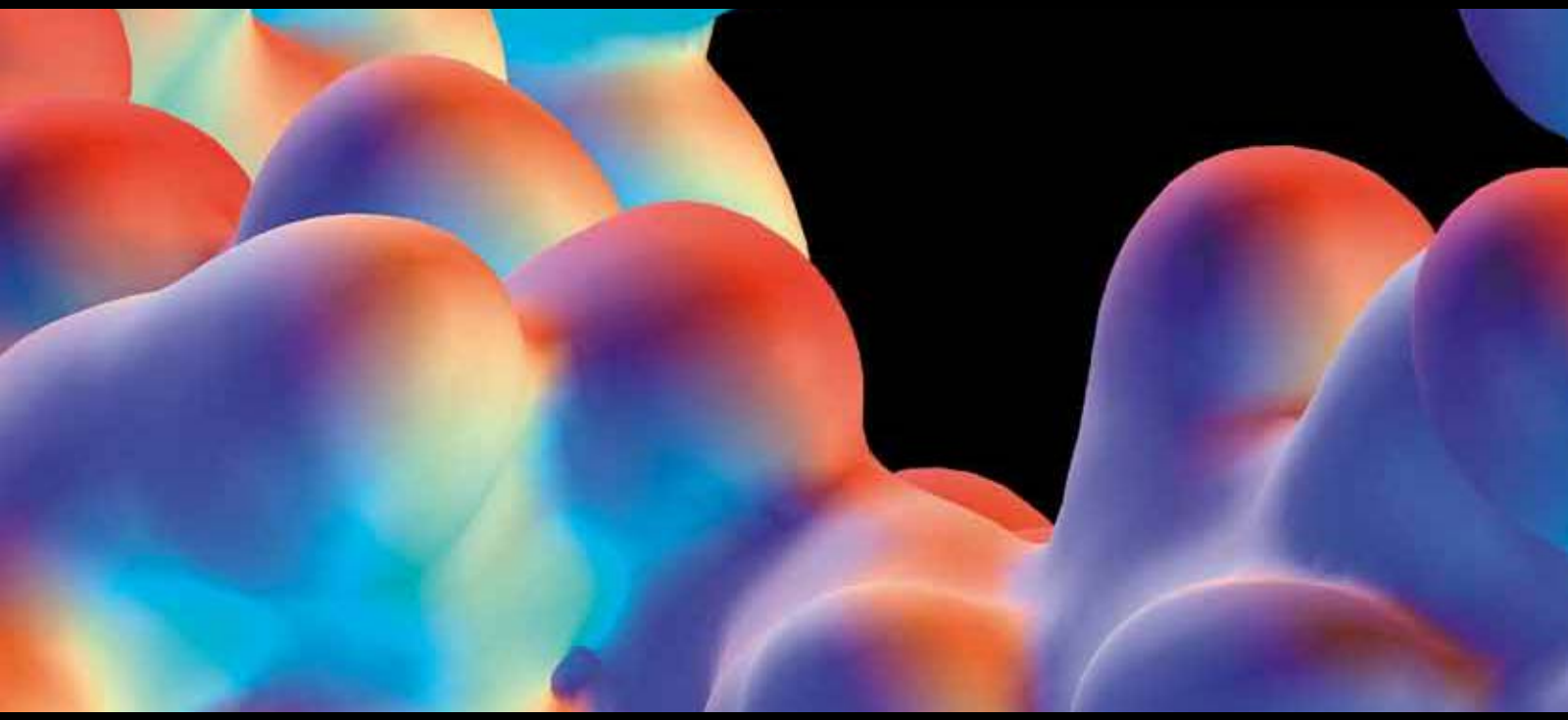
Enzyme Research

New Enzymes as Potential Therapeutic Targets for Trypanosomiases and Leishmaniasis

Guest Editors: Claudio Alejandro Pereira, Ariel Mariano Silber, and Elena Gonzalez-Rey



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Editorial Board

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Editorial

New Enzymes as Potential Therapeutic Targets for Trypanosomiases and Leishmaniasis

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Infections caused by the protozoan parasites *Trypanosoma cruzi*, *Trypanosoma brucei*, and *Leishmania* spp. are among the most relevant public health problems in the developing countries. Furthermore, climatic changes and migratory fluxes of the human population are broadening the former geographic restrictions of most of these diseases. Only a few therapeutic treatments are available for these infections, and main drawbacks of drugs in use are their low efficiency, high toxicity, and the emergence of strains resistant to available treatments. All these facts make the research on new drug targets and strategies for developing new drug therapeutic strategies a relevant issue.

In this special issue, we presented original research papers and reviews on the discovery of novel enzyme targets which contributed to having a picture of the state of the art on trypanosomatids' enzymes research oriented to therapeutic applications. The topics addressed in this special edition include a wide variety of enzymes and metabolic pathways: biosynthesis of galactofuranose (M. Oppenheimer et al.), prereplication machinery (S. G. Calderano et al.), glutamate metabolism (A. Magdaleno et al.), ornithine decarboxylases (J. J. Barclay et al.), sphingolipid biosynthetic (C. M. Koeller and N. Heise), phosphotransferase enzymes involved in cell energy management (C. A. Pereira et al.), Heme metabolic pathway (K. E. J. Tripodi et al.), phospholipases A (M. L. Belaunzaran et al.), topoisomerases (B. Banerjee et al.), P4 Nucleases (L. Rahbarnia et al.), enolases (L. Avilan et al.) and in silico prediction of drug targets by non-homologous isofunctional enzymes analysis (M. Rajao Gomes et al.).

I hope the information in this special issue will be useful not only for scientists in the area, but also as a status report on this critical issue for developing countries.

Claudio Alejandro Pereira
Ariel Mariano Silber
Elena Gonzalez-Rey

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Research Article

Specific and Nonhomologous Isofunctional Enzymes of the Genetic Information Processing Pathways as Potential Therapeutical Targets for Tritryps

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Leishmania major, Trypanosoma brucei, and *Trypanosoma cruzi* (Tritryps) are unicellular protozoa that cause leishmaniasis, sleeping sickness and Chagas' disease, respectively. Most drugs against them were discovered through the screening of large numbers of compounds against whole parasites. Nonhomologous isofunctional enzymes (NISEs) may present good opportunities for the identification of new putative drug targets because, though sharing the same enzymatic activity, they possess different three-dimensional structures thus allowing the development of molecules against one or other isoform. From public data of the Tritryps' genomes, we reconstructed the Genetic Information Processing Pathways (GIPPs). We then used AnEnPi to look for the presence of these enzymes between *Homo sapiens* and Tritryps, as well as specific enzymes of the parasites. We identified three candidates (ECs 3.1.11.2 and 6.1.1.-) in these pathways that may be further studied as new therapeutic targets for drug development against these parasites.

1. Introduction

Recent estimates indicate that more than one billion people, living in tropical and subtropical regions of developing countries, are at the risk of contracting diseases (which are mostly endemic at these places) caused by the protozoans *Leishmania major*, *Trypanosoma brucei* and *Trypanosoma cruzi* [1–3]. These three microorganisms, together known as the Tritryps (family Trypanosomatidae, order Kinetoplastida), also cause the death of thousands of people every year [4]. Despite all these facts, these infirmities are still considered as neglected diseases by the health agencies [5].

The control of the diseases caused by these parasites depends nowadays on chemicals, vaccines not being commercially available so far. Besides, there is a very limited set of pharmaceuticals available at this moment: most of them were discovered at approximately 50 years ago, and they also have disadvantages like high toxicity, low efficacy, or high costs; the development of resistance is also a possibility [6–8]. However, with the recent publication of the Tritryps' genomes [9–11], new opportunities allowed a better understanding of several biological processes that, up to this point, were poorly understood or even unknown in these organisms [7, 12].

Cellular functions are based on complex networks of chemical reactions that interact producing observable results. The rapid development of DNA sequencing techniques provided a huge amount of information leading to a new comprehension about the organization of cellular processes. First, by using annotation data, genes are classified in groups in accordance with their functions. Part of the gene products are enzymes, proteins that catalyze cellular reactions, making part of complex biochemical pathways. In the postgenome era, the study of these processes is gaining an importance, to improve the comprehension of the dynamics and regulation of these pathways, as well as the discovery of previously unknown steps [13, 14].

The reconstruction of biochemical pathways is considered to be one essential step in the study of cellular processes [15]. Applications of these reconstructions may vary from

the drawing of the biological system to the generation of testable hypotheses about the structure and working of the pathway and from the elucidation of complex properties not inferred by the simple description of the individual components to the recognition of potential drug targets against pathogenic organisms via the identification of essential steps in these processes [16]. Several methods and databases are available for the reconstruction of said pathways from genome information; one of the main resources for this task is the KEGG database [13, 17, 18]. One way to link the biological processes to the genomic information is through the EC numbers, which represent the reaction each enzyme catalyzes. There are other types of functional classifications, (reviewed by Ouzounis and collaborators [19]), but the EC classification system is certainly one of the most used by the scientific community.

Enzymes have a high degree of specificity for their substrates and are fundamental for any biochemical process. They act in an organized sequence, catalyzing successive reactions in enzymatic pathways, guaranteeing the maintenance of life in all organisms [20]. A particular group of enzymes, the nonhomologous isofunctional enzymes (NISE or analogous enzymes), executes the same function in different organisms, but without detectable similarity between their primary structures and, possibly, between their tertiary structures as well. Once analogy is detected between a pathogen's enzyme and its human counterpart, it may be possible to use this analog as a potential target for drug development, provided it belongs to an essential biochemical step of the pathogen. However, only a few studies have been done to identify and annotate isofunctional nonhomologous enzymes as such [21–25].

Maintenance of the genome depends on the efficiency and accuracy of DNA replication, as well as the repairing systems. Through a series of complex interactions, the genome is transcribed and in good part translated, in order to produce the RNAs and proteins necessary for the organism. These molecules form its structure or participate in important reactions. For these reasons, the pathways of DNA replication and repair, transcription and translation (some of the Genetic Information Processing Pathways (GIPPs)) comprise some of the most important processes for the organism survival [26, 27] and were thus chosen as targets of this study.

Analyses of genomic data from *L. major*, *T. brucei*, and *T. cruzi* have provided a global view of the protein-coding genes that produce enzymes belonging to important pathways through the identification of several processes in common between these parasites and other species. A thorough examination of all this information may allow the identification of steps of the GIPPs that are particularly accessible to potential therapeutic interventions. New drugs may be also developed from inhibitors of specific biochemical processes essential to the parasite but absent in their hosts. In this work, we employed computational methods to identify not only specific but also nonhomologous isofunctional enzymes in the genetic information processing pathways of the Tritryps, enzymes that could serve as interesting candidates for further studies aiming at their validation as drug targets.

2. Methodology

2.1. Predicted Protein Sequences of Tritryps. The dataset of predicted proteins of *Leishmania major*, *Trypanosoma brucei*, and *Trypanosoma cruzi* was obtained from TritrypDB (http://tritrypdb.org/tritrypdb/) as shown in Table 1.

2.2. Pathways and Enzyme Classes. A set of pathways (maps) referring to the replication and repair, transcription and translation processes was obtained from KEGG (http://www.genome.jp/kegg/pathway.html#genetic). This dataset contains a complete biochemical description of the pathways related to genetic information processing observed in different organisms. Functions comprising a certain pathway were extracted from these descriptions as a collection of EC numbers and were used as templates for the reconstruction of the correspondent pathways in Tritryps. Each pathway is associated with a set of proteins, usually a list of enzyme families with their EC numbers. KEGG has a total of 10 maps distributed among these pathways: 6 maps representing replication and repair; 2 maps symbolizing the transcription, but only one with an associated EC number; 2 translation maps of which only one has an associated EC number.

2.3. Clustering. To group homologous enzymes with the same activity, we used the AnEnPi pipeline (http://www.dbbm.fiocruz.br/AnEnPi/) [22], which was based on a previous study in which enzymes are considered analogous (i.e., with different evolutionary origins) according to differences in their primary structures [24]. After clustering, enzymes within a given cluster are considered homologous, while enzymes in different clusters (of the same function) are considered analogous. As the cut-off parameter used in AnEnPi is based on experimental data obtained from enzymes, other values should probably be employed for other types of proteins.

2.4. Protein Function Inference. Using another module of AnEnPi, we were able to infer function of the predicted proteins of trypanosomatids using the groups (or clusters) obtained after clustering. In this module, the EC number as-signment is based on the sequence similarity report from a BLASTP [28] procedure: predicted proteins of Tritryps (query) against the sequences of each individual AnEnPi cluster (subject), as described in detail in [22]. The cutoff employed for functional inference was the *e*-value of e^{-20}.

2.5. Genetic Information Processing Pathways Reconstruction and Search for NISE and Specific Enzymes. The reconstruction of the GIPPs was performed using the data inferred by the AnEnPi pipeline. After functional inference, enzymatic activities shared by Tritryps were disclosed using scripts written in Perl language. NISE and specific enzymes were obtained through an examination of the groups (or clusters) produced after clustering, where sequences of Tritryps and

Table 1: Organisms, dataset version, and number of predicted proteins of the Tritryps' genomes.

Organisms	Version	Predicted Proteins
L. major	http://tritrypdb.org/common/downloads/release-1.0/Lmajor/	8406
T. brucei	http://tritrypdb.org/common/downloads/release-1.0/Tbrucei/	10123
T. cruzi	http://tritrypdb.org/common/downloads/release-1.0/Tcruzi/	23031

H. sapiens were considered analogous if allocated in different groups and specific if absent in *H. sapiens*. The PDB database was searched for 3D structures resolved for these enzymes.

3. Results and Discussion

3.1. KEGG, Clustering, and Enzymatic Activity Inference. The Tritryps' genomes were first sequenced in 2005 [9–11], with all chromosomes well characterized (with the exception of *T. cruzi* due to the high degree of repetitions in its genome). However, some of the GIPPs still present gaps [29]. The computational reconstruction of these processes, in this work, is an attempt to obtain a better representation of them, with emphasis on the analogous and specific enzymes. These analogs are enzymes that, even with a small or no significant similarity between their primary structures (which reflect in differences in their 3D structure), are able to catalyze the same reaction [24]. For these reasons, recent efforts have been made to include this phenomenon in the functional annotations [21, 22, 30]. Inference of function, if based only on sequence similarity, may be insufficient since they are usually not able to detect nonhomologous isofunctional enzymes.

Tritryps share a series of features, like the presence of subcellular structures such as the kinetoplast and glycosomes. Each trypanosomatid is transmitted by a different vector, possessing distinct life cycles, tissue specificity, and pathogenies in their mammal host [31, 32]. In addition, they are considered "ancient" from an evolutionary perspective; in fact, they present peculiar mechanisms in some of the genetic information transmission processes. Many of these still have gaps to be filled [33]. In this context, we have compared the number of enzymatic activities shared among the three microorganisms (taking into account all pathways) and the unique activities based on the results obtained after clustering (Figure 1). It may be worth noticing that some activities found have the same isoform (or, more precisely, analog form) in the three microorganisms; this may serve as a basis (ideally and depending on several other factors) for one unique drug for the three pathogens or (much more likely) a family of related/similar molecules as drugs.

KEGG has its own annotation protocol, which to our knowledge is not described in detail anywhere; only its general lines are known [17, 34]. We opted to make a functional inference from all the predicted proteins of Tritryps, in order to have a unified and comparable data. For this, we performed a BLASTP of the available predicted proteins in the TriTrypDB against the obtained clusters. From this it was possible to infer functions not detected by

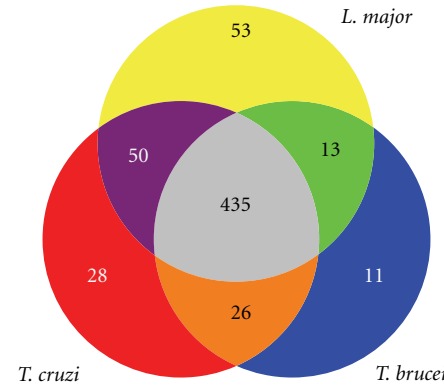

Figure 1: Venn diagram of shared enzymatic activities between the Tritryps. Yellow: *L. major* unique ECs; red: *T. cruzi* unique ECs; blue: *T. brucei* unique ECs; orange: between *T. cruzi* and *T. brucei*; green: between *L. major* and *T. brucei*; purple: between *T. cruzi* and *L. major*; gray: between all Tritryps.

KEGG, in almost all pathways studied. Even using a very restrictive cut-off (*e*-value $< 10^{-20}$), more enzymes were identified (data not shown), indicating the validity of this approach. In fact, even after using more restrictive *e*-values, like 10^{-40} or 10^{-80}, results did not differ for several ECs (data not shown). With these information, some of the GIPPs were reconstructed. The description of the enzymatic activities found by AnEnPi for each Tritryp is listed in Table 2.

3.2. Computational Reconstruction of the GIPPs. Figure 2 displays the computational reconstruction of the GIPPs using the map representing the aminoacyl-tRNA biosynthesis (map 00970) as an example. The other 7 maps, as well as the tables with the description of the enzymes highlighted in each map, are available in the Supplementary Material available online at doi:10.4061/2011/543912. In this map, all enzymatic activities detected by KEGG were also identified by AnEnPi for the Tritryps, with the exception of SepRS (EC 6.1.1.27). This enzyme participates of the alternative formation of Cys-tRNACys linking O-phosphoserine, a precursor of the aminoacid cysteine, to tRNACys. Then SepCysS (Sep-tRNA:Cys-tRNA synthetase—EC 2.5.1.73) converts O-phosphoseryl-tRNACys in cysteinyl-tRNACys. This alternative formation of Cys-tRNACys has been only detected in methanogenic archaea so far, where in some species the enzyme cysteinyl-tRNA synthetase (EC 6.1.1.16), which catalyzes the direct production of Cys-tRNACys, is lacking [35, 36]. However, we could not identify the second enzyme which completes the alternative formation of Cys-tRNACys,

Table 2: List of additional ECs found in each process from GIPPs.

