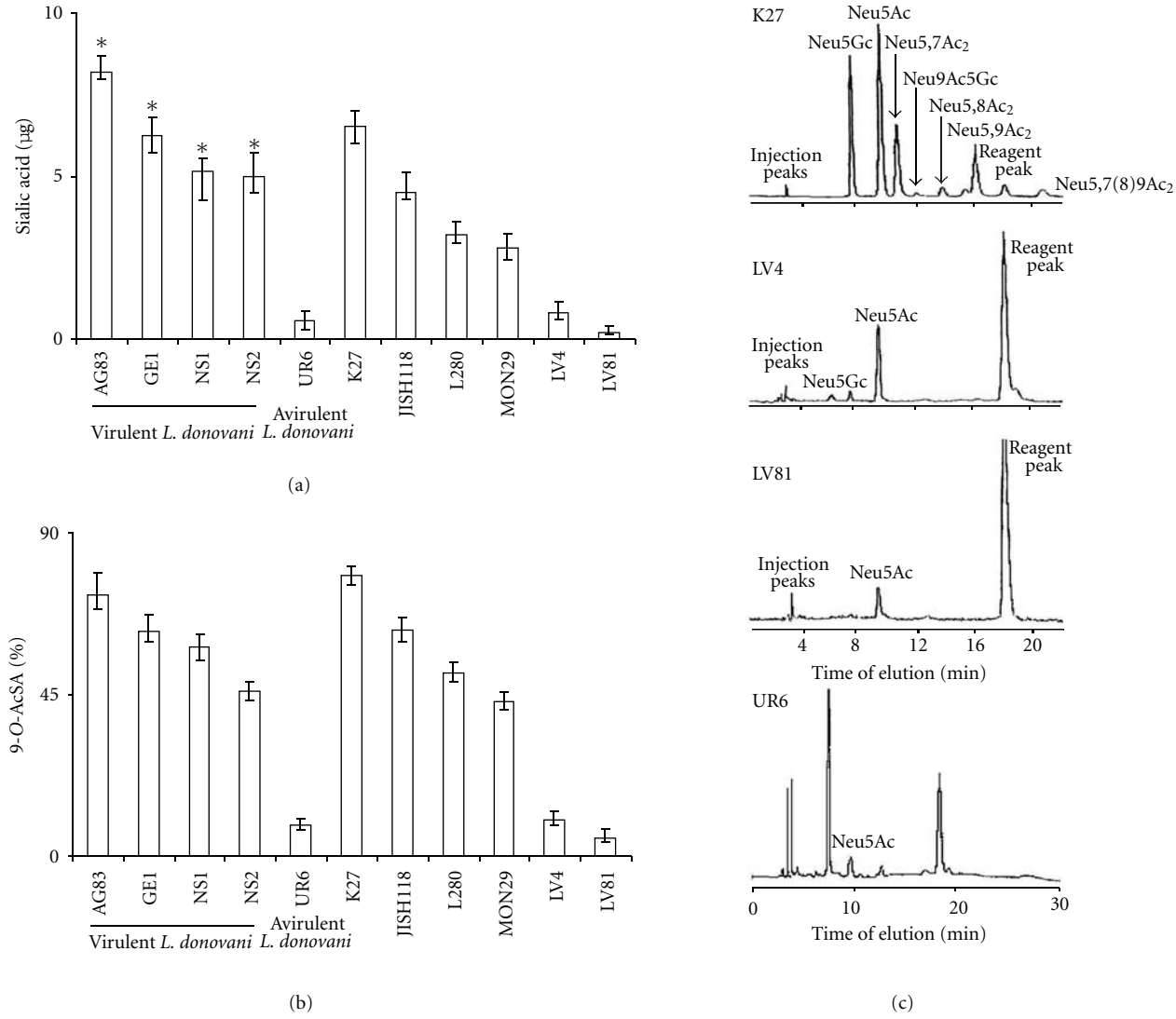

Figure 1: Identification of sialic acids on promastigotes of different *Leishmania sp.* (a) Fluorimetric estimation of enhanced sialic acids on promastigotes of virulent *Leishmania sp.* as compared to their minimal presence on avirulent UR6. Results are the mean ± S.D. where, ∗ (asterisk) means $P < 0.01$ as compared to UR6 were performed using as Graph-Pad Prism statistics software (Graph-Pad Software Inc., San Diego, CA, USA) as described in [68, 69]. (b) Differential distribution of 9-*O*-AcSA on promastigotes as detected fluorimetrically. Results are expressed as 9-*O*-AcSA (%), defined as the percent of 9-*O*-AcSA present in the total sialic acid content ($\mu g/5 \times 10^8$ promastigotes) as described in [68, 69]. (c) Representative HPLC chromatogram of sialic acids and its derivatives on promastigotes of a few virulent *Leishmania sp.* and avirulent UR6 of *L. donovani* (reproduced and adapted from [68, 69] with permission of the publishers, the Cambridge University Press, and Oxford University Press).

"avirulent" strain owing to its poor ability to infect and multiply within macrophages [70, 71]. Virulent AG83 revealed a 15-fold higher presence of total SA content as compared to avirulent UR6. Amongst six other virulent strains of *Leishmania sp.*, K27 shows highest amount of total SA as compared to the least presence on LV81 (Figure 1(a)). Our observations indicate a specific order of SA predominance as follows: K27 > JISH118 > L280 > MON29 > LV4 > LV81. Furthermore, 9-*O*-AcSA (%) demonstrates a similar trend of distribution in all the virulent strains indicating their differential species-specific distribution showing its minimal presence on avirulent UR6 (Figure 1(b)). Accordingly, K27, JISH118, L280, and MON29 are categorized as high SA-containing strains having enhanced 9-*O*-AcSA (9-*O*-AcSA^{high}) whereas LV4 and LV81 demonstrate considerably reduced SA. Liberated SA from K27 shows comigrating peaks corresponding to Neu5Ac, Ne5Gc, *N*-acetyl-7/8/9-*O*-acetylneuraminic acid, and *N*-glycolyl-9-*O*-acetylneuraminic acid (Tables 1 and 2, Figure 1(c)). LV4 and LV81 having reduced SA show similar patterns. Complete absence of the peak corresponding to 9-*O*-AcSA on avirulent UR6, confirms its undetectable presence (Figure 1(c), Table 1). Neu5Gc is detected on UR6, which is strongly bound to the surface, as after extensive washing of

Table 1: Quantitative analysis of sialic acids by fluorimetric-HPLC.

Strain	Designation and species	Disease form	HPLC of promastigotes ($\mu g/2 \times 10^9$)[$]		
			Neu5Ac	Neu5Gc	Neu5,9Ac$_2$
AG83	MHOM/IN/83/AG83 *(L. donovani)*	Visceral	0.80	ND	7.7% of total sialic acid
UR6	MHOM/IN/78/UR6 *(L. donovani)*	Avirulent (incapable of infection)	0.055	0.28	ND*
K27	MHOM/SU/74/K27 *(L. tropica)*	Cutaneous	15.12	9.76	5.16
LV4	MNYC/BZ/62/M379 *(L. Mexicana)*	Cutaneous	0.72	0.08	ND
LV81	MORY/BR/72/M1824 *(L. amazonensis)*	Diffuse	0.12	Trace amount	ND

*ND, not detectable.

[$]The quantitative measurement of the different derivatives of sialic acids has been represented by normalizing the cell number (2×10^9) and amount expressed as μg for the ease of comparison. Under the actual experimental conditions, analysis of K27, LV4, and LV81 was performed using 5×10^9 promastigotes. Similarly, the amount of sialic acids estimated on AG83 and UR6 promastigotes was detected in terms of ng under the specified assay conditions (reproduced and adapted from [59, 68, 69] with permission from publishers, the Oxford University Press, and Cambridge University press.)

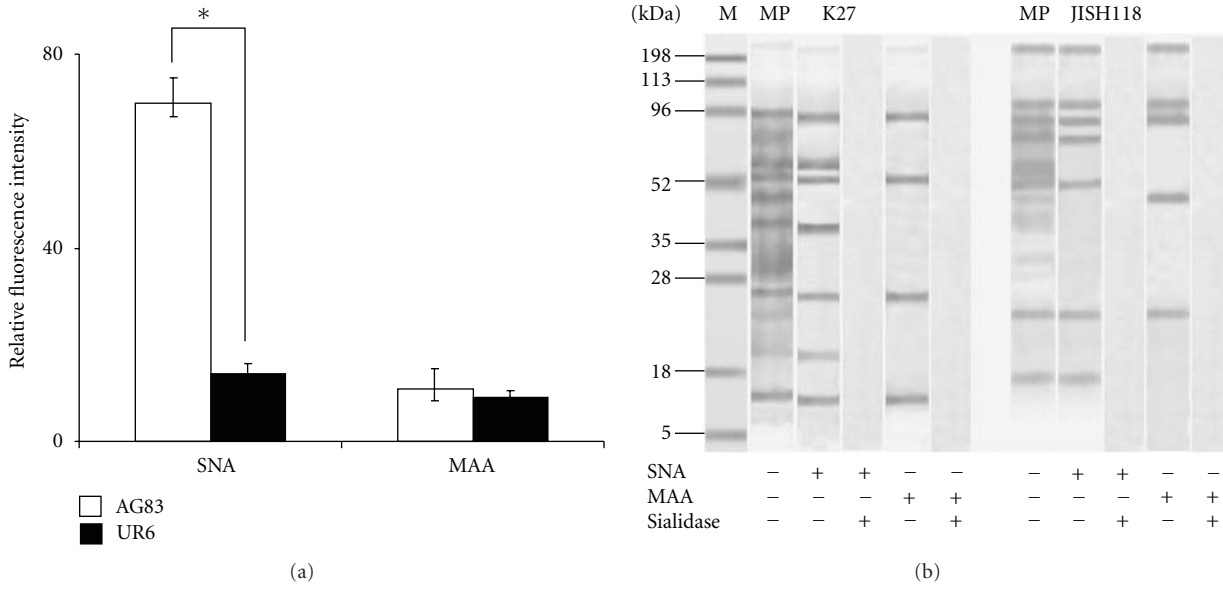

Figure 2: (a) Enhanced α2-6 linked SA on virulent AG83 as compared to avirulent UR6 promastigotes. Assay was performed using biotinylated SNA and MAA and the binding was analyzed by flow cytometry as described in [69]. "*" means-Results are the mean ± S.D. of 4 experiments performed in duplicate and * (asterix) means $P < 0.01$ as compared to UR6. (b) Linkage-specific sialoglycoproteins on virulent promastigotes of *Leishmania sp*. Membrane preparations of virulent K27, JISH 118 promastigotes and their corresponding desialylated membranes were blotted using biotinylated SNA and MAA as described in [68]. Total membrane proteins of different strains and molecular weight markers are denoted as Lanes MP and M, respectively (reproduced and adapted from [68, 69] with permission of the publishers, the Cambridge University Press, and Oxford University Press).