Pathway number	Map description	*T. cruzi*	*T. brucei*	*L. major*	EC description
Translation					
Map00970	Aminoacyl-tRNA biosynthesis	2.1.2.9	a	b	Methionyl-tRNA formyltransferase
		6.1.1.-	6.1.1.-	6.1.1.-	O-phosphoseryl-tRNA synthetase
Replication and repair					
Map03030	DNA replication	3.6.1.-	3.6.1.-	3.6.1.-	Hydrolases acting in phosphorus-containing anhydrides
Map03410	Base excision repair	3.1.-.-	3.1.-.-	3.1.-.-	Hydrolases acting on ester bonds
		3.1.11.2	3.1.11.2	3.1.11.2	Exodeoxyribonuclease III
Map03420	Nucleotide excision repair	2.7.11.22	2.7.11.22	2.7.11.22	Cyclin-dependent kinase
Map03430	Mismatch repair	3.6.1.-	3.6.1.-	3.6.1.-	Hydrolases acting in phosphorus-containing anhydrides
Map03440	Homologous recombination	3.1.-.-	3.1.-.-	3.1.-.-	Hydrolases acting on ester bonds
Map03450	Nonhomologous end-joining	2.7.11.1	2.7.11.1	2.7.11.1	Nonspecific serine/threonine protein kinase
		2.7.7.7	2.7.7.7	2.7.7.7	DNA-directed DNA polymerase
		3.1.-.-	3.1.-.-	3.1.-.-	Hydrolases acting on ester bonds
		4.2.99.-	4.2.99.-	4.2.99.-	Other carbon-oxygen lyases

[a]Previously identified by KEGG as entry "Tb11.01.7110".
[b]Previously identified by KEGG as entry "LmjF32.2240".

SepCysS. One possible explanation is that, while this pathway is essential to archaea (that do not possess the direct pathway for Cys-tRNACys formation), it is not for the Tritryps. Or yet, this enzyme has a particular gene sequence or structure, not yet examined experimentally.

The enzymatic activity represented by EC 2.1.2.9 (methyonyl-tRNA formyltransferase), which is also part of the aminoacyl-tRNA biosynthesis map, was identified by KEGG only for *L. major* and *T. brucei*; this activity was identified by AnEnPi in *T. cruzi*. This enzyme is responsible for adding the formyl radical to tRNAMet, which serves as the tRNA initiator of the polypeptide chain during translation in bacteria. It has the same function in eukaryotes, acting in mitochondria [27]. Since mitochondria have a bacterial evolutionary origin, their translational apparatus follow the bacterial model. Genomic data of the organisms studied in this work consists mainly of nuclear DNA. The occurrence of this enzyme in nuclear DNA is in agreement with the observed absence of tRNA genes in the mitochondrial DNA of Tritryps (kDNA), which are imported from the cytoplasm [37–39].

DNA in cells is often under attack by mutagens, oxygen radicals, and ionizing radiation, and even cellular processes can create mutagenic and cytotoxic DNA lesions which can be lethal to the cell. Organisms possess broad mechanisms of DNA repair to fix damaged DNA and in order to keep viability and genomic stability [40]. In this context, we identified four enzymatic activities with complete EC numbers (four digits) from three DNA repair pathways: base excision repair (EC 3.1.11.2), nucleotide excision repair (EC 2.7.11.22), and nonhomologous end-joining (EC 2.7.11.1 and EC 2.7.7.7) (Table 2).

The enzyme exodeoxyribonuclease III (3.1.11.2—Figure S3 and Table S4) is responsible to catalyze the degradation of double-stranded DNA acting progressively in a 3′ to 5′ direction, releasing 5′-phosphomononucleotides on base excision repair (BER) pathway. The enzymes of this pathway are conserved from bacteria to man, but mammalian enzymes frequently add in, within a larger structural framework, the catalytic core domains of bacterial enzymes [40, 41].

Cyclin-dependent kinase (EC 2.7.11.22) from nucleotide excision repair (NER) is linked to a complex called holo-TFIIH complex (Figure S4 and Table S5). This is a multiprotein complex required not only for transcription but also for nucleotide excision repair. This enzyme is responsible for the phosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II in the absence of promoter opening [42].

Nonhomologous end-joining (NHEJ) is a kind of recombination that links the ends from broken nonhomologous chromosomes. The core NHEJ components are conserved from yeast to mammals and consist of the XRCC4/DNA-Ligase IV complex and the Ku70/Ku80 heterodimer. Both protect exposed DNA of degradation. First, the catalytic subunit, formed by DN-APKcs (EC 2.7.11.1—nonspecific serine/threonine protein kinase) and Artemis, is recruited. The DNA-PKcs phosphorylate the Ku heterodimer and also the Artemis complex which corresponds to a nuclease. Interactions between such protein complexes approximate

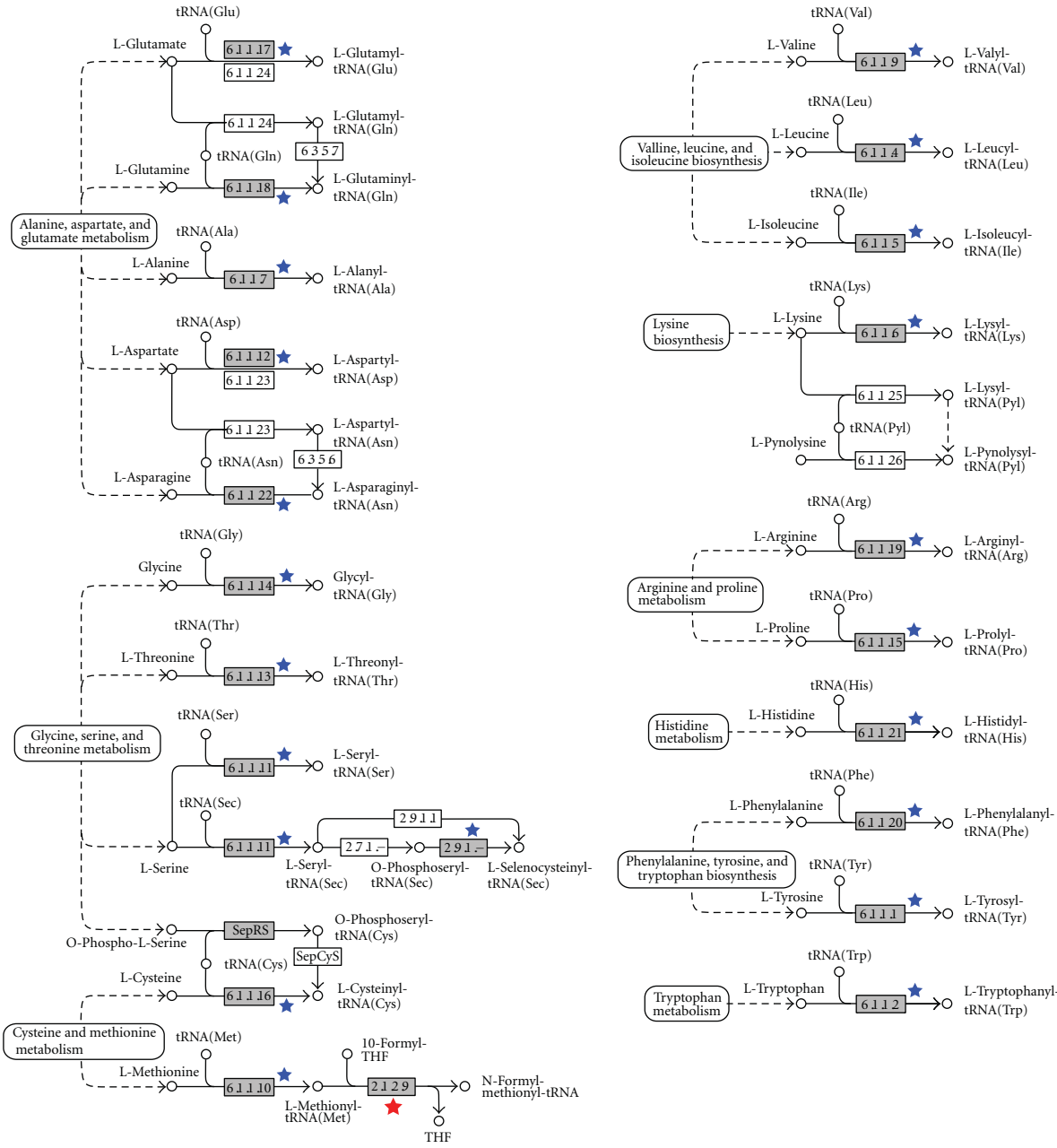

Figure 2: Aminoacyl-tRNA biosynthesis map. Gray boxes represent enzymes identified by AnEnPi for the Tritryps. White boxes are those that were not identified by KEGG neither by AnEnPi. Blue star: ECs identified by KEGG for the Tritryps. Red star: ECs identified by KEGG for *L. major* and *T. brucei*. Modified from http://www.genome.jp/kegg/pathway/map/map00970.html.

the chromosomal ends. Another enzyme whose participation is essential in such complex is the DNA-directed DNA polymerase (EC 2.7.7.7) which fills in the gaps when the ends are joined (Figure S6 and Table S7) [43–45].

3.3. Specific Enzymes and Functional Analogs between the Tritryps and Homosapiens as Potential Therapeutic Targets. Data produced by the genome projects of the Tritryps allowed researchers to establish new strategies to solve the problems caused by these diseases, which affect a great percentage of the world's population [46]. The majority of the proposed drugs so far were discovered many years ago and several of them are toxic, have low efficacy, and the risk of resistance development is also a possibility [7]. To search for functional analogs that could serve as potential candidates as drug targets, we looked for the presence of these enzymes between the Tritryps and *H. sapiens*, by comparing their primary structures. One case meeting these criteria was identified: the exodeoxyribonuclease III (EC 3.1.11.2) from BER pathway.

Exodeoxyribonuclease III is an exonuclease that cleaves the 5′ side of an AP (apurinic/apyrimidinic) site, acting in

the repair pathway by base excision [47]. In *Escherichia coli* this enzyme is a DNA-modifying enzyme, very frequently used in molecular biology, which degrades single-stranded DNA as a substrate. We searched for more information about the inhibitors of this enzyme in the BRENDA database (http://www.brenda-enzymes.org/). According to Hoheisel [48], double-stranded DNA was found to be a competitive inhibitor of the enzyme activity. Other known inhibitors are EDTA (Ethylenediamine tetraacetic acid) [49], Mn^{2+} at concentrations above 5 mM [50], NaCl [48], p-chloromercuribenzoate [51], PNA (Peptide nucleic acids) [52], and $ZnCl_2$ [51, 53].

Apurinic/apyrimidinic sites are very toxic to cells if not repaired. These sites can be generated by normal aerobic metabolism, UV light, or H_2O_2. Exodeoxyribonuclease III (*xthA* gene) can be considered a relevant target for Tritryps because it plays an essential role in the BER pathway, a key repair system to neutralize DNA oxidative stress. *E. coli xthA* mutant strains hold a residual AP endonucleolytic activity due to the protein encoded by the *nfo* gene, the endonuclease IV (Endo IV). Mutants of *nfo* or *xthA* genes are generally sensitive to oxidizing agents [54]. Some authors pointed out that Exo III is involved in the protection of *E.coli* cells against the toxic effects of UV light, H_2O_2 [54–57] and is necessary to induce DNA damage repair [58].

Moreover, we have also identified a potential therapeutic target unique for *L. major*, the DNA 3-methyladenine glycosilase II (EC 3.2.2.21). This enzyme consists in a glycosilase which breaks the bond between alkylated nitrogenated bases and their phosphate group, removing it and leaving an AP site [59, 60].

O-phosphoseryl-tRNA synthetase (EC 6.1.1.-), assigned to the Aminoacyl-tRNA biosynthesis map, was identified as a specific activity in Tritryps when compared with *H. sapiens*. This enzyme, today designated by the EC number 6.1.1.27, catalyzes the alternative formation of Cys-tRNACys [61], as previously described.

The TDR Targets database (http://tdrtargets.org/) integrates genetic and biochemical information to pharmacological data, all related to (primarily) tropical pathogens. The main objective is to assist the search for targets using an integrative platform [62]. None of the two ECs identified (EC 3.1.11.2 and EC 6.1.1.27) had any information related to the Tritryps in this database. This suggests that the approach used in this work may increase the number of possible drug targets. However, exodeoxyribonuclease III (EC 3.1.11.2) is assigned as a potential target in this database, but for other organisms. In addition, DNA 3-methyladenine glycosilase II (EC 3.2.2.21), which in this work was identified only in *L. major*, is also assigned as a potential target (again, for other organisms, not for *Leishmania*).

None of the enzymatic functions disclosed in this work has a resolved 3D structure in the PDB database for any of the Tritryps. Use of resolved 3D structures, as well as other types of information like functional studies, is paramount to advance research on these enzymes, to ensure that they are indeed possible targets for drug development. In the present work, we have studied only a part of the pathways assigned to the GIPPs in KEGG. We have left aside other important pathways such as those related to protein folding, sorting, and degradation, consisting in about 7 additional maps with several enzymes. Moreover, KEGG has already integrated more information and maps to the GIPPs, since it is updated weekly. In the future, a thorough reevaluation of the available data may disclose new cases of analogy and/or new specific enzymes.

The utilization of computers is constantly increasing in the field of drug discovery, because of the great potential in speeding up the identification of suitable targets and useful compounds and also (arguably the most important feature) in reducing costs. In this work, the development and utilization of computational methods allowed us to identify, in the genetic information processing pathways of Tritryps, specific and nonhomologous isofunctional enzymes (NISE). The identification of NISE allowed the construction of an enriched list of proteins (containing not only organism-specific enzymes) that must be further studied to be validated as drug targets. Among these studies, we can cite (i) the obtention of crystals of the selected proteins to allow the construction of 3D models by molecular modeling, (ii) molecular dynamics and docking studies, to obtain a refined representation of their structure, including movement and possibly other interacting molecules as well, and (iii) a series of functional studies to determine their kinetics, expression patterns, stability, essentiality, and so forth.

Acknowledgments

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Research Article

Molecular Cloning and Characterization of P4 Nuclease from *Leishmania infantum*

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Parasite of the genus *Leishmania* is reliant on the salvage pathway for recycling of ribonucleotides. A class I nuclease enzyme also known as P4 nuclease is involved in salvage of purines in cutaneous Leishmania species but the relevant enzymes have not been characterized in *Leishmania infantum* (*L. infantum*). The aim of this study was to clone and characterize the gene encoding class I nuclease in *L. infantum*. DNA extracted from *L. infantum* was used for amplification of P4 nuclease gene (Li-P4) by PCR. The product was cloned, sequenced, and expressed in *E. coli* for further characterization. Analysis of the sequence of Li-P4 revealed that the gene consists of an ORF of 951 bp. Sequence similarity analysis indicated that Li-P4 has a high homology to relevant enzymes of other kintoplastids with the highest homology (88%) to p1/s1 class I nuclease from *L. donovani*. Western blotting of antirecombinant Li-P4 with promastigote and amastigote stages of *L. infantum* showed that this nuclease is present in both stages of parasite with higher expression in amastigote stage. The highly conserved nature of this essential enzyme in *Leishmania* parasites suggests it as a promising drug target for leishmaniasis.

1. Introduction

Protozoan parasites of the genus *Leishmania* cause a spectrum of clinical disease, including cutaneous, mucocutaneous, and visceral leishmaniasis (VL). Approximately 12 million people are infected with this parasite worldwide with 1.5–2 million new cases occurring each year [1]. *Leishmania* parasites are dimorphic organisms which exist as promastigotes in extracellular stage and in the sandfly midgut, and as amastigote that lives intracellularly in the phagolysosomes of macrophages in the mammalian host cells [2, 3]. Unfortunately, currently available treatment regimens are nonselective drugs with significant toxicity and limited efficacy [4, 5]. On the other hand, efforts aimed at the development of vaccines have only achieved low levels of protection in trials in human subjects [6, 7]. Thus, there is an urgent need to identify novel molecular targets that can be exploited for drug development, vaccine design, or both. Purine salvage pathway seems as an attractive target for drug development against *Leishmania*. Trypanosomatid protozoa such as *Leishmania* are purine auxotrophs and are totally dependent upon their hosts to provide purine nucleotides for their survival, growth, and multiplication [8, 9]. It has shown that *Leishmania* promastigotes possess a unique class I nuclease, [10, 11] that involves the salvage of preformed purines through the hydrolysis of either 3′-nucleotides or nucleic acids [12–16]. An intracellular amastigote-specific protein, P-4, with class I nuclease activity has also been

identified in *L. pifanoi* by Kar et al. [17] and by our group in *L. major* [18]. However, there was no data about the presence and characteristics of this nuclease in *L. infantum*, the causative agent of infantile human leishmaniasis. The aim of this study was to clone and characterize the P4 nuclease from *Leishmania infantum.*

2. Materials and Methods

2.1. Parasite and DNA Extraction. In this study, Iranian strain *of Leishmania infantum* was used. Promastigotes were cultured at 26°C in RPMI 1640 medium with glutamine (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum (Sigma-Aldrich). Organisms were harvested in logarithmic phase and washed with phosphate buffer saline (PBS, pH 7.2). Parasites were disrupted in lysis buffer (50 mM NaCl, 50 mM EDTA, 1% SDS, 50 mM Tris-HCl, pH 8.0) and incubated overnight with proteinase K (100 mg/mL, Sigma-Aldrich) at 37°C. DNA was then purified by phenol-chloroform extraction and ethanol precipitation.