promastigotes, the washes show negligible amounts of SA. The topology of parasites cannot be decoded without the knowledge of different forms of molecules present on them as specific chemical modifications may influence their biological function; hence there is need for the assessment of the linkage specificity of SA. Predominance of α2-6-linked SA in all virulent strains demonstrates higher binding with SNA and recombinant siglec-2 having a preferential specificity for α2-6 linked SA (Figure 2(a)) corresponding to the presence of distinct sialoglycoproteins (α2-6- and α2-3-linked) on different promastigote membranes (Figure 2(b)) as compared to UR6. All virulent strains demonstrate higher binding with Achatinin-H as compared to UR6 and this binding is almost nullified when the promastigotes were de-*O*-acetylated using recombinant *O*-acetyl esterase from *Haemophilus influenzae* ([73], Figures 3(a) and 3(b)). The reagent used is a re-combinant form of 9-*O*-acetyl hemagglutinin esterase of in-fluenza C virus. It has been originally cloned in an SV40 vector [73] to construct a gene consisting of the influenza C virus HE1 domain fused to the eGFP gene. The esterase specifically cleaves the 9-*O*-acetyl groups. Enhanced number of receptors (9-*O*-acetylated sialoglycotopes) is found on all different virulent promastigotes (Figure 3(c)); K27 having the highest (1.94×10^7) and LV81 with lowest number (1.42×10^4) of receptors. In contrast, avirulent UR6 shows a basal level binding signifying that this unique sialoglycotope

Table 2: Quantitative analysis of sialic acids by GC/MS.

Derivative ($\mu g/5 \times 10^9$)	*L. tropica* (K27)		
	Retention time (min)	R_{Neu5Ac}	Mass fragment ions A-F (m/z)
Neu5Ac	14.10	1.00	668, 624, 478, 400, 317, 298, 173
$Neu5,9Ac_2$	15.80	1.12	638, 594, 478, 400, 317, 298, 175
Neu5Gc	24.60	1.74	756, 712, 566, 488, 386, 317, 261
	L. amazonensis (LV81)		
Neu5Ac	14.80	1.00	668, 624, 478, 400, 317, 298, 173
	L. mexicana (LV4)		
Neu5Ac	14.80	1.00	668, 624, 478, 400, 317, 298, 173
Neu5Gc	25.60	1.74	756, 712, 566, 488, 386, 317, 261

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is a potential marker for virulent strains. Additionally, the presence of distinct 9-*O*-acetylated sialoglycoproteins on different *Leishmania sp.* corroborates similar finding (Figure 3(d)). Although we have provided strong evidence in support of the presence of 9-*O*-AcSA and linkage specific SA, there are a few limitations in this field of work due to lack of reagents/probes. First of all, there are only a few lectins which can bind only to linkage-specific 9-*O*-AcSA. Achatinin-H, in spite of having a narrow binding specificity towards 9-*O*-AcSA derivatives α2-6 linked to subterminal *N*-acetylgalactosamine (9-*O*-AcSAα2-6GalNAc), binds only to the terminal modifications on glycoproteins [59, 61, 62]. Hence, sialoglycolipids having possible 9-*O*-AcSA escapes detection. Additionally, the labile nature of *O*-acetylation due to sensitivity towards alkaline pH and high temperature which are two main inherent problems also restricts the use of many other chemical/analytical methods for elaborate study. Besides under physiological conditions, *O*-acetyl esters from C7 to C8 spontaneously migrate to C9-OH group unless already substituted. Therefore, specific identification of *O*-acetylation at 7, 8, or 9 position is quite difficult. Accordingly, the binding of Achatinin-H towards 7-*O*- or/and 8-*O*-Ac sialic acids cannot be excluded. Therefore, presence of such linkages in *O*-acetylated sialoglycoproteins may be present on parasites. Furthermore, unavailability of analytical tools with distinct binding affinity for 9-*O*-acetylated α2-3 and/or α2-8 linked SA limits our study. Accordingly, the existence of 9-*O*-acetylated α2-3 and/or α2-8 linked SA cannot be ruled out. Additionally, the lack of chemically synthesized inhibitor for the *O*-acetylated sialoglycotope forces the use of only bovine submandibular mucin containing maximal amount of 9-*O*-AcSA, which poses another limitation. Taking into consideration the lim-itations of the probes, there remains scope for further ex-ploration of the promastigote surface. However, it may be envisaged that a varying distribution of SA on various virulent *Leishmania sp.* causing different forms of the disease probably imply their relevance to pathogenesis. In contrast, minimal or undetectable presence of SA especially 9-*O*-AcSA on avirulent UR6 also provides evidence in signifying their probable association with virulence. Hence we hypothesize this 9-*O*-acetylated sialoglycotope to be one of the many existing markers of virulence in *leishmania* biology.

8. Role of 9-*O*-AcSA in Entry of Virulent Promastigotes within Host Macrophages

Recognition and entry of parasites within the host cell is the first step towards the establishment of a successful infection. This section highlights the role of 9-*O*-AcSA on virulent promastigotes in infectivity [68]. The 9-*O*-AcSAs on virulent promastigotes modulate macrophage-promastigote interactions. The entry of virulent AG83 promastigotes via 9-*O*-AcSA is maximal at 37°C as compared to 4°C and 25°C, respectively, indicating the influence of temperature. The phagocytic index exhibits a linear rise with varying promastigote : macrophage ratio and demonstrates 98% internalization indicating phagocytic saturation beyond two hours. Increased phagocytic index for virulent promastigotes (Figure 4(b)) compared to avirulent UR6 signify its virulence. The enhanced internalization of virulent promastigotes indicates the influence of 9-*O*-AcSA in promastigote-entry. De-*O*-acetylated virulent promastigotes demonstrate a substantial decrease in infectivity (%) and phagocytic index exhibiting a good correlation with each other, further proving the significance of 9-*O*-AcSA in parasite-entry (Figures 4(a)–4(c)). Desialylated virulent promastigotes also exhibit reduced infection suggesting the contribution of SA in parasite entry to some extent (Figure 4(c)).

Metacyclogenesis is the process of differentiation of the infective metacyclic promastigotes that determines the virulence of a particular *Leishmania sp.* [74, 75]. The increased proportion of metacyclic promastigotes in a 5 to 6 day stationary phase culture decides the effective virulence of a particular strain. Distribution of different virulence factors (LPG and gp63) on virulent parasites and their absence in avirulent UR6 hints towards their role in metacyclogenesis [75–77]. Different structural modifications in the glycan composition of LPG play an important role in altered metacyclogenesis of *L. major* and *L. donovani* [75–77]. Reduced metacyclogenesis in avirulent UR6 shows decreased proportion of FSC^{low} metacyclic population (R1)

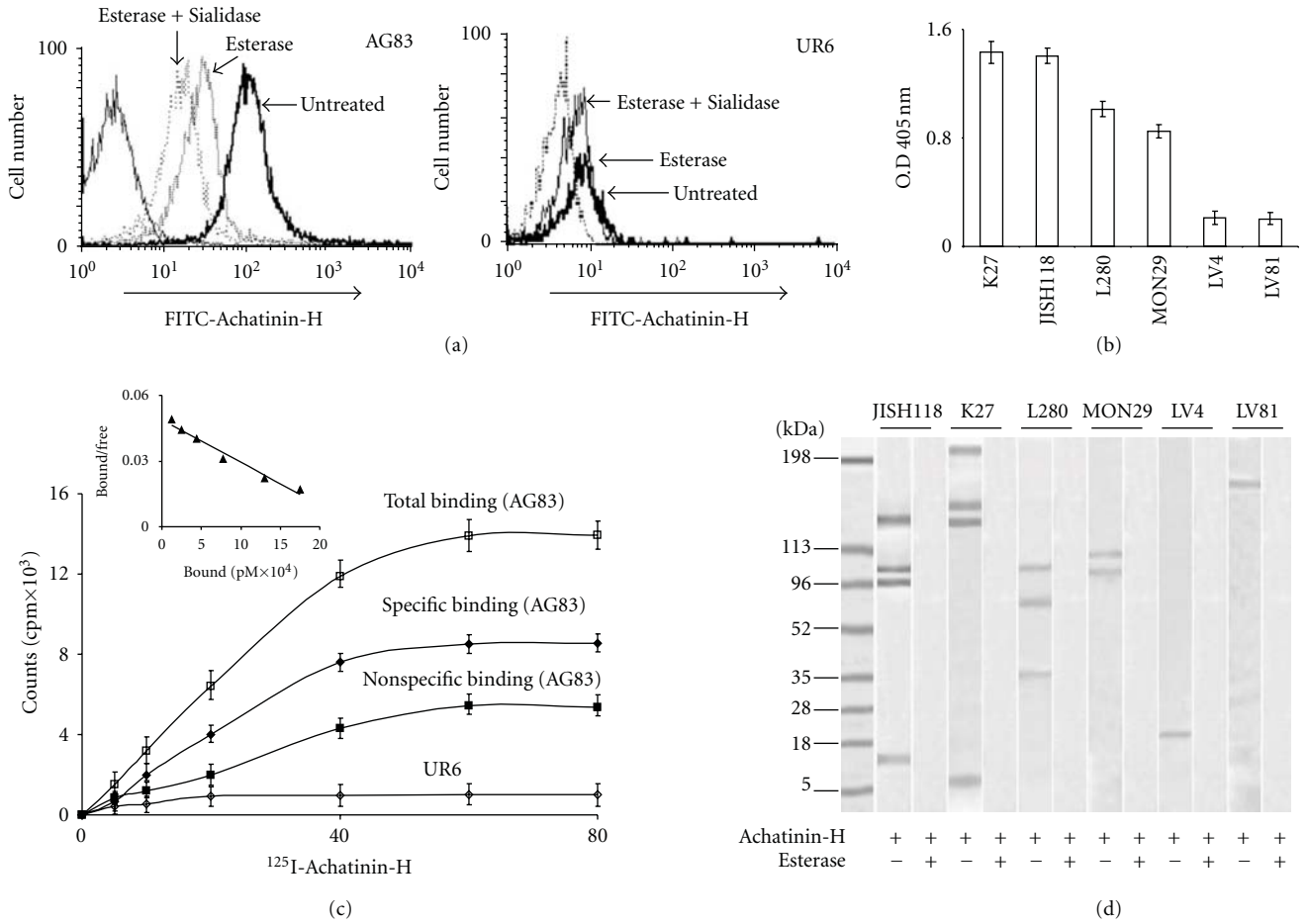

Figure 3: Enhanced 9-*O*-AcSA on *Leishmania sp.* with increased α2-6 linked SA. (a) Differential presence of 9-O-AcSA on the surface of virulent and avirulent promastigotes. The binding of FITC-Achatinin-H with promastigotes AG83 and UR6 was analyzed before and after de-*O*-acetylation followed by subsequent desialylation using sialidase from *Arthrobacter ureafaciens* by flow cytometry as described in [68, 69]. (b) Demonstration of 9-*O*-AcSA by ELISA. Membrane lysates of the respective strains were incubated separately with Achatinin-H and the binding was recorded colorimetrically as described in [68]. (c) Increased number of surface 9-*O*-AcSA containing sialoglycotope on AG83 and their minimal distribution on UR6 promastigotes. The receptors (*O*-acetylated sialoglycoproteins) on AG83 were estimated by determining the specific binding of AG83 (black diamond) by subtracting the nonspecific binding (black square) using excess unlabelled Achatinin-H from total binding (white square) . UR6 (white diamond) evidenced a basal level of specific binding. *Inset:* scatchard plot showing the binding of ^{125}I-Achatinin-H with 9-*O*-AcSA containing sialoglycoproteins present on AG83 promastigotes as described in [69]. (d) Presence of 9-O-acetylated sialoglycoproteins as detected by Western blot on virulent promastigotes of different *Leishmania sp.* The specificity of binding was examined by using membrane proteins of de-*O*-acetylated promastigotes as described in [68, 69] (reproduced and adapted from [68, 69] with permission of the publishers, Oxford University Press, and Cambridge University Press, resp.).

in stationary phase as compared to their higher proportion in virulent AG83, indicating reduced metacyclogenesis in avirulent strains (Figure 4(d)). Interestingly, the proportion of metacyclics increases from logarithmic to stationary phase cultures in virulent AG83. In contrast in UR6, there is no significant increase in the proportion of metacyclics in the different phases of culture. Higher proportions of metacyclic population of AG83 show enhanced distribution of 9-*O*-AcSA as compared to their negligible presence on metacylics of UR6 (Figure 4(e)). This observation demonstrated that not only there was reduced proportion of metacyclics in avirulent UR6 but even this small proportion showed minimal presence of 9-*O*-AcSA. This hints towards a probable link, between metacyclogenesis and the presence of 9-*O*-AcSA. The direct influence of 9-*O*-AcSA in the process of metacyclogenesis remains to be investigated and is a matter of future research.