2.2. PCR Amplification. A pair of primers was designed based on P4 gene sequence previously reported for cutaneous leishmaniasis (CL) strains: forward, 5′-CATATGTGGGGCTGCGTGGGTCACAT-3′ and reverse, 5′-TACTCGAGCACCTCGCTTCGGACG-3′ [19]. Each PCR reaction contained 200 ng DNA, 10 p mol each of forward and reverse primers, 1.5 mM Mgcl2, 200 μM dNTPs, 1 × PCR buffer, 2 unit of *Pfu* DNA polymerase (Fermentas) and up to 25 μL d H2O. PCR amplification was carried out in 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 60 sec, and extension at 72°C for 60 sec with a final extension cycle at 72°C for 20 min. PCR products were electrophoresed on 1.5% agarose gel and stained by ethidium bromide. The DNA bands were visualized under an ultraviolet light (UV transilluminator) and documented.

2.3. Gene Cloning. The PCR product was purified by PCR product purification kit (Roche) and ligated into the pGEM-T easy vector (Promega). The ligation reaction was transformed into DH5α (Promega) competent cells and plated on Luria-Bertani (LB) agar, containing ampicillin (50 mg/mL), 5-bromo-4 chloro-3-indolyl-β-D-galactoside (X-gal: 20 mM), and Isopropyl thio-β-D-galactoside (IPTG: 200 mg/mL). The white colonies containing recombinant plasmid were selected [20] for plasmid extraction and PCR screening [21]. Then cloning was verified by restriction digestion and sequencing.

2.4. Expression and Purification. The pGEM vector containing Li-P4 gene was digested with Nde I and Xho I and the insert was purified, subcloned into the Nde I - Xho I digested pET28a (Novagen) expression vector and transformed into the *E. coli* BL21 (Novagen). The bacteria containing pET28a-Li-P4 was cultured in LB broth medium and grown until OD = 0.5. Expression of recombinant P4 was induced by addition of 25 μL of 1 mM IPTG then incubated for further 4 h at 37°C and analyzed by SDS-PAGE [22]. For purification of recombinant Li-P4, *E. coli* BL21 containing expression vector was cultured in 1 liter LB broth medium, and following induction with IPTG, the pellet was collected by centrifugation. The bacterial sediment was disrupted in 5 mL lysis buffer (PH = 8, 50 mM NaH2PO4, 300 mM NaCl) by sonication, centrifuged for separation of soluble and insoluble fraction and analyzed by SDS-PAGE. Since the recombinant protein was expressed as 6 His-tag fusion, the Ni-NTA column (Qiagen) was used for purification. Briefly, Li-P4 inclusion body was dissolved in lysis buffer containing 8 M urea and passed through Ni-NTA column that previously equilibrated with related buffer. The column was washed and recombinant protein was released and collected using 250 mM Imidazol. The purified protein then was dialysed against PBS and used for further experiments.

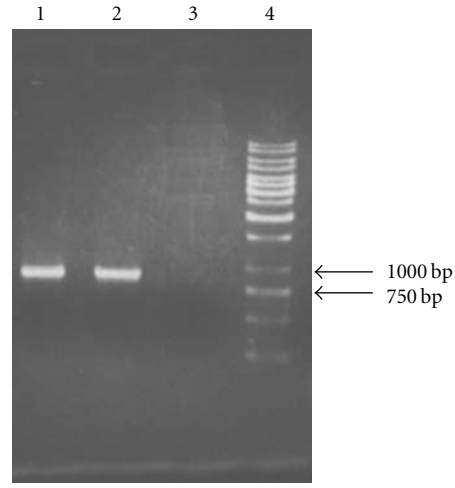

Figure 1: Amplification of P4 nuclease gene of *Leishmania infantum*. Lane 1 and Lane2: 951 bp Li-P4 gene PCR product, Lane 3: no DNA, and Lane 4: 1 Kb DNA Ladder.

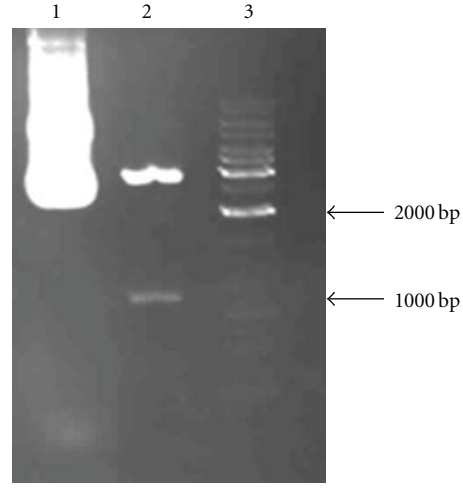

Figure 2: Cloning of Li-P4 nuclease gene in pGEM-t vector. Lane 1: undigested recombinant plasmid, Lane 2: Eco RI-digested recombinant plasmid, Lane 3: 1 kb DNA ladder.

```
L. infantum       HML LA E I ARR QLD DK NK E K I DAMA E V F AQS G P F P S S P DMVQA A C WAD D V K L WR Q R A M G S W  60
L. donovani       HML LA E I AHR QLD DK NK E K I DAMA E V F AQS G P F P S S P DMVQA A C WAD D V K R WR Q Y A M A T W  60
L. major          HML LA E I AHR QLN DE NK E K L DAMA H A F AQS G P F E S S P DMVQA A C WAD D V K R WG Q Y A M A T W  60
L. pifanoi        HML LA E I ARR QLD DK NK E K I QKMA A V F S DS G P F P T S P DMVQA A C WAD D V K L WR Q R G M S T W  60
L. amazonensis    HML LA E I ARR QLD L E NE E K I E LMA A V F S GS G P F P MS P S MVQA A C WAD D V K L WR Q Y A M S T W
                  ******** :***:  : *: **: :  **  . *:  ****   **. ************  *  *  . * . : *

L. infantum       H YF DA P YNP E N IN I T DA I A T VNA V T V S RNM I  SAL K NT K A P LYML N F AW AN L V H I F G D L H Q  120
L. donovani       H F F AA P YNP E N IN I T DA I DT VNA V T V S LDM I  S SL K NT K A P LYML N F AW AN L V H I F G D L H Q  120
L. major          H F FDK P YNP E D IN I T DP V A T VNA V T V S RNM V  T SL R RT N A P LY L L N F AW VN L V H I L G D L H Q  120
L. pifanoi        H YYDK V YNP E N IN I T DP V N T VNA L T A S RNM V  T SL K K S K A P LY L L N F AW VN L V H I F G D L H Q  120
L. amazonensis    H F YAM P YNP G N IN I T DP V N T VNA V T V C LDM V  T SL K NS K A P LY L L N F AW VN L V H I F G D L H Q  120
                  * : :    ***  : *****. :  ****: * . .  : * : : * : . : : ****: ***** . ****** : * * * *

L. infantum       P LHT I S R Y S S E Y P HG D K G G Q P LHT I V R G T S L S P HG D K E A I C T G T P P R Y Q R P L S Y T D L F A L  180
L. donovani       P LHT I S R Y S S E Y P HG D K G G N L I Q V M V G R K S L R L HA L W D N I C T G A P P R Y Q R P L S Y T D L F A L  180
L. major          P LHT T S R Y S S E Y P HG D K G G N E V E V Q V G K R K V N L HA V W D N I C S G T P P R Y K R P L S Y T D L F A L  180
L. pifanoi        P LHT I S R Y T T A Y P HG D Q G G N A V S V R A G R R K V K L HA L W D N I C T G A P P R Y Q R P L S Y T D L F A L  180
L. amazonensis    P LHT I S R Y T T A Y P HG D Q G G N A I S V R V G G K K V K L HA L W D N I C S A T P P R Y Q R P L S H T D L F A L  180
                  **** ***: :  *****: **:  :  .  .    . :  *.  :  **: . : **** : ***** : ** * ** *

L. infantum       A A T A D R L L E T Y I F P E A L R T L V D V M A I H E E S H M F A V N T S Y P G V T P G A T L S E A Y L A R C K R V A  240
L. donovani       A A T A D R L L E T Y I F P E A L R T L V D V M A I H E E S H M F A V N T S Y P G V T P G A T L S E A Y L A R C K R V A  240
L. major          A A T A D R L L E T Y T F P E A L R T L V D V V A I H E E S H M F A V N T S Y P G V T P G A T L S D A Y L A R C K R V A  240
L. pifanoi        S A T A D R L V E T Y T F S E E L R T L V S V K A I H E E - Y M F A V N T S Y P G V T P G A T L S D A Y L D K C K R V A  239
L. amazonensis    S A T A D G L V E T Y T F S E A L E T L V D V M A I H E E S Y M F A V N T S Y P G V T P G G T L S R A Y L D K C K R V A  240
                  : **** * :*** * . * * . ***. * ***** : ************** . *** *** : * * * *

L. infantum       E A R L T L G G Y R L G  252
L. donovani       E A R L T L G G Y R L G  252
L. major          E A R L T L G G Y R L G  252
L. pifanoi        E A R L T L G G Y R L G  251
L. amazonensis    E A R L T L G G Y R L G  252
                  ************
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Figure 3: Alignment of deduced amino acid sequence of *L. infantum* amastigote P4 with class I nuclease from *L. donovoni* (GeneBank Accession no. ABE69185.1), *L. major* (Accession no. XP_001684745.1), *L. amazonensis* (Accession no. AAO65599.1), and *L. pifanoi* (Accession no. AAD48894.2). 5 conserved domains along with several semiconsereved domains between this nuclease are indicated by continuous stars.

Results of purification were controlled by %10 SDS-PAGE gels.

2.5. Production of Rabbit Antiserum against Recombinant Li-P4. An adult rabbit (New Zealand White) was immunized subcutaneously with 200 mg of purified rL-iP4 emulsified in an equal volume of complete Freund's adjuvant (CFA) (Sigma-Aldrich), followed by a subcutaneous boosting 2 weeks later with 100 μg of recombinant P4 protein in incomplete Freund's adjuvant (IFA) (Sigma-Aldrich). Two weeks later, the rabbit was boosted intravenously with 50 μg of rLiP4, and blood was collected 1 week later.

2.6. Western Blotting. For determination of P4 nuclease, lysates of promastigotes and amastigotes of *L. infantum* were incubated with rabbit antiserum raised against rLi-P4 followed by incubation in 1/5000 dilution of HRP conjugated goat antirabbit IgG (Sigma-Aldrich). After washing to remove unbound antibodies, membranes were incubated in 1/5000 dilution of HRP conjugated goat antirabbit IgG (Sigma) and processed for ECL. The protocol for detection of *Leishmania* elongation factor-1a (as an internal control) in lysates of promastigotes and amastigotes using mouse monoclonal anti-EF-1a and HRP-conjugated antimouse IgG was as described previously [23].

3. Results and discussion

3.1. Gene Cloning. After culture, the *Leishmania infantum* was subjected to DNA extraction and Li-P4 gene amplification. PCR amplification of P4 nuclease gene from *L. infantum* results in a PCR product of 951 bp that was in expected size (Figure 1). The PCR product was cloned in the pGEM-T easy vector using T-A cloning method and confirmed by restriction digestion (Figure 2).

3.2. Sequencing and Multiple Alignments. Li-p4 was amplified from *L. infantum* amastigote using forward and reverse primers based on the sequence previously reported for cutaneous leishmaniasis (CL) strains. The PCR product was

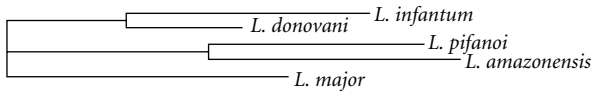

Figure 4: Phylogenetic tree of P1/S1 nuclease gene sequences among species of *Leishmania*. This tree shows the relatedness between P4 nuclease from *L. infantum* and P1/S1 nuclease *L. donovani.*

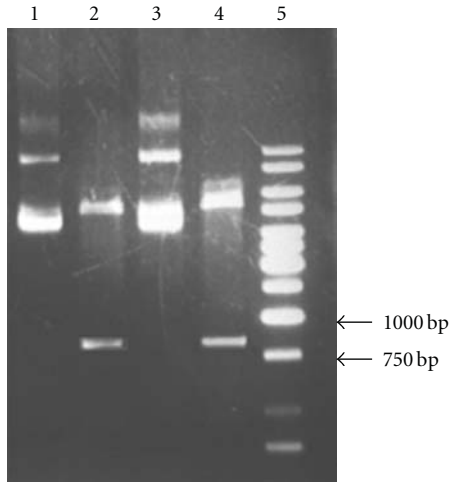

Figure 5: Cloning Li-P4 nuclease gene in pET 28a vector. Lane 1 and 3: recombinant plasmid, undigest, Lane 2 and 4: Nde I - Xho I digested recombinant plasmid, and Lane 5: 1 Kb DNA Ladder.

cloned into the pGEM-T easy vector and sequenced. The nucleotide sequence was submitted to the GeneBank/NCBI Data Base under accession number ABY27514.1. Result of sequencing revealed that the gene consists of an ORF of 951 base pairs with a predicted molecular mass of 33 kDa. Structural analysis using Signal P software showed a signal sequence consisting of the first 30 amino acids and two putative N-linked glycosylation sites (at amino acid residues 108 and 251). A search of the SWISSPRO database with the predicted amino acid sequence revealed a significant similarity to a number of proteins belonging to the Class I nuclease family as shown by the alignment using the ClustalW program (Figure 3) [24]. The protein with highest similarity to P4 nuclease was P1/S1 secretory nuclease of *L. donovani* (GenBank accession no. ABE69185.1) [23] (Figure 4). Other proteins with significant similarity were: p1/s1 nuclease of *L. major* (accession no. XP_001684745.1) [18], single-strand-specific nuclease from *L. pifanoi* (accession no. AAD48894.2), and P4 nuclease of *L. amazonensis* (accession no. AAO65599.1); identities and positivities between Li-P4 nuclease and each of these proteins are shown in Table 1. Analysis of the sequences for conserved domains using the NCBI protein blast tool showed that in spite of some heterogeneity in primary structure, all of these proteins contained a conserved nuclease domain specific to class I nuclease family.

3.3. Expression and Purification of Recombinant P4 Nuclease. For expression of recombinant Li-P4, the PCR product was

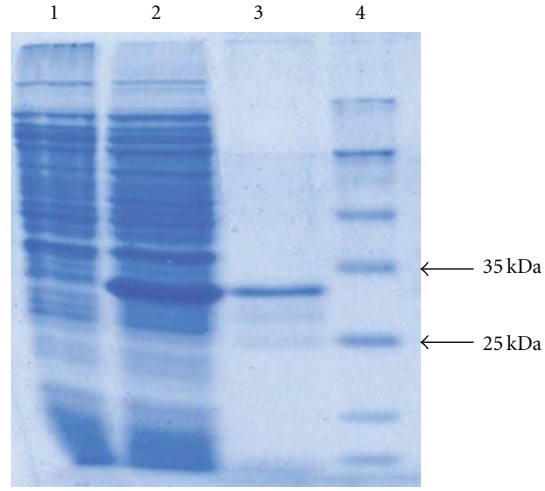

Figure 6: SDS-PAGE analysis of recombinant Li-P4 produced in BL21, Lane 1: bacterial lysate before induction, Lane 2: bacterial lysate after induction with IPTG, Lane 3: purified recombinant Li-P4, and Lane 4: molecular weight marker.

Table 1: Similarity of predicted amino acid sequence of Li-P4 nuclease with different promastigote-specific P1/S1 nuclease of various *Leishmania* species.

Leishmania species	Identity %*	Positivity %**
P4 nuclease (*Leishmania infantum*)	100	100
P1/S1 secretary nuclease (*Leishmania donovani*)	89	92
p1/s1 nuclease (*Leishmania major*)	81	89
single strand-specific nuclease (*Leishmania pifanoi*)	77	85
P4 nuclease (*Leishmania amazonensis*)	75	85

* Identity is referred to the extent to which two sequences are invariant.
** Positivity is referred to the extent to which two sequences possess amino acids with the same physicochemical properties.

subcloned in the NdeI-XhoI site of pET28a (Novagen) in frame with C-terminal 6His-tag and transformed into the *E. coli* Bl21 (Figure 5). Induction of recombinant protein expression by IPTG resulted in a high level of expression. The molecular mass was found to be 33 kDa, which was slightly higher than the native mature protein due to the addition of a Hexa-His-tag sequence. Purification of recombinant protein was performed by affinity chromatography on Ni-NTA resin, and fractions were analyzed by SDS-PAGE (Figure 6).

3.4. Differential Expression of P4 Protein in the Amastigotes. To examine further developmental regulation of expression of P4 nuclease in *L. infantum*, polyclonal rabbit antisera raised against recombinant Li-P4 were used to probe western blots containing lysates of promastigotes and amastigotes of *L. infantum*. As shown in Figure 7(a), a strongly reactive band of the expected mass (33 kDa) was observed in lysates of amastigotes (lane 3) but appeared fairly in lysates of procyclic or metacyclic promastigotes (lane 1 and 2, resp.).

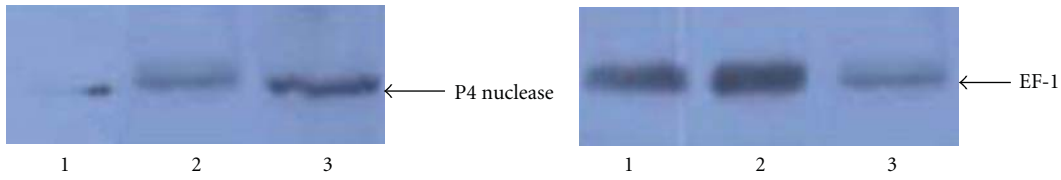

Figure 7: Analysis of P4 gene expression in different life cycle stages of *L. infantum*. (a) Promastigote and amastigote cell lysates were analyzed by immunoblotting using rabbit anti-recombinant Li-P4. Lane 1: Log phase promastigotes. Lane 2: Stationary phase promastigotes. Lane 3: Amastigotes. (b) The same blot as in a was probed with rabbit anti Elongation Factor 1-alfa antibody. The results indicate higher expression of P4 nuclease in amastigote stage of parasite.

To ensure that equivalent amounts of promastigote and amastigote proteins were loaded and transferred for detection, a similar blot was incubated with monoclonal antibody against EF-1α (Upstate Biotechnology, USA), a protein that is expressed in all life cycle stages (Figure 7(b)). As it is shown in Figure 7(b), the abundance of EF-1α in promastigote samples (lane 1 and 2) was similar to that one in amastigote lysates (lane 3).

P-4 is a single-stranded specific class I nuclease that was identified initially in *L. amazonensis* [16] and then characterized in *L. pifanoi* by Kar et al. [17]. Recently, we isolated a class I nuclease gene from amastigotes of *L. major* [18]. In the present study we characterized this nuclease in *L. infantum*. Similarity analysis revealed that the sequence of corresponding protein from *L. infantum* has high sequence homology to the P4 nuclease of *L. donovani*, *L. major, L. Pifanoi,* and 3′-nucleotidase/nuclease enzymes previously described in different trypanosomatids [11]. The alignment results indicated the presence of 5 main conserved domains between these nucleases from different species.

The class I nuclease from promastigote stage of some *Leishmania* species have been extensively studied [8, 13, 19, 25, 26]. It has shown that expression of this nuclease upregulated significantly in response to purine starvation [13, 17] which is consistent with the opinion that class I nuclease enzyme plays a vital role in providing purines for growth and development of *Leishmania* parasites. Since there is no a homologue enzyme with similar properties in mammalian tissues, this enzyme can serve as a chemotherapeutic target for selective targeting of *Leishmania* during infection [11]. Considering that *Leishmania* is present as amastigotes in mammalian tissues and this stage is responsible for disease manifestations, the higher expression of the class I nuclease in amastigote stage further supports the use of this enzyme as a target.

4. Conclusion

In conclusion, the results of the present study revealed that Li-P4 nuclease belongs to the class I nuclease group of enzymes that are highly expressed in amastigote stage of *L. infantum* and could be exploited as target for chemotherapy.

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Research Article

Trypanosoma cruzi Coexpressing Ornithine Decarboxylase and Green Fluorescence Proteins as a Tool to Study the Role of Polyamines in Chagas Disease Pathology

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Polyamines are essential for *Trypanosoma cruzi*, the causative agent of Chagas disease. As *T. cruzi* behaves as a natural auxotrophic organism, it relies on host polyamines biosynthesis. In this paper we obtained a double-transfected *T. cruzi* parasite that expresses the green fluorescent protein (GFP) and a heterologous ornithine decarboxylase (ODC), used itself as a novel selectable marker. These autotrophic and fluorescent parasites were characterized; the ODC presented an apparent Km for ornithine of 0.51 ± 0.16 mM and an estimated V_{max} value of 476.2 nmoles/h/mg of protein. These expressing ODC parasites showed higher metacyclogenesis capacity than the auxotrophic counterpart, supporting the idea that polyamines are engaged in this process. This double-transfected *T. cruzi* parasite results in a powerful tool—easy to follow by its fluorescence—to study the role of polyamines in Chagas disease pathology and in related processes such as parasite survival, invasion, proliferation, metacyclogenesis, and tissue spreading.

1. Introduction

Protozoan parasites cause disease, death, and severe social and economic losses, particularly in developing countries. *Trypanosoma cruzi* is the causative agent of Chagas disease, which affects 16–18 million people mainly in Latin America [1, 2].

T. cruzi is a digenetic parasite whose life cycle involves an insect vector (from Reduviidae family) and a vertebrate host. The parasite undergoes deep morphological, biochemical, and physiological changes as an adaptive response to the extreme environmental variations that it faces around the life cycle. The epimastigote form (E) is a proliferative and noninfective extracellular stage, present in the insect gut. At the insect rectum, it differentiates to metacyclic trypomastigote (MT), an infective stage present in the contaminated insect faeces, which enter the vertebrate host through the insect wound or via mucosal membranes. Once inside, the trypomastigotes invade different kinds of cells, including macrophages, fibroblasts, and muscle cells [3]. There, they transform to amastigote stage and replicate to reach a critical number of parasites per cell, then they differentiate to blood stream trypomastigotes (BT), host cells burst, and release trypomastigotes, which either infect other cells or get sucked up by a bug vector [4–6]. The recent construction of stable green fluorescent protein (GFP) expressing *T. cruzi* strains has resulted in a useful tool to follow processes as cell invasion and infection progression [7, 8].

Polyamines are ubiquitous cell compounds essential for macromolecular biosynthesis [9, 10] and normal cell survival, proliferation and differentiation [11–13]. Several studies related to polyamine metabolism in pathogenic protozoa

have been carried out with the aim to affect the proliferation, infectivity, or differentiation of these organisms and find new therapeutic targets against parasitic diseases [1, 14, 15]. With this kind of approach it was discovered that the acute infections of *Trypanosoma brucei brucei* in mice as well as the human African sleeping sickness, caused by *T. brucei gambiense*, can be treated by α-difluoromethylornithine (DFMO), the specific and irreversible ornithine decarboxylase (ODC) inhibitor [16–18].