9. Enhanced 9-*O*-AcSA in Different *Leishmania sp.*, Nitric Oxide (NO) Resistance, and Modulation of Host Responses

The presence of SA especially 9-*O*-AcSA on virulent *Leishmania sp.,* its role in entry of promastigotes into macrophages and their undetectable presence on avirulent UR6 intimates

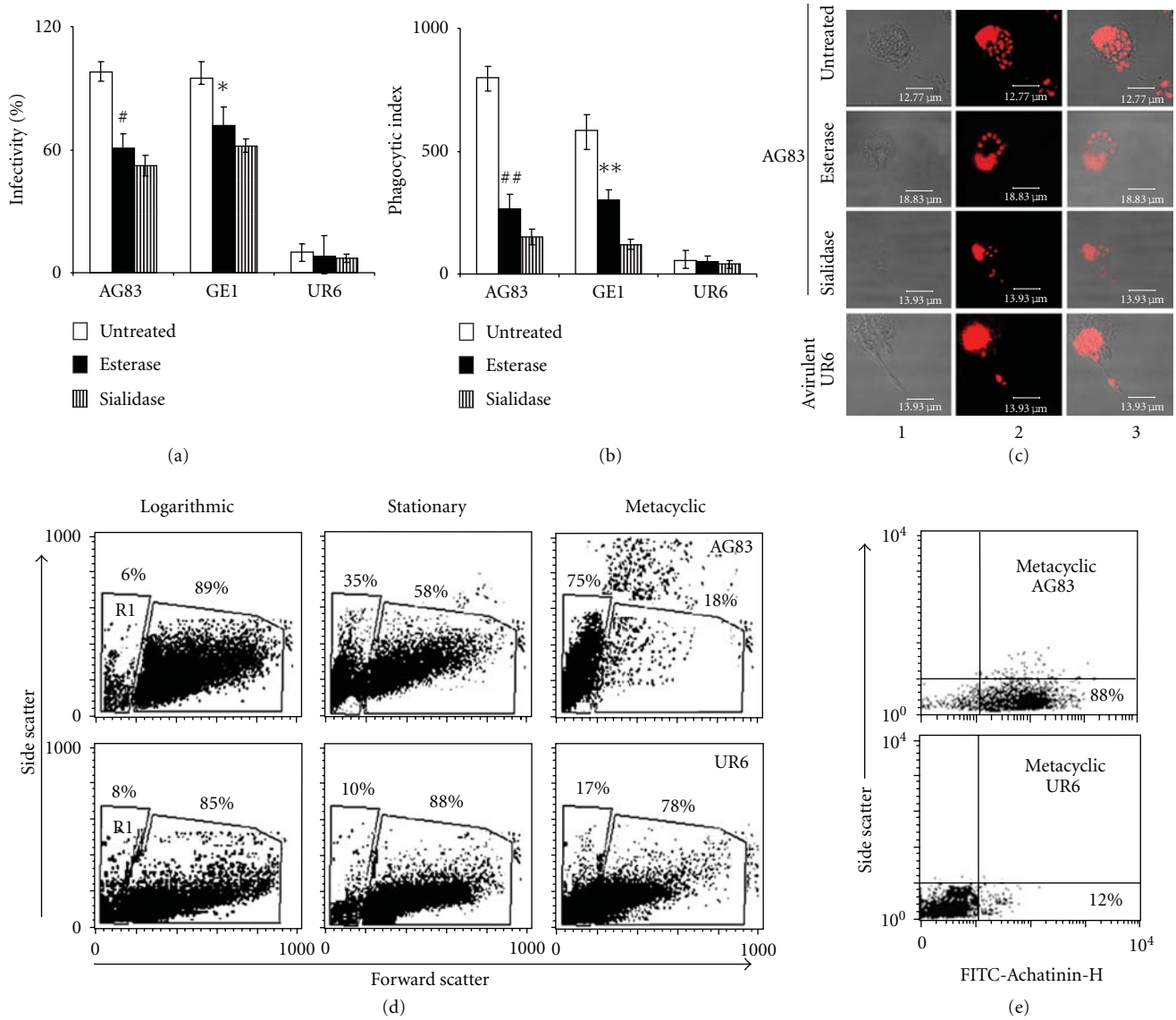

Figure 4: Enhanced entry of virulent AG83 promastigotes having increased metacyclogenesis and higher distribution of surface 9-*O*-AcSA with macrophages. (a) Decreased infectivity (%) of virulent promastigotes after de-*O*-acetylation and desialylation. The infection assays were performed using macrophage : promastigote ratio 1 : 10, for 2 h at 37°C using untreated (white square), esterase (black square), and sialidase (lined square) treated AG83, GE1 promastigotes as described in [69]. The reduction in infectivity (%) of de-*O*-acetylated virulent strains was compared against untreated control. In parallel, UR6 promastigotes with minimal sialic acids were similarly treated. $^{\#}$ denotes $P < 0.01$ for AG83 and * denotes $P < 0.05$ for GE1. (b) Reduced phagocytic index of virulent promastigotes of L. donovani after de-*O*-acetylation (black square) and desialylation (lined square) as compared to untreated controls (white square). Similar experimental conditions were used as described in legends of Figure 4(a) [69]. In parallel desialylated promastigotes were also used. $^{\#\#}$ denotes $P < 0.01$ for AG83 and ** denotes $P < 0.01$ for GE1. (c) Photomicrographs demonstrating enhanced entry of virulent *L. donovani* promastigotes within macrophages. Virulent AG83 and avirulent UR6 promastigotes were treated with esterase and sialidase for the assay under optimized conditions and the results were compared with untreated promastigotes by confocal microscopy. Column 1, phase photomicrograph. Column 