The naturally occurring polyamines in most cell types, including mammalian cells, are putrescine (actually a diamine precursor), spermidine and spermine, while in trypanosomes this last polyamine was only found in trace amounts [11, 19, 20]. There are some differences related to polyamine biosynthetic enzymes between mammalian cells and parasites; however, polyamines are essential for both kinds of organisms. In fact, only two kinds of wild-type cells, Halobacteriales and Methanobacteriales, have been described with the ability to grow in the absence of a detectable polyamine concentration [21, 22].

The main route of putrescine biosynthesis is through the ornithine decarboxylation using the enzyme ornithine decarboxylase (ODC; EC 4.1.1.17), key enzyme capable of regulating cell growth and differentiation [22–24]. Many plants, bacteria and some specific mammalian tissues possess an alternative pathway by arginine decarboxylase (ADC; EC 4.1.1.19) where arginine is converted to putrescine via the intermediate agmatine [25–29].

The next step in polyamine biosynthesis is catalyzed by spermidine synthase (SpdS; EC 2.5.1.16) that transfers an aminopropyl group from the donor decarboxylated S-adenosylmethionine (dcAdoMet) to putrescine, producing spermidine. Spermine synthase (SpmS; EC 2.5.1.22) transfers a second aminopropyl group from dcAdoMet producing spermine. This enzyme is generally absent in the protozoan parasites; however, *T. cruzi* would be an exception since its genome presents two aminopropyltransferase genes, one of them a putative SpmS. This function has been proposed *in silico* but not functionally validated yet [1, 30].

The biosynthesis of polyamines is irreversible, but there is a route for polyamine back-conversion involving two steps: a spermidine/spermine N-acetyltransferase (EC 2.3.1.57) and polyamine oxidase (PAO; EC 1.5.3.13) [31, 32].

In trypanosomatids, spermidine plays a central role because it is the precursor of the derivative N1, N8-bis-glutathionyl-spermidine (trypanothione). This compound, unique in these protozoan organisms, is essential to maintain the redox equilibrium in the parasite cell and plays the metabolic and antioxidant functions of glutathione [33–35]. For this and other reasons, it is not surprising that polyamines are essential for survival and proliferation of *T. cruzi* [36–40]. However, *T. cruzi* is one of the extremely few eukaryotic organisms auxotrophic for polyamines. We have previously demonstrated that *T. cruzi* epimastigotes are unable to synthesize putrescine *de novo* because they contain neither ODC nor ADC enzymatic activity, due to the absence of ODC and ADC genes [38, 41, 42]. This conclusion is also supported by data from the *T. cruzi* genome project [30]. Polyamines seem to be crucial for the other *T. cruzi* stages, particularly in trypomastigote internalization process in, at least, some cellular types, and infection progression as well as for other related parasites infection [6, 43–46]. Since parasites live inside other organisms, numerous essential metabolites can be obtained directly from the host without the need to synthesize them [47]. It seems to be the case for polyamines, where its uptake systems from the parasite together with the ODC enzymatic activity from the host are a concerted and critical system for *T. cruzi* survival and host infection.

In this work we obtained a double-transfected *T. cruzi* strain that expresses the green fluorescent protein (GFP) and a heterologous ODC enzyme. The latter is encoded by the ODC gene, an auxotrophy rescuing gene used here as a selectable marker. Then we selected a Y-GFP-ODC clone with high expression of both GFP [7] and ODC activity [38]. These autotrophic and fluorescent parasites were characterized as well as the heterologous ODC enzyme activity. As *T. cruzi* is a naturally auxotrophic organism for polyamines, the autotrophy acquisition could provide information about the role of polyamines in processes such as parasite survival, invasion, proliferation, metacyclogenesis and tissue spreading, under different conditions of polyamines availability natural or induced with polyamine inhibitors; the green fluorescent protein allows to easily follow those processes microscopically. Therefore, the double-transfected *T. cruzi* parasite is a powerful new tool to study the role of polyamines, naturally provided by the host ODC, in the parasite cell cycle. This would help to better understand Chagas disease pathology and to find targets for new therapies.

2. Materials and Methods

2.1. Chemicals. Brain heart infusion, tryptose, and yeast extract were obtained from Difco Laboratories (Detroit, MI, USA). Minimal essential medium (SMEM), heat-inactivated fetal calf serum (FCS), and amino acids were from GIBCO BRL (Gaithersburg, MD, USA); vitamins, bases, haemin, polyamines, the ODC cofactor pyridoxal 5′-phosphate (PLP), protease inhibitors, HEPES buffer, antibiotics, cycloheximide and Rhodamine-conjugated phalloidin were purchased from Sigma (St. Louis, MO, USA). L-[1-^{14}C]ornithine (57.1 Ci/mol) was from NEN Life Science Product, Inc (Boston, MA). The inhibitor α-difluoromethylornithine (DFMO) was a gift from Merrell Dow Research Institute (Cincinnati, OH).

2.2. T. cruzi Epimastigote Cultures. Epimastigote culture of Y strain previously transfected with the green fluorescent protein (GFP) gene (kindly provided by Dr S. Schenkman, Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo (São Paulo, SP, Brazil)) (Y-GFP) was maintained at 28°C in rich media BHT, while the derived culture, Y-GFP retransfected with ornithine decarboxylase (ODC) gene, Y-GFP-ODC population (Y-GFP-ODC strain), and the selected clone (Y-GFP-ODC clone) were cultured at 28°C in a semisynthetic medium (SDM79) which contains only traces of polyamines [38]. Haemin

(20 mg/L), 10% heat-inactivated fetal calf serum and the antibiotics streptomycin (100 μg/mL), penicillin (100 U/mL) and the selective drug G418 (250 μg/mL) were added to all cultures.

To determine the growth kinetics of each strain, a part of Y-GFP culture was maintained in BHT while others were placed in SDM79 medium in absence or presence of 1 mM putrescine or in Diamond in absence or presence of 2 mg/mL glucose. Y-GFP-ODC strain and the derived clone were maintained in SDM79 medium without or with the addition of 5 mM α-difluoromethylornithine (DFMO). Parasite growth was followed by cell counting. Cultures were diluted to 10–20 $\times$ 10^6 cells/mL every 48–72 h, when the stationary phase was reached.

2.3. Parasite Transfection. Epimastigote forms of Y strain had been previously transfected with pROCKGFPNeo and characterized [7, 48]. This recombinant plasmid contains the GFP coding region and sequences that allow a stable DNA integration at the β-tubulin loci [7]. A selected clone from this Y-GFP strain was retransfected with the recombinant vector $pODC_7$, as previously described [38]. $pODC_7$ contains the complete open reading frame (ORF) of ODC gene from *C. fasciculata* [49] cloned into the expression vector pRIBOTEX [50] that integrates at the ribosomal genes loci [38, 51].

After transfection, parasite culture was diluted with rich medium and incubated for 72 h at 28°C to allow its recovery before adding the restrictive culture conditions, consisting in 500 μg/mL Geneticin (G 418) (selection by antibiotic resistance) and semisynthetic medium SDM79 (selection by polyamine autotrophy development). Subsequent growth was followed by cell counting. Samples of *T. cruzi* cultures before and after transfection were taken to analyze cellular morphology, fluorescence, and ODC activity. Drug selection was completed 50 days after electroporation as indicated by the mock-transfected parasites (transfected with empty pRIBOTEX) and the appearance of ODC activity. This time was slightly longer than that seen in each of the previous single-transfection cases (the *T. cruzi* transfection with GFP [7] and with ODC [38], in both cases selected by resistance to 250 μg/mL G418).

2.4. Selection of T. cruzi Y-GFP-ODC Clones. After selection of the double-recombinant Y-GFP-ODC *T. cruzi* strain, cloned subpopulations were obtained by diluting part of the culture to a final density of 0.1 cells/0.2 mL of SDM79 medium supplemented with 10% FCS and 250 μg/mL of G418 and plated in 96-well plates. They were incubated at 28°C in humid chamber for 4-5 weeks and analyzed with a fluorescence or confocal microscope. GFP-expressing clones were selected and transferred into 1 mL of the same medium in culture bottles and incubated at 28°C. Once they reached the plateau the cultures were expanded to 5 mL and ODC activity was assayed (see Section 3).

2.5. Preparation of Cell Extracts and ODC Enzymatic Assays. Cell extracts were obtained and the enzymatic activity was measured by the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine as previously described [52]. Briefly, parasites were collected at the exponential phase of growth. They were washed and lysated by two cycles of freeze-thawing and sonication, in buffer A (50 mM Hepes, pH 7.4, 1 mM DTT, 0.5 mM EDTA and 0.1 mM pyridoxal 5′-phosphate) supplemented with protease inhibitors and 0.5% Nonidet P40. Cell lysates were centrifuged for 10 min at 10000 $\times$g. Supernatant fractions were tested for ODC activity by incubation at 37°C in presence of 2 mM L-[1-^{14}C] ornithine (5 uCi/mL). The enzymatic reaction was performed for the indicated times and stopped by the addition of 0.5 N perchloric acid. The radioactive CO_2 released during the reaction was trapped on a piece of Whatman 3 MM paper soaked with 2 M KOH and then measured in a scintillation counter. Protein concentration was measured, using BSA as standard, according to Bradford [53].

2.6. Kinetic Parameters of Heterologous ODC. Ornithine decarboxylation was carried out at different times, substrate and inhibitor concentrations. The determination of the kinetic constants apparent Km and V_{max} values for ornithine of the enzyme expressed in Y-GFP-ODC were calculated from Lineweaver-Burk plots. Studies of inactivation by DFMO were carried out as previously described [38, 54].

2.7. Trypanosoma cruzi Metacyclogenesis. For *in vitro* differentiation of *T. cruzi* epimastigotes, we have used a method previously described [55, 56] with some modifications. Briefly, 25 $\times$ 10^6 epimastigotes from 15-day-old cultures from Y-GFP and Y-GFP-ODC strain and clone grown in Diamond or SDM79 media, respectively, were harvested by centrifugation at 600 $\times$g for 15 min at 15°C. The parasites were then incubated for 2 h at 37°C in 50 μL of triatomine artificial urine (TAU) medium (190 mM NaCl, 17 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 8 mM phosphate buffer, pH 6.0). After that, parasites were diluted (1 : 100 dilution) in TAU3AAG medium (TAU supplemented with 10 mM L-proline, 50 mM L-sodium glutamate, 2 mM L-sodium aspartate, and 10 mM D-glucose) and maintained for 24 or 48 h in culture flasks at 28°C [55, 56]. Epimastigotes treated with α-difluoromethylornithine (DFMO) or Putrescine (Put) during metacyclogenesis were obtained by addition of 5 mM DFMO or 0,2 mM Put in both TAU and TAUAAG media at the same conditions as controls.

Mixed parasitic forms corresponding to epimastigotes and metacyclic trypomastigotes (E-MT suspensions) were harvested by centrifugation at 600 $\times$g for 15 min and used to infect Vero cells monolayers using 24-well plates containing glass coverslips in D-MEM supplemented with 3% FBS and antibiotics (infection medium). 24 h after infection, cells were washed, fixed, and stained with Rhodamine-phalloidin for the fluorescence microscopy analysis (see below). The metacyclogenesis capacity of these suspensions was estimated by counting the percentage of infected cells. Parasites were directly localized as green structures inside cells, whereas host cell extension was visualized in red, as indicate in the following.

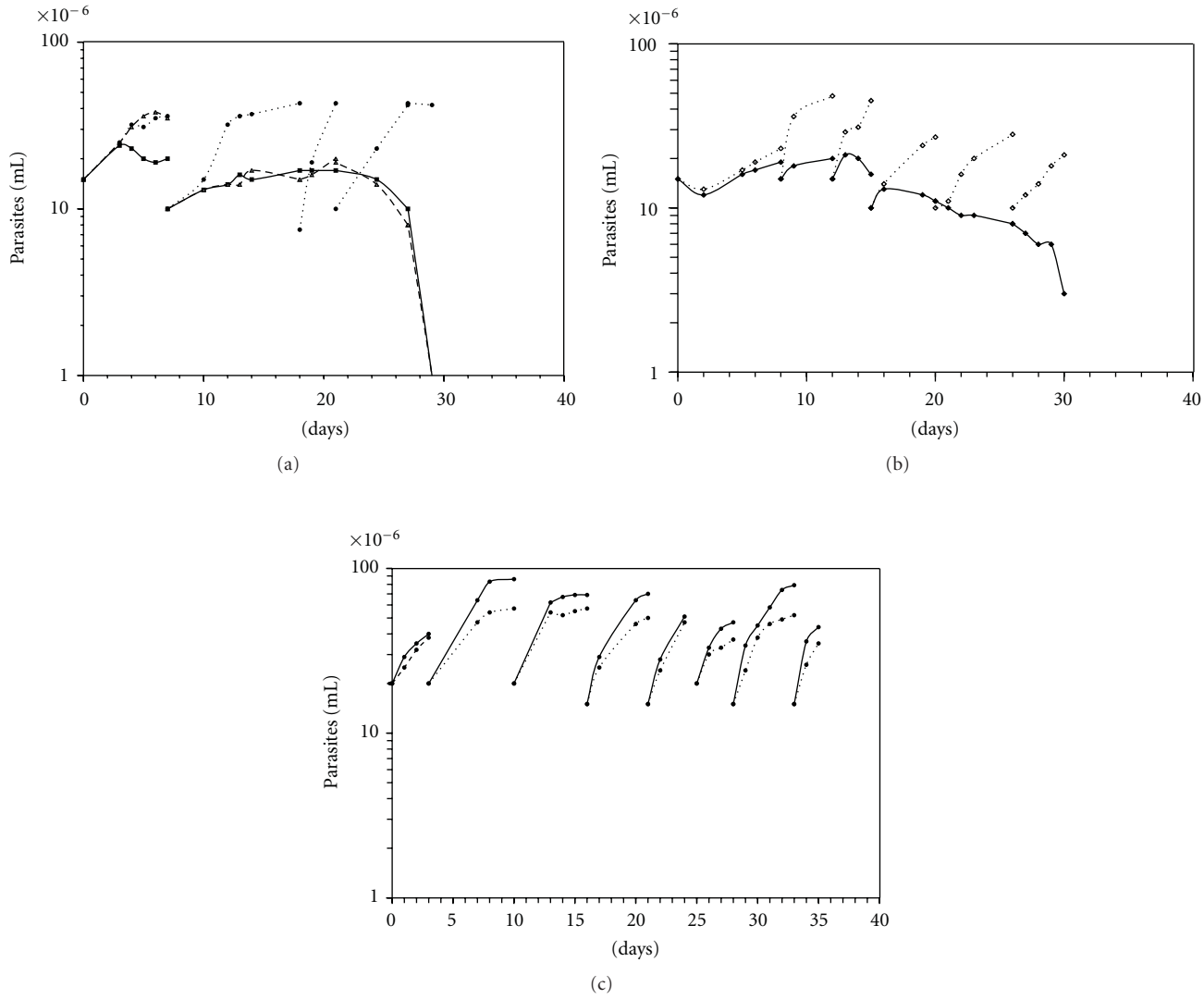
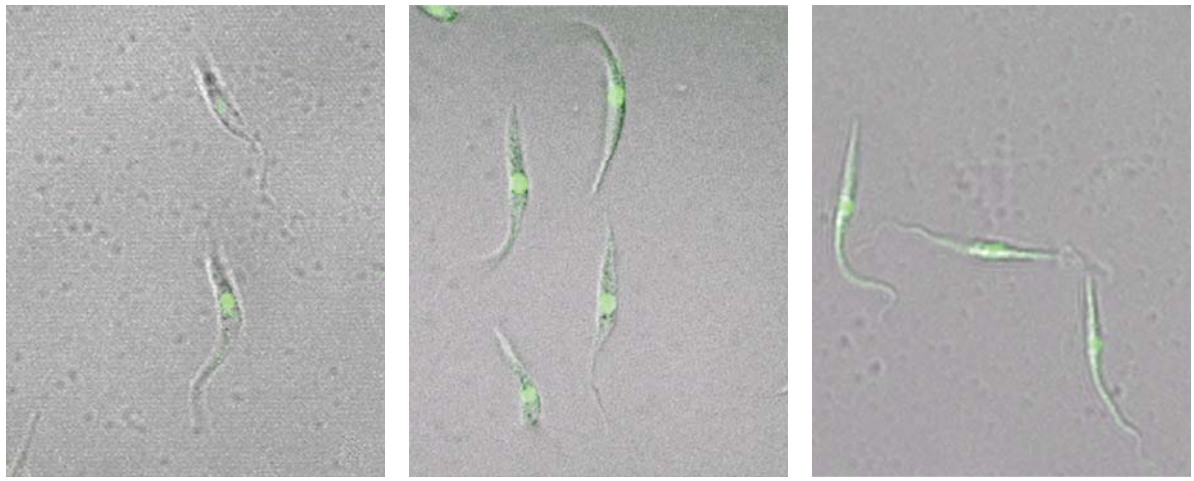


Figure 1: Growth kinetics of Y-GFP and Y-GFP-ODC clone cultured at different conditions. Cultures carried out under the indicated conditions were diluted to 10–20 × 10^6 cells/mL when the stationary phase was reached. (a) Y-GFP cultured in rich media BHT (- -●- -), Diamond (—■—), and in Diamond supplemented with 2 mg/mL glucose (- -△- -). (b) Y-GFP cultured in the semisynthetic medium SDM79 without (—♦—) or with (- -◊- -) 1 mM putrescine. (c) Y-GFP-ODC clone cultured in SDM79 (—●—) or in SDM79 supplemented with 5 mM α-difluoromethylornithine (DFMO) (- -●- -). All cultures were continuously maintained in presence of 250 μg/mL G418.

2.8. Fluorescence Microscopy. For Rhodamine-phalloidin staining, Vero cells were washed three times with PBS to remove free extracellular parasites, fixed with 3% paraformaldehyde solution in PBS for 10 min at RT, washed with PBS, and blocked with 50 mM NH_4Cl in PBS. Subsequently, cells were permeabilized with 0.05% saponin in PBS containing 0.5% BSA and then incubated with Rhodamine-conjugated phalloidin for 1 h at 37°C to label F-actin. Cells were washed three times with PBS and mounted with Mowiol before fluorescence microscopy analysis. Confocal images were obtained with an Olympus FV1000 confocal microscope and the FV 10-ASW 1.7 program (Olympus, Japan). Images were processed using Adobe 7.0 software (Adobe Systems).

3. Results

3.1. Growth Kinetics of Y-GFP and Y-GFP-ODC Strain and Clone. Y-GFP strain cultured in the rich medium BHT, supplemented with G418, maintained the proliferative capacity for long periods of time having a doubling time of 2-3 days and a constant maximal cell density of ~40 × 10^6 parasites/mL (Figure 1(a)). On the other hand, Y-GFP cultured in Diamond, the rich medium habitually used for maintaining this strain [7, 8], grew slowly with a doubling time of approximately 7 days. The maximal cell density (~37 × 10^6 parasites/mL) was scarcely reached at the first-second passage (Figure 1(a)). To discard that the low proliferation rate was due to low availability of glucose, an important source of



(a) (b) (c)

Figure 2: Fluorescence microscopy of Y-GFP (a) and Y-GFP-ODC strain (b) and the selected Y-GFP-ODC clone epimastigotes (c) (1000x).

energy for *T. cruzi* [57], we followed the Y-GFP growth in Diamond supplemented with 2 mg/mL glucose. There were no differences in the kinetic characteristics between both conditions. When Y-GFP strain was cultured in the semi-synthetic medium SDM79 (containing only traces of polyamines), the kinetics of growth (Figure 1(b)) was similar to that observed in Diamond. After 10–15 days of culture under these three conditions, Y-GFP parasites progressively stopped growth, started decreasing, and finally were lysed. However, when Y-GFP was cultured in SDM79 supplemented with 1 mM putrescine the culture could recover the proliferation rate (Figure 1(b)).

The double-transfected Y-GFP-ODC clone grew continuously in SDM79, even better than Y-GFP in BHT, with a doubling time of 2-3 days and a maximal cell density of $\sim 80 \times 10^6$ parasites/mL (Figure 1(c)). Similar results were obtained for the Y-GFP-ODC strain (not shown). The continuous proliferation of the double-transfected Y-GFP-ODC strain and clone in absence of polyamines indicates that the original auxotrophy for diamines of *T. cruzi* was reversed after trans-fection with the heterologous ODC gene, as it was previously described for monotransfected strains [38].

α-difluoromethylornithine (DFMO), the specific and irreversible inhibitor of ODC, altered the growth rate of Y-GFP-ODC clone, particularly the maximal cell density (lower than 55×10^6 parasites/mL) (Figure 1(c)). The partial effect of DFMO on the proliferative rate could be explained in different ways [58, 59] that will be discussed later.

The three cultures were analyzed by confocal microscopy. There were ~80% of parasites expressing GFP in Y-GFP strain, while the Y-GFP-ODC double-transfected strain and the selected clone presented values of ~95%, similar to those described by Pires et al. [7]. The cell morphology of the three cultures was similar (Figure 2).

3.2. ODC Enzymatic Activity in Double-Transfected T. cruzi Epimastigotes. The ODC activity was measured at 72 h and 10, 20, 30, 40, and 50 days after transfection of Y-GFP *T. cruzi* epimastigotes with the heterologous ODC. We have found that double-transfected Y-GFP-ODC cells expressed high levels of ODC activity after 50 days following transfection (Figure 3(a)). It was different from previous reports, where a transient expression (between 48 h and 7 days) was detectable in ODC single-transfected parasites; that expression decreased markedly during the following weeks and recovered at 30–40 days after transfection [38]. This difference could be related to the cell machinery readaptation to transcribe-translate a new foreign and active gene, doing this already with GFP and the neomycin phosphotransferase (neomycin resistance).