2, detection of propidium iodide-stained fluorescence. Column 3, overlap of 1 and 2. (d) Increased proportion of metacyclic promastigotes in stationary, phase of virulent AG83 as compared to UR6. Promastigotes of logarithmic, stationary and metacyclic (after purification by PNA-negative agglutination) stages of AG83 and UR6 was assessed by flow cytometry to demonstrate the percent of metacyclics (FSC^{low}, R1 population) in as represented in FSC versus SSC plots. (e) Increased distribution of 9-*O*-AcSA on metacyclic promastigotes of AG83 as compared to UR6. Flow cytometric analysis of metacyclic promastigotes obtained from stationary phase cultures of AG83 and UR6 after subsequent enrichment through PNA-negative selection were incubated with FITC-Achatinin-H to detect the presence of 9-*O*-AcSA as described in [69]. (reproduced and adapted from [69] with permission of the publishers and the Cambridge University Press).

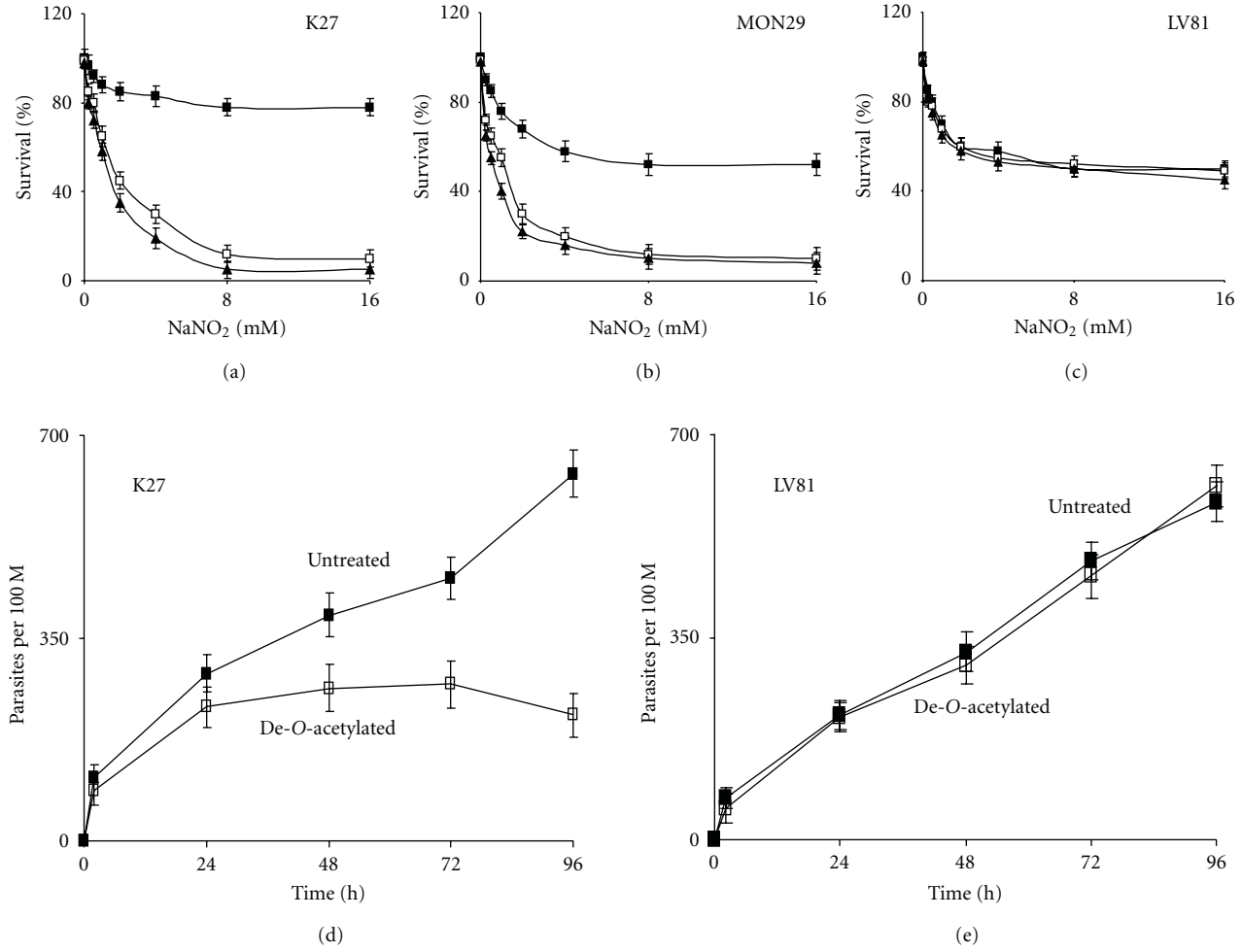

Figure 5: Increased NO resistance and enhanced intracellular survival of 9-*O*-AcSA^{high} promastigotes as compared to their de-*O*-acetylated forms. Untreated K27 (a), MON29 (b), and LV81 (c) with 9-*O*-AcSA (black square), esterase (white square), and sialidase (black triangle) treated promastigotes were exposed to $NaNO_2$ and their viability (%) was estimated by MTT assay as described in [68]. Enhanced intracellular survival of 9-*O-AcSA*^{high} promastigotes within macrophages (ϕ). Promastigotes of K27 (d) and LV81 (e) before (black square) and after de-*O*-acetylation (white square) were incubated with macrophages at a ratio of 1 : 10, for 0–96 h at 37°C and the interaction was quantified in terms of phagocytic index as described in details in [68] (reproduced and adapted from [68] with permission of the publishers and the Oxford University Press).

their significance in parasite biology. Being intracellular obligatory parasites they have the ability to withstand the oxidative stress exerted by the host [33, 34, 37, 45, 46]. Promastigotes of all the six virulent strains demonstrate a differential viability when exposed to $NaNO_2$, indicating their inherent ability to resist NO for combating the host defense system. A decrease in viability of de-*O*-acetylated parasites (Figures 5(a) and 5(b)) indicates a probable association of NO-resistance and 9-*O*-AcSA. Thus removal of *O*-acetyl group by deacetylation hints the possible role of this particular modification of SA. Desialylated promastigotes exhibit an additional reduction in survivability. The parasite (LV81) with least amount of SA and 9-*O*-AcSA demonstrates unchanged viability after exposure to NO before and after de-*O*-acetylation and desialylation suggesting a species-specific role of the 9-*O*-acetylated sialoglycotope (Figure 5(c)). Infection of de-*O*-acetylated promastigotes of 9-*O*-AcSA^{high} strains show a maximal reduction in the number of intracellular parasites (Figures 5(d) and 5(e)). This indicates that 9-*O*-AcSA influence proliferation of promastigotes after which they are subjected to probable intracellular killing in the absence of the 9-*O*-acetylated sialoglyctope. LV4 and LV81 do not show any significant effect further indicating the specific-specific effect.

Host responses are a key to intracellular killing or multiplicity of *Leishmania*. Interestingly, supernatants of postinfected 9-*O*-AcSA^{high} promastigotes (K27, JSIH118, L280 and MON29) demonstrate a negligible accumulation of NO, indicating the effect of 9-*O*-AcSA in bestowing a survival benefit (Figure 6(a)). In contrast supernatants from de-*O*-acetylated 9-*O*-AcSA^{high} promastigotes show increased accumulation of NO further suggesting that 9-*O*-AcSA probably imparts a survival benefit, in the absence of which host leihmanicidal responses are triggered.