The ODC specific activity in the double-transfected Y-GFP-ODC was around 39 ± 4 nmol/h/mg prot. Once the Y-GFP-ODC clone was selected, it showed an ODC activity with little variation during the course of the experiment reaching values of 590 nmol/h/mg prot (Figure 3(b)). The ODC activity in the Y-GFP strain was, as expected, negligible in all assays.

3.3. Properties of the Heterologous ODC Expressed in the Selected Y-GFP-ODC Clone. The kinetic parameters of ODC expressed in the Y-GFP-ODC clone were calculated from a Lineweaver-Burk plot (Figure 4(a)). The estimated V_{max} value was 476.2 nmoles/h/mg of protein, and the apparent Km for ornithine was 0.51 ± 0.16 mM. While the Km value was similar to those obtained previously for other transfected *T. cruzi* strains [38] and for *C. fasciculata* [60], the V_{max} was higher than those obtained in previous assays of single-transfection with ODC gene in DFMO-sensitive strains. Instead, the V_{max} value obtained in Y-GFP-ODC clone was similar to that observed for ODC-transfected strains after reaching DFMO resistance [38, 58, 59].

The assays of *in vivo* ODC turnover (determined by stopping protein synthesis with 50 μg/mL cycloheximide and measuring the remaining enzymatic activity at different

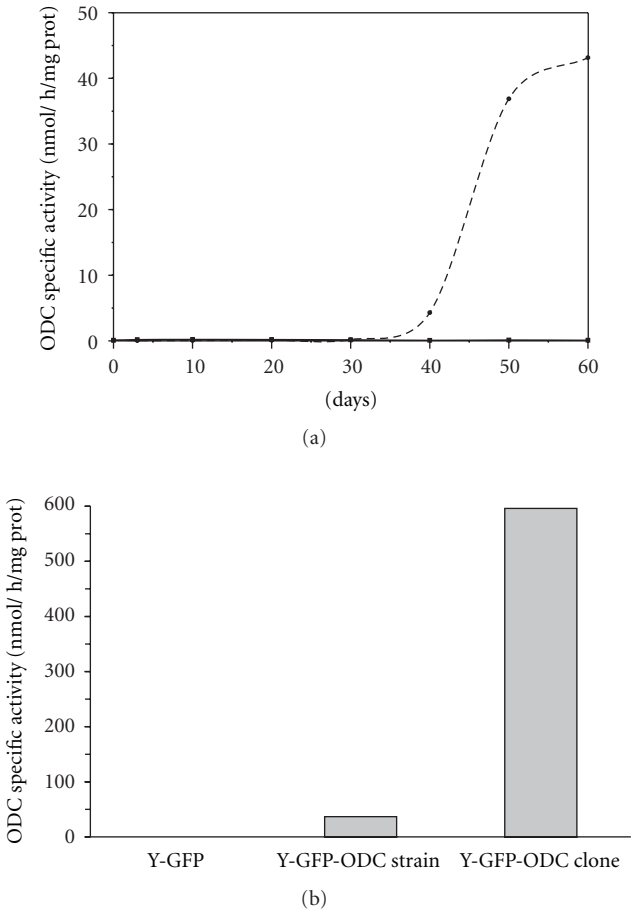


Figure 3: Ornithine decarboxylase (ODC) specific activity in Y-GFP and Y-GFP-ODC cell extracts. (a) ODC specific activity was measured in cell extracts obtained at different times after transfection; (—■—) Y-GFP strain; (- -●- -) Y-GFP-ODC strain. (b) ODC activity in extracts of exponentially growing cultures at 60 days after transfection and clone selection. All values are the average of at least three experiments.

times) showed that the ODC expressed in Y-GFP-ODC clone was very stable, with a half-life longer than 6 h (data not shown), remarkably longer than the half-life of the ODC expressed in *Crithidia fasciculata*, its natural cellular context [60]. This notorious change in stability has already been observed in experiments of single transfection in other strains of *T. cruzi* [38].

When Y-GFP-ODC clone was cultured in presence of DFMO, the inhibitor was effectively internalized into the cell causing a notable ODC activity inhibition (Figure 4(b)). Additional in vitro assays showed that DFMO inhibits the ODC reaction in a dose-response way, with an IC_{50} lower than 0.13 mM (Figure 4(c)).

Based on these observations, the best explanation of why DFMO caused only a moderated effect on Y-GFP-ODC clone proliferation (Figure 1(c)) seems to be the high V_{max} of ODC activity that correlates with a high number of active ODC molecules, making Y-GFP-ODC clone resistant to DFMO.

3.4. T. cruzi Metacyclogenesis in Y-GFP and Y-GFP-ODC Parasites. We next compared the capacity of Y-GFP-ODC (strain, and clone) and the auxotrophic Y-GFP to differentiate from epimastigotes (E) to metacyclic trypomastigotes (MT) forms using an *in vitro* protocol previously described [55, 56]. Y-GFP, Y-GFP-ODC strain and Y-GFP-ODC clone were incubated with TAU medium for 2 h and subsequently with TAU3AAG medium for 48 hrs to induce *T. cruzi* differentiation (TAU treatment). The MT generated for each cell culture was estimated using the E-MT suspensions to infect Vero cell monolayers during 24 h and comparing with their controls, in Diamond or SDM79 media for Y-GFP or Y-GFP-ODC, respectively.

After the infection time, the cells were washed, fixed and incubated with Rhodamine-phalloidin to localize actin cytoskeleton of host cell by fluorescence microscopy, process that allows the clear observation of cellular limits, whereas *T. cruzi* parasites were observed directly by the presence of GFP protein. Figure 5(a) shows, at two differential amplification levels, confocal images of Vero cells infected by MT generated by epimastigotes of Y-GFP or Y-GFP-ODC strain under TAU treatment. Quantification studies indicated that the percentage of infected cells increased when comparing parasites obtained from Y-GFP-ODC metacyclogenesis with the Y-GFP control (Figure 5(b), black bars); even more, in some cases it was possible to observe more than one Y-GFP-ODC parasite per cell. Although the magnitude of this increment was not as high as to be statistically significant, the tendency was constant. Supporting this result, we observed that the presence of DFMO in the metacyclogenesis assay (see details in Materials and Methods) impaired the infection of Y-GFP-ODC strain and clone, while it has no evident effect over Y-GFP (Figure 5(b), dashed bars) because the inhibitor target is not present in this strain. Conversely, the percentage of infected cells was increased when Put was added to Y-GFP parasites during the differentiation process (Figure 5(b), grey bars) showing similar values to those found in Y-GFP-ODC parasites. Those results indicate that polyamines supply favours the E to MT conversion.

There is an important number of morphological and biochemical events that occur during metacyclogenesis process. Although the molecular causes of those changes are largely unknown, these experiments clearly show the participation of polyamines in that process. Recently published results showed that spermidine can regulate the transcription of cell cycle and autophagy genes in numerous organisms [13]. Our results show that the availability of polyamines during *T. cruzi* differentiation favours the global process, increasing the infective capacity of E-MT suspensions. As our results were obtained by indirect infection of Vero cells, the major infective capacity, reached in the presence of endogenous or exogenous source of polyamines, could be explained in different ways. Polyamines could, for example, activate the expression of genes that participate in differentiation from E to MT or those that code to membrane molecules necessary for host cell interaction and invasion, improving the quantity or quality of the process, respectively.

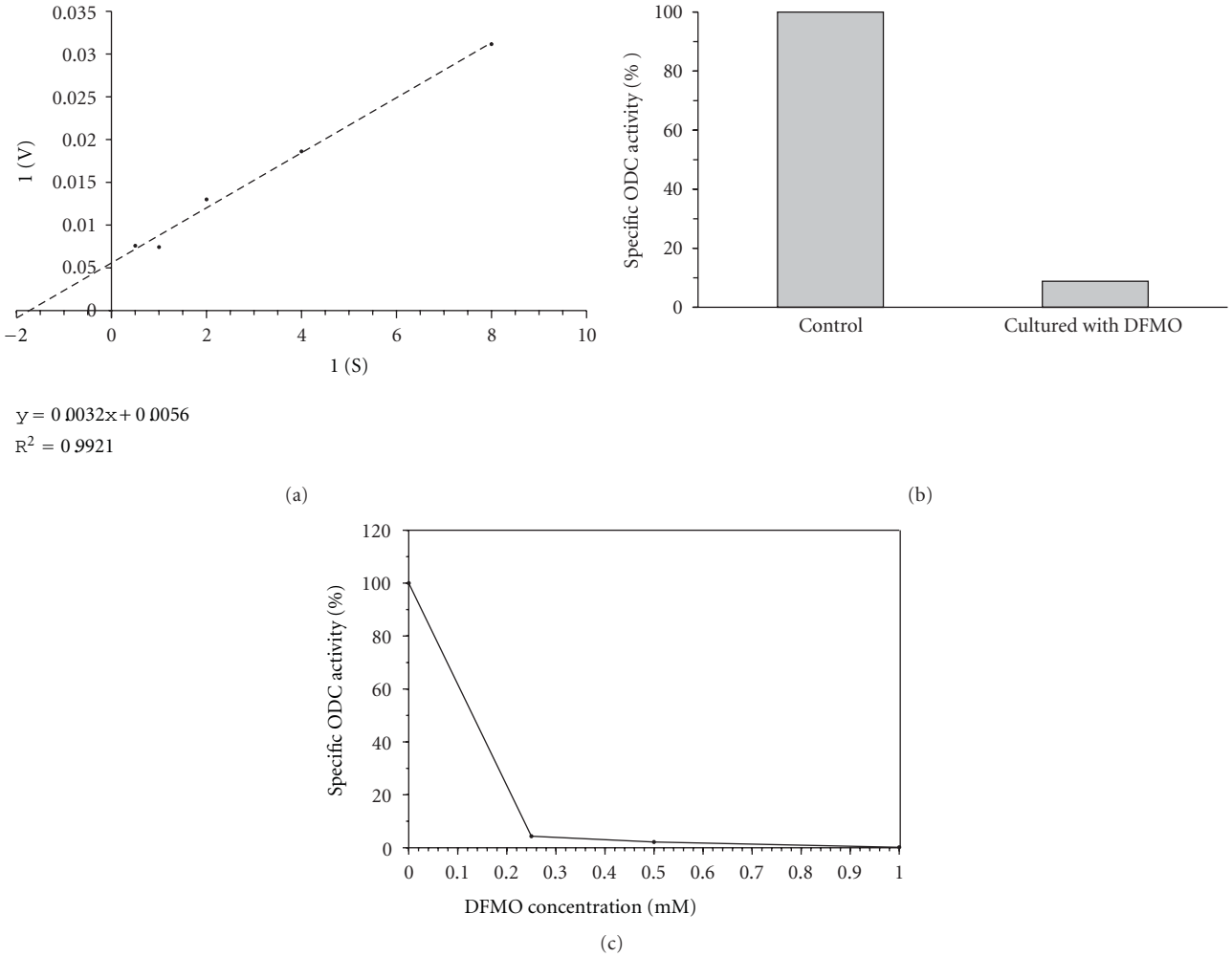


Figure 4: ODC activity characterization. (a) Plot of Lineweaver-Burk. ODC activity was assayed *in vitro* at different concentrations of the substrate ornithine to estimate apparent Km and V_{max}. (b) The Y-GFP-ODC clone was cultured in absence or presence of 5 mM α-difluoromethylornithine (DFMO) for 24 hs, then cells were harvested, washed and ODC activity was measured. (c) ODC activity was carried out in the presence of DFMO at different concentrations. Values in B and C are given as percentages of maximal activity. All other details as indicated in Materials and Methods. These values are the average of two independent experiments.

4. Concluding Remarks

Polyamines availability shows to be an important requirement for *T. cruzi* proliferation, invasion of mammalian host cells, intracellular multiplication, and infection progression, but the way that they act is still uncertain [1, 44, 61–63]. Unlike other trypanosomatid parasites, *T. cruzi* does not synthesize polyamines by an endogenous ODC and relies on the polyamine availability from the parasite external milieu [38, 41]. However, some evidence suggests that polyamines are not as free and available as it could be predicted by polyamine abundance in the host; polyamines could be conjugated or stored in reservoirs so they are not homogeneously distributed and equally available around all host cells and fluids or under different treatments [37, 46]. The polyamine content in the host relies on diet and, specially, in its ODC enzyme, whose activity is highly regulated at multiple levels [64]. Different treatments that alter the host ODC activity modulate, in turn, polyamine intracellular content—ergo its availability for *T. cruzi* intracellular stages—as much as polyamine concentration in extracellular fluids, the natural medium for bloodstream stages. In this context, the host ODC as well as the parasite uptake system would be potential targets, as by self or as a complementary target increasing the effect of antitrypanosomal therapies. As there has been some discrepancies about the possible presence of arginine decarboxylase (ADC) activity under specific metabolic conditions [41, 42, 65–68] as well as there are clear evidence about the presence of ADC in at least some kind of cells that could be infected by *T. cruzi* [27–29], this other possible way to obtain polyamines should be taken into account when the role of polyamines among the *T. cruzi* biological cycle is studied. In any case, the growth curves and the results obtained in metacyclogenesis assays support the idea that polyamines are engaged in parasite proliferation and in differentiation process. Therefore, this double transfected *T. cruzi* becomes

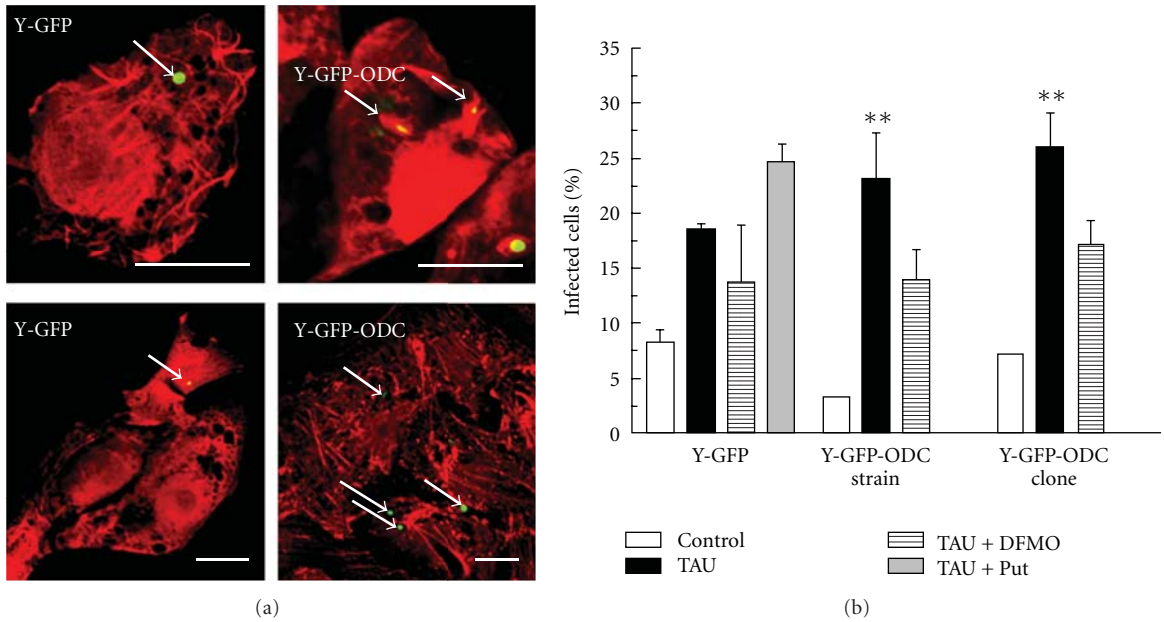


Figure 5: Metacyclogenesis of Y-GFP and Y-GFP-ODC strain and clone. After metacyclogenesis protocol (see details in Materials and Methods), the total mixed suspensions of E and MT from each condition (Control, TAU, or TAU + DFMO) were used to infect Vero cells for 24 h followed by detection of the host cell actin cytoskeleton as indicated under Materials and Methods. (a) Confocal images showing Vero cells infected for 24 h with *T. cruzi* Y-GFP and Y-GFP-ODC clone obtained from TAU medium. (b) Percentage of infected Vero cells with *T. cruzi* Y-GFP, *T. cruzi* Y-GFP-ODC strain, and *T. cruzi* Y-GFP-ODC clone at indicated conditions. Data represent the mean ± SEM of at least two independent experiments (number of counted cells ≈100). Significantly different from control: $^{**}P < .01$, Bars: 10 μm.

a powerful and easy-to-follow tool to study the role of polyamines in Chagas disease pathology. In particular, the expression of GFP gene in this double-mutant (or in new mutants for other genes), which allows a simple visualization of *T. cruzi* cellular limits, represents an advantage compared to the detection techniques frequently used (such as *T. cruzi* nuclear and kinetoplast DNA Hoechst staining or indirect immunofluorescence) to localize and characterize the host cell parasitophorous vacuole (TcPV) as well as the transit and escape of *T. cruzi* from the TcPV. Further experiments of infection in cell monolayers and animal models will be done to confirm and extend the knowledge about the role of polyamines in host cell invasion and infection progression.

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Review Article

Trypanosome Prereplication Machinery: A Potential New Target for an Old Problem

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Approximately ten million people suffer from Chagas disease worldwide, caused by *Trypanosoma cruzi*, with the disease burden predominately focused in Latin America. Sleeping sickness is another serious health problem, caused by *Trypanosoma brucei*, especially in sub-Saharan countries. Unfortunately, the drugs currently available to treat these diseases have toxic effects and are not effective against all disease phases or parasite strains. Therefore, there is a clear need for the development of novel drugs and drug targets to treat these diseases. We propose the trypanosome prereplication machinery component, Orc1/Cdc6, as a potential target for drug development. In trypanosomes, Orc1/Cdc6 is involved in nuclear DNA replication, and, despite its involvement in such a conserved process, Orc1/Cdc6 is distinct from mammalian Orc1 and Cdc6 proteins. Moreover, RNAi-mediated silencing of trypanosome Orc1/Cdc6 expression in *T. brucei* decreased cell survival, indicating that Orc1/Cdc6 is critical for trypanosome survival.

1. Introduction

Currently, approximately ten million people have Chagas disease worldwide, with the disease burden being centered in Latin America. In 2008, approximately ten thousand people died from this disease. Sleeping sickness is another serious health problem, particularly in sub-Saharan countries; in the first half of the twentieth century, this disease practically decimated entire communities in central Africa [1]. After many surveillance programs, 2009 marked the first time in 50 years that less than ten thousand new cases of sleeping sickness were reported in Africa (http://www.who.org/).

The causal agents of Chagas disease and sleeping sickness are, respectively, the protozoan parasites *Trypanosoma cruzi* and *T. brucei* of the Kinetoplastida order. Two subspecies of *T. brucei*, *T. b. rhodesiense*, and *T. b. gambiense*, are responsible for acute sleeping sickness in Eastern and Southern Africa and chronic sleeping sickness in Western and Central Africa, respectively. Chronic sleeping sickness accounts for approximately 95% of all reported cases.

These protozoa have life cycles that alternate between a mammalian host and an insect host. *T. cruzi* epimastigotes are a noninfective life cycle stage of the parasite that proliferate by binary fission in the guts of *Triatoma infestans* insects, which are more commonly known as kissing bugs. These epimastigotes then transform into the infective metacyclic form in the insect hindgut. Then, when the insect vector bites a mammalian host, they eliminate the metacyclic parasites in their feces. This allows the parasites to penetrate the wounded skin and enter into the mammalian host's circulatory system. Within the bloodstream, the metacyclic parasites transform into trypomastigotes, which then infect mammalian cells and transform into amastigotes. Amastigotes are spherically shaped and proliferate inside the infected cells until transforming into nonreplicative trypomastigotes. The life cycle is completed when an insect vector bites an infected mammalian host and takes up trypomastigotes within the blood that then transform into epimastigotes inside the insect gut.

In contrast to *T. cruzi*, the life cycle of *T. brucei* spp. is entirely extracellular. In this case, an infected tsetse fly (*Glossina* genus) bites a mammalian host, transferring metacyclic trypomastiogte forms into the circulatory system. Metacyclic trypomastiogtes transform into bloodstream

trypomastigotes that then proliferate in the hemolymphatic system as trypomastigotes, which are slender in form; next, the parasites transform in a non-proliferative form that is stumpy in appearance. When a new tsetse fly bites an infected mammalian host, these non-proliferative, stumpy parasites are taken up by the fly and transform into proliferating procyclic forms in the fly midgut. Parasites then migrate to the salivary glands of the insect where they transform into epimastigotes that can proliferate by binary fission. Finally, the epimastigotes transform into infective metacyclic trypomastigotes, which are then injected into a new mammalian host during the tsetse fly's bite.

As shown above, Chagas disease is transmitted through the infected feces of triatomines, whereas sleeping sickness is transmitted through the infected saliva of tsetse flies. However, other transmission modes are shared by both species, and include transmission through blood transfusions, vertical or mother-to-child transmission, and accidental infections in the laboratory.

Diagnosis is based on the presentation of clinical symptoms and signs, direct parasitological testing of blood or cerebrospinal fluids (in the case of *T. brucei* infection), serological tests and/or by parasite DNA detection using polymerase chain reactions (PCR). To detect *T. cruzi* by PCR, samples are assayed for the presence of minicircle kinetoplast DNA and a 195-bp reiterated DNA sequence [2, 3]. One serological test that is used for sleeping sickness diagnosis is the card agglutination test for trypanosomiasis (CATT); it detects *T. b. gambiense*-specific antibodies [4]. For Chagas disease, *T. cruzi*-specific antibodies can be detected with assays using either crude or recombinant antigens [5, 6]. However, the use of serological tests to infer Chagas disease cure is controversial, as antibodies against parasite antigens can remain in circulation for long periods of time [7].

The absence or presence of mild symptoms is associated with the chronic phases of both diseases. However, the acute symptoms of Chagas disease can be diagnosed by a trained physician and include swelling, nausea, diarrhea, vomiting, liver or spleen enlargement, fever, headaches, and chest or abdominal pain. About 40% of patients develop chronic disease with heart or colon dilation after 10–20 years of infection. Similarly, sleeping sickness is also characterized by two distinct stages. In the acute phase or hemolymphatic stage, symptoms include headache, malaise, weight loss, fatigue, fever, and vomiting. In the second phase, also known as the neurological phase, parasites are present within the cerebral spinal fluid and brain and cause many neurological and physiatric symptoms including anxiety, disruption of the sleep-wake cycle, behavior changes, and motor features such as muscle tremors and walking difficulties [8]. If untreated, sleeping sickness is fatal. In the absence of treatment, it is estimated that *T. b. gambiense* infection is fatal within three years [9] and that *T. b. rhodesiense* infection is fatal within six to eight months [10, 11].

Drugs used to treat Chagas disease and sleeping sickness have undesired toxic side effects and are not effective against all parasite life cycle stages or parasite strains because of drug resistance. However, effective drugs exist to treat the acute phases of both diseases. Despite the presence of numerous side effects, benznidazole (BNZ) (N-benzyl-2-nitroimidazole-1-acetamide) and nifurtimox (NF) (4-([5-nitrofurfuryledene]amino)-3-methylthiomorpholine-1,1 dioxide) are effective against Chagas disease. Both were introduced into clinical use in the 1970's [12]. While NF increases the production of free radicals believed to cause trypanosome death [13, 14], BNZ disrupts protein synthesis and the respiratory chain of *T. cruzi* (for a review see [15]). However, these drugs are ineffective during the chronic phase of the disease. In the latter phases of Chagas disease, the course of treatment is dependent on the patient's symptoms; medicines or surgery are recommended for patients with heart complications, while diet changes and possibly surgery are suggested for patients with digestive complications.

To treat sleeping sickness, four medicines are currently commonly used. Pentamidine and suramin are considered first-stage drugs. Pentamidine has been used since 1940 and is the first-choice drug to treat the initial stages of *T. b. gambiense* infection; it is administered as an intramuscular injection. The exact antiprotozoal mechanisms of action of pentamidine are still unknown, perhaps because it acts against many targets including mitochondria and DNA (reviewed at [16, 17]). Suramin has six negative charges that allow it to interact electrostatically with many trypanosomal enzymes, including enzymes involved glycolysis and the pentose phosphate pathway [18]. As resistance against suramin has been observed in *T. b. gambiense*, suramin is currently only used for cases of *T. b. rhodesiense* infection (for a review see [16]).

More toxic that pentamidine and suramin, melarsoprol, eflornithine, and a combination of eflornithine and NF (for melarsoprol-refractory patients) are used to treat patients in the neurological phase. Eflornithine, alpha-difluoromethylornithine, is administered intravenously and infusion and irreversible inhibits polyamine biosynthesis acting at ornithine decarboxylase (EC 4.1.1.17). It is only effective against *T. brucei gambiense* infections [19]. In spite of its toxic side effects, melarsoprol is the most widely used drug during the second stage of sleeping sickness; further, it is the only drug available to treat *T. b. rhodesiense* infections. Melarsoprol is derived from arsenic, and it is believed that melarsen oxide is its active metabolite *in vivo.* Although it is unknown how melarsoprol kills parasites, parasite lysis following melarsoprol exposure was demonstrated [20].

Although there is a clear need for new drugs to treat trypanosome-induced diseases, few drugs and clinical trials have been initiated recently. The absence of standard protocols to evaluate drug efficacy and the absence of clinical parasitological markers contribute to the difficulty of launching new treatment initiatives. In the case of Chagas disease, novel drugs with less toxic effects and shorter administration times and drugs to treat chronic disease should be prioritized. Therefore, a standard protocol for drug screening against acute *T. cruzi* infections was proposed to evaluate drug efficacy both in *in vitro* and *in vivo* models compared to BNZ. Although it is very important to evaluate the effect of these compounds during chronic infection, it is not possible due to the difficulty in evaluating parasite clearance using current methods [21]. Most recently, studies

have focused on the azolic compounds itraconazole and posaconazole that inhibit ergosterol synthesis. In mouse models, these drugs have been shown to cure both acute and chronic Chagas disease [22]. In addition, posaconazole has activity against *T. cruzi* both *in vivo* and *in vivo*.

Regarding sleeping sickness, new drugs should have lower toxic side effects, treat both *T. b. gambiense* and *T. b. rhodesiense* infections, and treat late-stage *T. b. rhodesiense* infection. Pafuramidine maleate (DB289) is a new drug in clinical trial for the treatment of sleeping sickness [23]. However, as it cannot cross the blood-brain barrier, it can only be used for early stage treatment. Despite this, it has an advantage over pentamidine because it can be orally administrated.

As illustrated above, the need for new drugs and novel drug targets to treat both Chagas disease and sleeping sickness is evident. These drugs should target fundamental pathways within these parasites. In spite of the conserved nature of essential pathways among eukaryotic organisms, we identified a trypanosome prereplication machinery component that is fundamental for replication and that is distinct from eukaryotic prereplication machinery; this component is necessary for origin selection and the establishment of the DNA replication fork [24]. These data indicate that this enzyme is a potential drug target for the treatment of both Chagas disease and sleeping sickness.

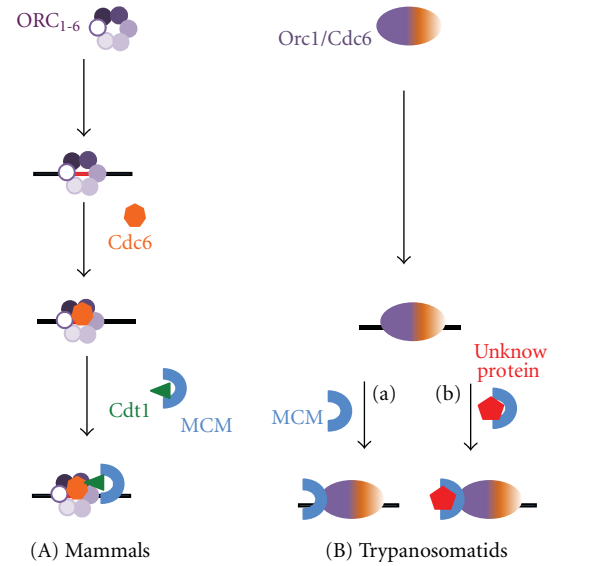


FIGURE 1: Schematic representation of the prereplication machinery from mammals (A) and trypanosomatids (B). (a) and (b) indicate the two possible mechanisms for MCM recruitment to the replication origin. (a) Orc1/Cdc6 recruits the MCM complex. (b) An unknown protein recruits the MCM complex to the replication origin.

2. Eukaryotic Prereplication Machinery

DNA replication in eukaryotes begins with the assembly of the prereplication complex on regions along chromosomes known as replication origins [25, 26]. The prereplication complex assembly (depicted in Figure 1) begins when the origin recognition complex (ORC), comprised of six different subunits (Orc_{1-6}) [25, 27], recognizes the replication origins and allows the recruitment of cell division cycle Cdc6 and Cdt1 proteins to the complex. Together, the ORC, Cdc6 and Cdt1 proteins recruit the mini-chromosome maintenance (MCM) complex, which is comprised of six subunits (Mcm_{2-7}); the MCM complex harbors the helicase activity that is essential for DNA replication [28]. Once the prereplication machinery is bound to the replication origins, these origins are licensed, meaning that the replication process can initiate at these origins through the binding of regulatory factors and DNA replication fork components during the onset of S-phase.

3. Structural Aspects of the ORC and Cdc6 Prereplication Machinery Components

The ORC complex was first purified from *S. cerevisiae* extract as proteins that bound to replication origins [29]. *S. cerevisiae* ORC (413 KDa) is a heterohexamer that contains six proteins named according their relative molecular mass: Orc1 (120 KDa), Orc2 (72 KDa), Orc3 (62 KDa), Orc4 (56 KDa), Orc5 (53 KDa), and Orc6 (50 KDa). Orc_{1-5} orthologs were identified in a wide range of organisms, suggesting that these genes are present in all eukaryotic organisms. Of the six Orcs, Cdc6 is most notably similar to Orc1 (see Figure 2). Therefore, Orc1 is more closely related to Cdc6 than to any other ORC component [30].

Among the ORC subunits, Orc1, Orc4, and Orc5 belong to the AAA+ family, proteins that exhibit ATPase activity and function in multiple cellular activities [31]. Orc2 and Orc3 contain an ATPase-like domain; Orc6 lacks the AAA+ domain and shows no structural similarity to the other Orc proteins. The AAA+ domain contains the Walker A and Walker B motifs and regions named sensor 1 and sensor 2. These regions are typical in proteins that act as "Clamp-loaders", proteins that encircle DNA and bind other factors and serve as processivity-promoting factors in DNA replication in an ATP-dependent manner. Walker A and B motifs and sensor 1 and 2 regions are responsible for ATP binding and hydrolysis [31]; ATP binding and hydrolysis trigger a conformational change within the ORC that allows for the serial recruitment of proteins during prereplication complex assembly at replication origins [32].

Cdc6 (58 KDa in *S. cerevisiae*) was first isolated from thermo-sensitive mutants and identified as an important factor during the beginning of DNA replication [33]. Cdc6 also has the AAA+ domain, containing the Walker A and Walker B motifs and sensor 1 and 2 regions [30]. Similar to Orc1, Cdc6 exhibits intrinsic ATPase activity *in vitro* [34].

4. Recruitment of the Prereplication Machinery

The molecular bases that enable the recruitment of prereplication machinery have been described in yeast and they are dependent on ATP binding and ATP hydrolysis (Figure 3). As mentioned previously, the first step in prereplication machinery assembly is the binding of the ORC to the

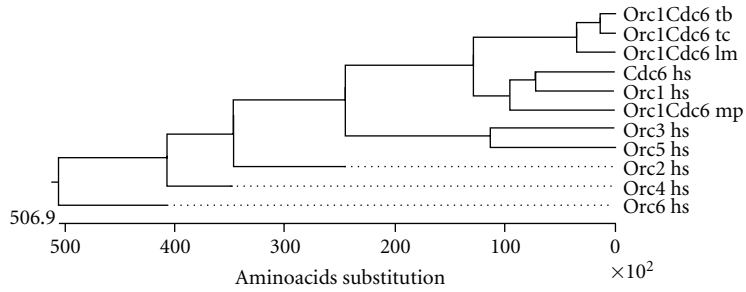


Figure 2: Phylogenetic tree of human Orc1 to Orc6, human Cdc6, and Orc1/Cdc6 from *Trypanosoma cruzi* (Orc1Cdc6 tc), *Trypanosoma brucei* (Orc1Cdc6 tb), *Leishmania major* (Orc1Cdc6 lm), and *Methanoplanus petrolearius* (orc1Cdc6 Mp), an *Archaea* species. The phylogenetic tree was generated using the ClustalW alignment method.

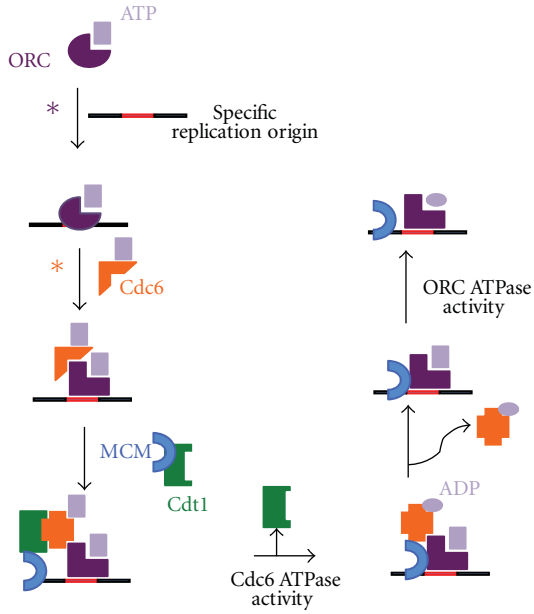


Figure 3: Schematic representation of prereplication machinery recruitment. Figure 3 shows that Cdc6 and ORC ATPase activities are important for the release of these molecules from the complex. Asterisks show the steps where ORC ATPase activity (purple ∗) or Cdc6 ATPase activity (orange ∗) is important for the prevention of the assembly of the prereplication machinery at nonspecific replication origins.

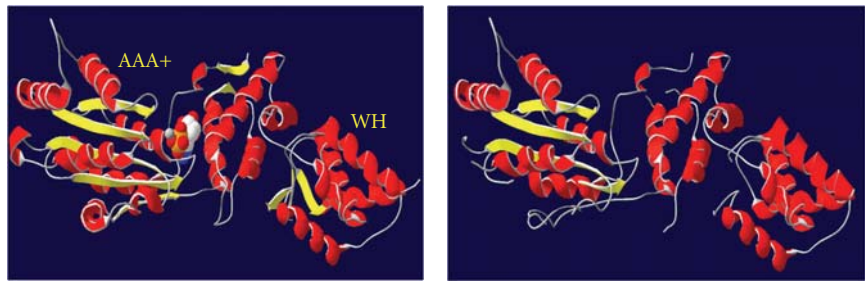
replication origins, a step that depends on the binding of the ORC to ATP. ORC-double-stranded DNA interactions are specific and inhibit the ATPase activity of the ORC. In this way, the high ATPase activity of the ORC inhibits stable ORC interactions with nonspecific DNA. However, in the presence of a specific replication origin, the ATPase activity of the ORC is inhibited, and it undergoes a conformational change; the conformation change stabilizes the ORC-DNA interaction and allows the ORC to bind Cdc6 (reviewed in [35]). The stability of ORC-Cdc6-DNA interactions is also regulated by the ATPase activity of Cdc6, which is high in the presence of nonspecific DNA, destabilizing the complex [34]. ORC-Cdc6-DNA interactions trigger a conformational alteration in Cdc6 that allows for the Cdc6-Cdt1 interaction to occur [36]. Therefore, the ATPase activity of both the ORC and Cdc6 work to select specific replication origins.

Once bound to DNA, the ORC and Cdc6 are able to interact with Cdt1, which brings the MCM complex to the replication origin. At this moment, the low activity of the Cdc6 ATPase that is important to establish ORC-Cdc6-DNA interaction is also required to recruit the MCM complex onto the replication origins (reviewed in [37]). After MCM complex recruitment, the Orc1 ATPase enables MCM-DNA interaction [38]. Once the MCM complex is recruited and established at the replication origins, the ORC, Cdc6 and Cdt1 do not need to be bound to replication origin sites any longer. At this point, prior to the activation of DNA replication, these proteins disassociate from the replication origins in an ATPase-dependent manner [39].

These data clearly demonstrate the extreme importance of the ATPase activities of the different components of the prereplication machinery in the stabilization and assembly of the prereplication machinery onto DNA replication origins. In the selection of replication origins, the assembly of the prereplication complex onto replication origins, the recruitment of the MCM helicase, and in the dissociation of the prereplication complex from the replication origins to avoid a new round of DNA replication in the same cell cycle, the balance between high and low ATPase activity is critical for precise DNA replication.

5. Trypanosome Orc1/Cdc6: The Prereplication Machinery Component and Its ATPase Activity

Although eukaryotic cells, trypanosomes have a prereplication machinery component that is different from eukaryotes and is instead closer to *Archaea* species. Genomic databases of trypanosomatids show that these organisms do not contain sequences in their genome that code for Orc1–Orc6 subunits, Cdc6 or Cdt1. Additionally, several *Archaea* sp. have one or more copies of proteins that exhibit high sequence homology with both Orc1 and Cdc6; these proteins are often called Orc1/Cdc6 proteins [40–42] (Figure 2). Trypanosomatids have annotated a gene for only one of the six subunits of the ORC, Orc1, which is also homologous to Cdc6. It is annotated as Orc1 [43] and



(a) (b)

Figure 4: (a) Shows the archaea Orc1/Cdc6 structure together with ATP. The AAA+ and WH domains are indicated. (b) Shows the predicted structure of *T. cruzi* Orc1/Cdc6, obtained using the 3D-PSSM program (http://www.sbg.bio.ic.ac.uk/~3dpssm/index2.html).

we named it Orc1/Cdc6. In the TIGR Parasites Database (http://www.tigr.org/), two sequences were annotated as Orc1 (Tc00.1047053511159.20 and Tc1047053508239.10) in the genome of *T. cruzi* (representing gene alleles). Single sequences were annotated as Orc1 in the genomes of *T. brucei* (Tb11.02.5110) and *T. brucei gambiense* (Tbg972.11.8220). The two Orc1 protein sequences from *T. cruzi* are 98.2% identical. The Orc1sequences from *T. cruzi* and *T. brucei* are 77.1% and 77.8% identical. Trypanosome Orc1/Cdc6 was confirmed as a prereplication machinery component because it replaced yeast Cdc6 in a yeast phenotypic complementation assay; further, the silencing of trypanosome Orc1/Cdc6 expression by RNA interference in *T. brucei* impaired nuclear DNA replication [24].

Analyses of structural alignment using the Phyre server, a web-based method for protein folding recognition, showed a higher structural similarity of trypanosome Orc1/Cdc6 to archaea Orc1/Cdc6 [24]. Also, analyses of the predicted tridimensional structure of *T. cruzi* Orc1/Cdc6 by 3D-PSSM (http://www.sbg.bio.ic.ac.uk/~3dpssm/index2.html), which determines the most probable folds based on the occurrence of motifs present in the secondary structure, showed that the probable structural of *T. cruzi* Orc1/Cdc6 is homologous to *Archaea* Orc1/Cdc6 (Figure 4). These analyses suggest that trypanosome Orc1/Cdc6 is closer to *Archaea* prereplication machinery than to mammalian prereplication machinery. Primary sequences of trypanosome Orc1/Cdc6 also contain Walker A and B motifs, related to ATP/GTP binding, and sensor 1 and 2 regions that are involved in ATP hydrolysis; these features are typical of prereplication machinery components that exhibit the AAA+ ATPase fold [44, 45]. In fact, both *T. cruzi* and *T. brucei* Orc1/Cdc6 exhibit ATPase activity, which, in the presence of increased concentrations of ATP follows a Michaelis-Menten (MM) kinetic model. This ATPase activity increases in the presence of nonspecific DNA [24], suggesting that, similar to yeast, trypanosome Orc1/Cdc6 ATPase activity might be involved in the selection of specific replication origins. As mentioned previously, there is no Cdt1 homologous protein in the trypanosomatid genome database. Therefore, in these organisms, the prereplication machinery might be assembled by the recruitment of the MCM complex by Orc1/Cdc6. Alternatively, the prereplication machinery could be assembled by an unknown protein that could bind Orc1/Cdc6 and recruit the MCM complex onto the replication origins. This assembly scheme is depicted in Figure 1(b). It is important to note that further studies should confirm if this schematic representation is correct. Recently, the group of Dr. Richard McCulloch identified at least one further ORC-like factor (pers comm.) in *T. brucei*. Further studies should also be conducted to determine if Orc1/Cdc6 ATPase activity is important for the assembly of prereplication machinery as well as for the release of the prereplication complex proteins from the replication origins. Nevertheless, the silencing of trypanosome Orc1/Cdc6 expression by RNAi in *T. brucei* negative affected cell survival [24]. This data strongly indicates that Orc1/Cdc6 is extremely important for trypanosome survival and identifies Orc1/Cdc6 as a potential target for drug design.