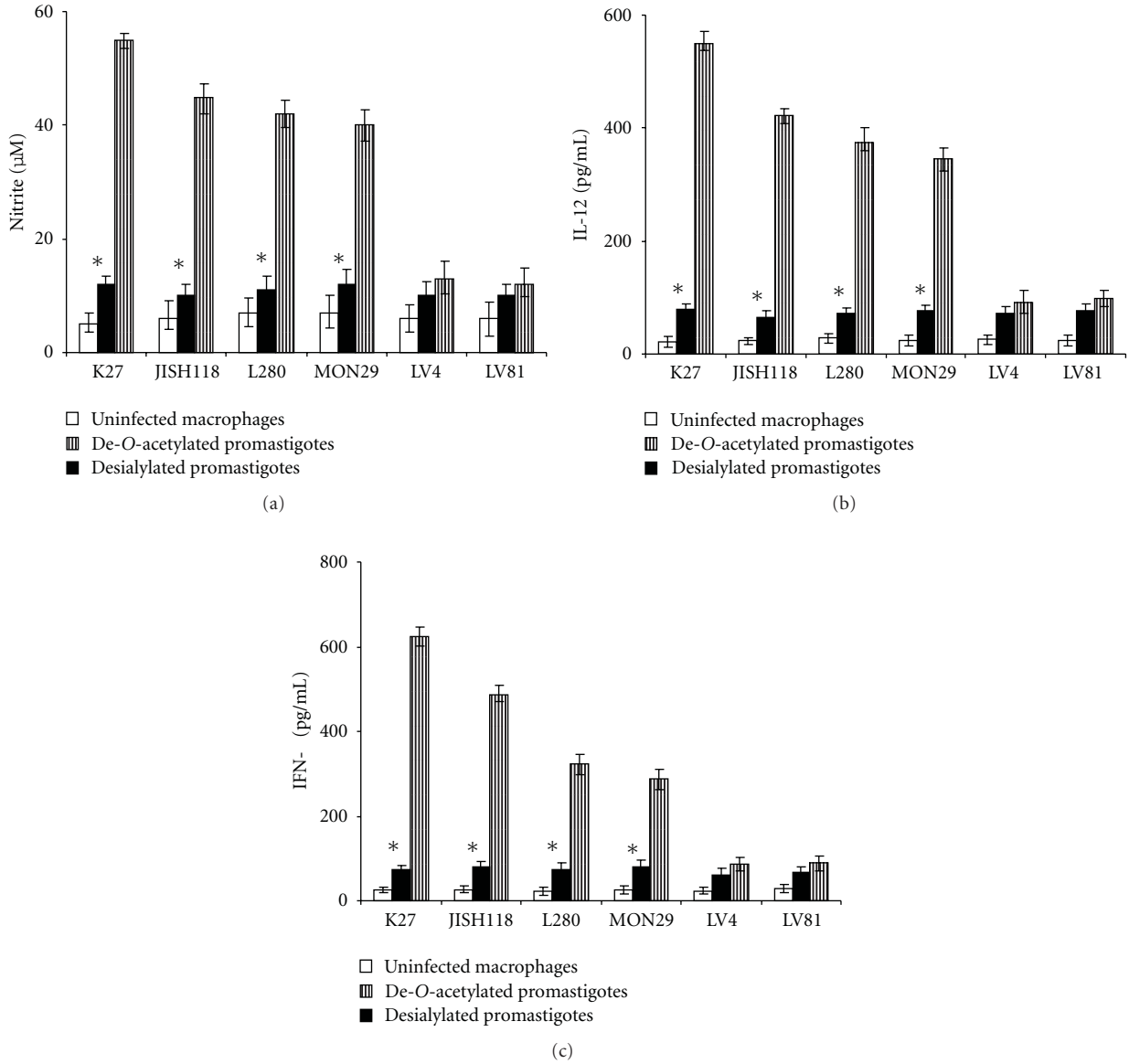

Figure 6: Inhibition of host leishmanicidal responses after infection of 9-*O*-AcSA^{high} promastigotes within macrophages as compared to their de-*O*-acetylated forms. (a) Reduced levels of NO production subsequent to infection with 9-*O*-AcSA^{high} promastigotes. Macrophages were incubated with promastigotes before (black square) and after de-*O*-acetylation (lined square) and liberated NO were estimated as described in [68]. Naïve macrophages served as controls (white square). (b)-(c) Decreased production of IL-12 and IFN-γ after infection with 9-*O*-AcSA^{high} promastigotes. Macrophages were infected with promastigotes before (black square) and after de-*O*-acetylation (lined square) and cytokines were detected in the supernatants by ELISA as described in detail in [68]. Macrophages without infection served as controls (white square; reproduced and adapted from [68] with permission of the publishers and the Oxford University Press). Asterix (*) denotes $P < 0.01$ for macrophages infected with 9-O-AcSA^{high} NO-resistant promastigotes versus de-*O*-acetylated susceptible promastigotes under similar conditions.

In leishmaniasis, production of a TH1 response surpassing the TH2 response is beneficial to the host [49]. Post-infection with de-*O*-acetylated 9-*O*-AcSA^{high} promastigotes demonstrate increased levels of IL-12 and IFN-γ, the signature TH1 cytokines (Figures 6(b) and 6(c)) indicating that the effective modulation of the host responses affects the *leishmania* biology via 9-*O*-AcSA acting as suitable ligands whose expression supports parasite entry. This may be one of the ways amongst many waiting to be explored.

10. Critical Evaluation and Perspective

The present scenario of leishmaniasis affecting millions in different tropical and subtropical countries causes immeasurable death and destruction. The increasing drug unresponsiveness of these manipulative parasites and the advent of several drug resistant clinical isolates have made the situation graver. Differential distribution of SA in different virulent *Leishmania sp.* shows enhanced 9-*O*-AcSA levels

in-dicating a balance between total SA and the percent of 9-*O*-AcSA under different disease conditions which indicate their probable association with the disease biology. In spite of the presence of significant levels of sialic acids in the culture medium, the minimal levels of SA and undetectable levels of 9-*O*-AcSA on avirulent UR6 indicate that the avirulent strain is unable to adsorb sialic acids from the environment as efficiently as virulent AG83, which suggest a probable link between this sugar and virulence. Additionally, the preferential adsorption of Neu5Gc by avirulent UR6 promastigotes in contrast to virulent AG83 raises questions on the preferential adsorption of this sugar by UR6 which paves the path for future investigations. It may also be envisaged that these acquired sialic acids could possibly fit within the well-defined glycocalyx of the parasite but the mechanism remains to be investigated. But whatever may be the consequencess it would lead to a probable change in the surface architecture that probably create newer sites of mimicry or recognition or immunomodulation. Demonstration of distinct bands corresponding to different sialoglycoproteins also indicates that probably the adsorbed sialic acids modify specific surface molecules. However, due to the lack of sialic acid biosynthetic machinery known the exact mechanism remains to be investigated. Furthermore, identification of these specific proteins by proteomic/glycoproteomic approaches on the parasite surface bearing terminal 9-*O*-AcSA would open up newer targets for exploiting parasite biology in future. Additionally these parasite surface sialoglycoconjugates may be harnessed for the production of sialoglycotope-specific antibodies which could be effectively used. Present day research for combating this disease involves the identification and characterization of novel molecular markers that not only controls parasite survival within host cells but also elicits a favorable TH1 bias essential for the curative response. The presence of 9-*O*-AcSA on virulent strains as compared to their undetectable presence in avirulent strains suggests their importance in virulence. Furthermore the possible influence of 9-*O*-AcSA in enhancing parasite entry within macrophages, NO-resistance and host modulation suggests their essential role in parasite biology. However, the direct association of 9-*O*-AcSA with differentiation, multiplication, and proliferation within macrophages requires detailed exploration of different facets of parasite biology. Whether these acquired sialic acids change their conformation to form a new set of molecules that play a crucial role in the intracellular parasite biology is a matter of future research. Moreover, the study needs to be extended in the *in vivo* system which remains to be explored. Such studies are presently ongoing. Importantly, 9-*O*-AcSA would also help to differentiate the strains based on their virulence interfering in the outcome of the disease. However, elucidation of the detailed mechanism influencing these facets requires investigation. These observations also raise the possibility that differential opsonization of pathogens using sialylated glycoconjugates may possibly be a general phenomenon exercised by many other parasites which will dictate the future research and is likely to be promising. Moreover, since these sialoglycotopes also occur in the metacyclic population of virulent *Leishmania sp.* they will probably serve as important determinants for early detection of the infection.

Abbreviations

CL:	Cutaneous leishmaniasis
gp63:	Glycoprotein 63
LPG:	lipophosphoglycan
MAA5	*Maackia amurensis agglutinin*
NO:	Nitric oxide
Neu5Gc:	*N*-glycolylneuraminic acid
SNA:	*Sambucus nigra agglutinin*
SA or Neu5Ac:	Sialic acids or *N*-acetylneuraminic acid
VL:	Visceral leishmaniasis
9-*O*-AcSA or Neu5,9Ac$_2$:	9-*O*-acetylated sialic acid
9-*O*-AcSGPs:	9-*O*-acetylated sialoglycoproteins.

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