6. ATPase Activity Inhibitors

Different proteins harboring ATPase activity are being studied as potential drug targets through the inhibition of their ATPase activity [46–49]. The inhibition of the ATPase activity of heat-shock protein 90 (HSP90) is a main target for cancer treatment. HSP90 is a chaperone that acts in the folding, stabilization, and assembly of several proteins that are involved in many biological processes [50, 51]; further, it is also responsible for the maintenance of cancer cells by facilitating the function of oncoproteins allowing malignant transformation [52]. Because HSP90 from tumor cells has a higher activity compared to HSP90 from normal cells [53], its inhibition effects in tumor cells are higher than in normal cells [53], making HSP90 a good target for drug design [46, 54]. Geldanamycin, a natural compound, was the first compound described with antitumor effects through the inhibition of HSP90 ATPase activity [55–57]. Geldanamycin binds to the N-terminal ATP-binding pocket of HSP90, thereby blocking its ATPase activity [58]. Since these findings have been reported, several studies have focused on HSP90 as a target, and several drugs such as 17-DMGA (alvespimycin) and 17-AAG/tanespimycin have reached phase I and phase II clinical trials, respectively [46].

Another important health problem is antibiotic resistance in the treatment of bacterial infections [59]. Different proteins with ATPase activity are being targeted for high-throughput screening in order to find new inhibitors with potential antibiotic effects. Bacterial RecA [60], DNA gyrase [48], and essential replicative DNA helicase [49] are some of the ATPase proteins upon which effort is being concentrated. RecA is a bacterial protein involved in DNA repair, which interacts with single-stranded DNA and through its ATPase activity allows for both recombinational DNA repair and horizontal gene transfer, processes that are essential for the acquisition of drug resistance genes [47]. Inhibitors of RecA ATPase activity that act by binding the ATP-binding site are being screened, indicating the potential development of antibiotics capable of preventing bacterial drug resistance [60].

DNA gyrase and the replicative DNA helicase are proteins involved in bacterial DNA replication and are essential for bacterial survival. Both have ATPase activity indispensable for their function [48]. DNA gyrase is a type II topoisomerase capable of introducing negative supercoils during DNA replication. Although it is comprised of two subunits (A and B), only B subunit has ATPase activity. The aminocoumarin antibiotic class, from which novobiocin (4-Hydroxy-3-[4-hydroxy-3-(3-methylbut-2-enyl)benzamido]-8-methylcoumarin-7-yl3-O-carbamoyl-5,5-di-C-methyl-α-l-lyxofuranoside) is licensed for treatment of human infections, binds to the B subunit inhibiting the ATP hydrolysis required for DNA supercoiling and preventing cell growth [48]. The DNA replicative helicase, DNA B, is responsible for opening double-stranded DNA through ATP hydrolysis, which makes the DNA available to the replication machinery for the duplication of the bacterial genome. Two compounds, flavonoid myricetin and triaminotriazine, are able to inhibit the ATPase activity of DNA B helicase in *Escherichia coli* and *Pseudomonas aeruginosa*, respectively, preventing cell growth [49]. However, as these drugs exhibit some cytotoxicity in mammalian cell culture, others inhibitors are being screened as possible drug candidates [49].

As discussed above, inhibitors of ATPase activity are being studied as possible drugs candidates; some, such as novobiocin, are even already being used for human treatment. This raises the possibility of searching for specific inhibitors of *T. brucei* and *T. cruzi* Orc1/Cdc6, as these proteins do not have a closely related protein in humans.

7. Final Considerations

In addition to its indispensable role as a DNA replication initiator, the ORC is known to affect diverse cell processes including chromosome segregation, cytokinesis, cell cycle regulation, and gene expression [61]. It is now time to analyze whether trypanosome Orc1/Cdc6, and its ATPase activity are also involved in non-DNA replication functions. If so, it would better justify additional efforts to search for an ATPase inhibitor against trypanosome Orc1/Cdc6.

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Review Article

Biosynthesis of Galactofuranose in Kinetoplastids: Novel Therapeutic Targets for Treating Leishmaniasis and Chagas' Disease

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Cell surface proteins of parasites play a role in pathogenesis by modulating mammalian cell recognition and cell adhesion during infection. β-Galactofuranose (Gal$f$) is an important component of glycoproteins and glycolipids found on the cell surface of *Leishmania* spp. and *Trypanosoma cruzi*. β-Gal$f$-containing glycans have been shown to be important in parasite-cell interaction and protection against oxidative stress. Here, we discuss the role of β-Gal$f$ in pathogenesis and recent studies on the Gal$f$-biosynthetic enzymes: UDP-galactose 4′ epimerase (GalE), UDP-galactopyranose mutase (UGM), and UDP-galactofuranosyl transferase (GalfT). The central role in Gal$f$ formation, its unique chemical mechanism, and the absence of a homologous enzyme in humans identify UGM as the most attractive drug target in the β-Gal$f$-biosynthetic pathway in protozoan parasites.

1. Galactofuranose

β-Galactofuranose (β-Gal$f$) is the five-member ring isomer of galactose (Figure 1). This rare sugar was initially found in several human bacterial pathogens including *Mycobacterium tuberculosis*, *Escherichia coli*, *Salmonella typhimurium*, and *Klebsiella pneumoniae* [1–4]. In *M. tuberculosis*, β-Gal$f$ is found in the arabinogalactan layer where it links the peptidoglycan and mycolic acid layers [1]. In *E. coli* and *K. pneumoniae*, it is present in the O antigen, while in *S. typhimurium* it is found in the T antigen [2–4]. In all of these organisms, the enzyme UDP-galactopyranose mutase (UGM) serves as the sole biosynthetic source of β-Gal$f$ as it is responsible for converting UDP-Gal$p$ to UDP-Gal$f$ (Figures 2 and 3) [5–10]. UDP-Gal$f$ serves as the precursor molecule of β-Gal$f$, which is attached to the various components of the cell surface by galactofuranosyl transferases (GalfTs) (Figure 2) [11, 12]. UGMs and GalfTs are not found in humans, therefore, they have been examined as potential drug targets.

Deletion of the genes coding for UGM or GalfTs has shown that these proteins are essential in *M. tuberculosis*, highlighting the importance for Gal$f$ in bacteria [13]. Studies have also been conducted to identify inhibitors for *M. tuberculosis* UGM [14–17]. These studies showed that specific inhibitors of *M. tuberculosis* UGM were able to prevent mycobacterium growth and, therefore, validated Gal$f$ biosynthesis as a drug target against mycobacteria [14].

β-Gal$f$ has also been shown to be present in fungi [18–21]. In the human pathogen *Aspergillus fumigatus*, it is found in four components of the cell wall: galactomannan, glycoprotein oligosaccharides, glycophosphoinositol (GPI) anchored lipophosphogalactomannan (LPGM), and sphingolipids [18, 22]. Deletion of the UGM and the Gal$f$ transporter genes in Aspergillus resulted in attenuated virulence, increased temperature sensitivity, and thinner cell walls [23, 24]. Gal$f$ is also present in protozoan parasites and is a virulence factor [25]. In *Leishmania* spp., it is present in the lipophosphoglycan (LPG) and in glycoinositolphospholipids (GIPLs). In *T. cruzi*, Gal$f$ is found in the GIPLs and glycoprotein oligosaccharides [26, 27]. This paper focuses on current knowledge on the biosynthetic pathway of β-Gal$f$

and its role in the pathogenesis of *T. cruzi* and *Leishmania* spp.

1.1. Overview of T. cruzi and Leishmania spp. T. cruzi is the causative agent of Chagas' disease, which often develops severe cardiac complications in patients with the chronic form of the disease [28]. In the *T. cruzi* life cycle, the parasite undergoes three developmental stages as it is transmitted from the insect vector (triatomine bug) to mammals: trypomastigote (vector feces and mammalian bloodstream), epimastigote (vector midgut), and amastigote (mammalian smooth muscle) [29]. *Leishmania* spp. are the causative agents of leishmaniasis, which can manifest in three forms—visceral, cutaneous, or mucocutaneous—depending on the species [30]. In the *Leishmania* spp. lifecycle, there are two stages: the amastigote (mammalian host macrophages) and the promastigote stage (vector (sand fly) midgut) [30].

Current treatments are limited due to toxic side effects and cost, therefore new drugs are needed [31–33]. Lifecycle progression of both *T. cruzi* and *Leishmania* spp. is associated with changes in the carbohydrate composition on the cell surface. These changes are important for mediating host-pathogen interactions Gal*f* levels and Gal*f*-containing glycans are shown to be modulated throughout the parasite life cycles and are important for pathogenesis [26, 34–36]. As Gal*f* biosynthesis has been shown to be an attractive drug target for other pathogens, enzymes involved in this pathway may also prove to be ideal drug targets for the treatment of Chagas' disease and leishmaniasis.

2. Biosynthesis of Gal*f* in Kinetoplastids

The biosynthesis of Gal*f* begins with the uptake and metabolism of galactose (Gal). Gal is an epimer of glucose that differs only by the orientation of the hydroxyl group at the carbon 4 position. Gal is a component of lactose in milk, is present in grains and beets, and can be utilized for energy after conversion to glucose (Glc). Gal is also a major component of glycans, present in proteins and lipids in most organisms, ranging from bacteria to mammals. The metabolism of Gal occurs via the Isselbacher or Leloir pathways (Figure 2). In the Leloir pathway, Gal is converted to glucose-6-phosphate (Glc-6-P), an intermediate in glycolysis (Figure 2(a)). After Gal is transported into the cytoplasm by hexose transporters it is phosphorylated by galactokinase (GalK). Phosphorylation of Gal prevents its transport out of the cell. Gal-1-phosphate (Gal-1-P) is then coupled to uridyl diphosphate by galactose-1-phosphate uridyltransferase (GalPUT) yielding two products, UDP-Gal and Glc-1-phosphate (Glc-1-P). UDP-Gal is converted to UDP-glucose (UDP-Glc) by UDP-glucose-4-epimerase (GalE). Glc-1-P is isomerized to Glc-6-P by phosphoglucomutase (PGM) [37, 38]. In the Isselbacher pathway, Gal-1-P can be directly converted to UDP-Gal by the enzyme UDP-sugar-pyrophosphorylase (USP) (Figure 2(b)) [39]. These pathways contribute to the pool of UDP-Gal required for the biosynthesis of the glycocalyx.

In *Leishmania* spp., galactose has been shown to be obtained from the environment by hexose transporters through radioactive labeling assays, and both the Leloir and Isselbacher pathways function to maintain proper levels of UDP-Gal [40]. The Isselbacher pathway is present in *L. major* due to the wide substrate specificity of USP, which can convert many sugars to the corresponding UDP-sugar including glucose, galactose, galacturonic acid, and arabinose [41]. The wide range of substrate specificity has been explored by crystallographic studies and has been attributed to a larger active site that can alter conformations of residues involved with sugar binding and the flexibility of the sugar-binding loop [42]. Deletion of the USP gene in *L. major* showed that the protein is nonessential and demonstrates that since the Leloir and Isselbacher pathways are redundant, proteins involved with the formation of UDP-Gal are not essential for *Leishmania* spp. survival [41, 43]. In *T. cruzi* and *Trypanosoma brucei*, galactose cannot be obtained from the environment because it is not recognized by the hexose transporters; therefore, these parasites rely on the action of GalE from the Leloir pathway for the direct conversion of UDP-Glc to UDP-Gal for galactose [37, 44, 45]. In both *T. cruzi* and *L. major*, UDP-Gal is converted to UDP-Gal*f* by UGM (Figures 2(c) and 3) [7]. UDP-Gal*f* is the substrate for several UDP-galactofuranosyl transferases, which decorate many glycoproteins and glycolipids on the cell surface of *T. cruzi* and *L. major*.

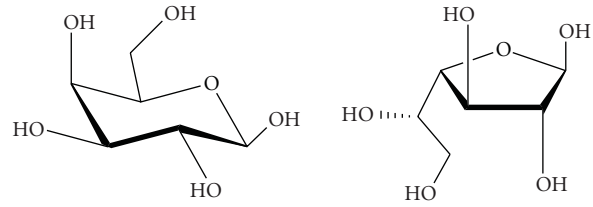

Figure 1: Structures of β-Galactopyranose and β-Galactofuranose.

2.1. Galactofuranose-Containing Proteins and Lipids. Gal*f* is found in many major components of the glycocalyx of *Leishmania* spp. and *T. cruzi*. In *Leishmania* spp., and Gal*f* is found in the lipophosphoglycan (LPG) and in glycoinositolphospholipids (GIPLs), while in *T. cruzi*, Gal*f* is found in the GIPLs and glycoprotein oligosaccharides (Figure 4) [26, 27]. In this section, we will describe the structure and role in pathogenesis of known Gal*f*-containing glycoconjugates.

2.1.1. Lipophosphoglycan (LPG) from Leishmania . LPG from *Leishmania* spp. has four components: a phosphoinositol lipid, a core oligosaccharide, phosphoglycan (PG) repeat units, and a cap (Figure 4(a)). β-Gal*f* is found in the core structure where it plays a role in connecting the PG repeat units to the phospholipid [35, 46]. LPG has been found to be important for adhesion to the sandfly midgut, resistance to the human complement C3b, protection from oxidative stress, and prevention from phagosomal transient fusion [47–50].

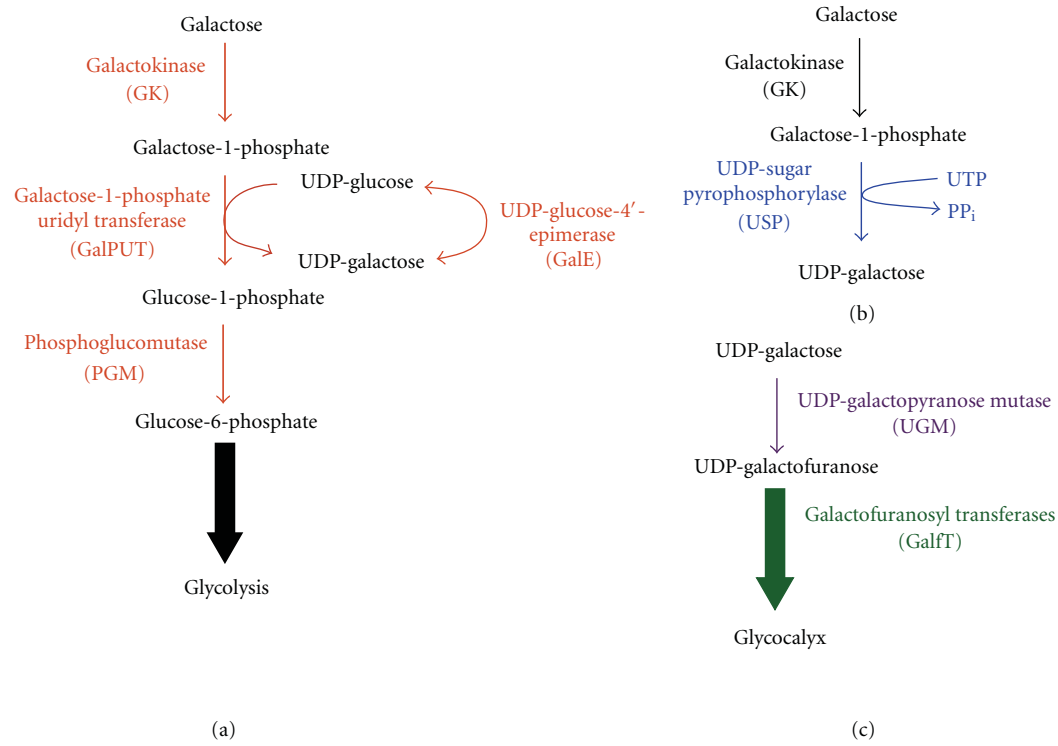

Figure 2: Biosynthetic pathways of Gal*f*. (a) In the Leloir pathway, Gal is transported to the cytoplasm where it is converted to galactose-1-phosphate by galactokinase (GK). Galactose-1-phosphate uridyl transferase (GalPUT) and UDP-Glc 4′ epimerase (GalE) are involved in the synthesis of UDP-galactose. (b) Alternatively, galactose can be directly converted to UDP-galactose by the Isselbacher pathway by UDP-sugar pyrophosphorylase (USP). (c) UDP-Galactose is then converted to UDP-Gal*f* by UDP-galactopyranose mutase (UGM), and UDP-Gal*f* is subsequently added to the glycocalyx by Galactofuranosyl transferases (GalfT).

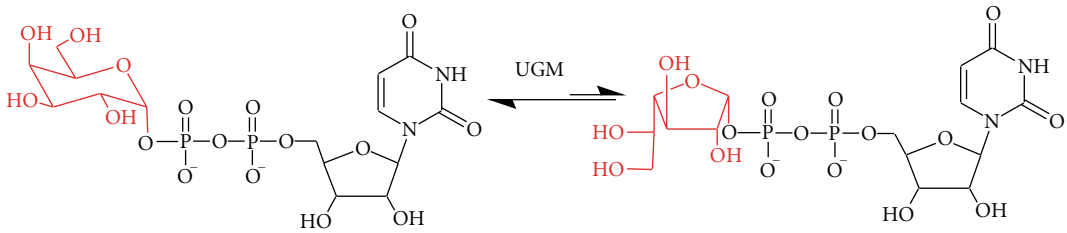

Figure 3: Reaction catalyzed by UDP-Galactopyranose mutase (UGM).

2.1.2. Glycoinositolphospholipids (GIPLs). GIPLs are free glycosylated phospholipids found in many kinetoplastids. Those found in *Leishmania* spp. and *T. cruzi* are considered unique due to the presence of β-Gal*f* (Figures 4(b) and 4(c)) [26, 51–54]. GIPL structure is species and strain dependent and varies in expression levels throughout the life stages of the parasite [55–58]. GIPLs from *Leishmania* spp. are thought to be precursor molecules for the synthesis of the LPG core structure [59]. *L. major* GIPL-1 has been shown to be involved in parasite-host interactions and is thought to play an important role in establishing infection [57, 60].

GIPLs from *T. cruzi* include a class of phospholipids previously identified as lipopeptidophosphoglycans (LPPGs) [61–63]. The LPPGs were originally considered a separate class from the GIPLs due to the presence of contaminating amino acids during their purification; these amino acids have since been identified as part of the NETNES [27, 64]. The importance of GIPLs in *T. cruzi* is revealed by studies that show that it plays a role in antigenicity, both with rabbit and human sera [36, 53]. The antigenicity is thought to be primarily due to the terminal β-Gal*f* residues either from the GIPLs or the O-linked mucins, as removal of β-Gal*f* results in decreased levels of antigenicity [36, 53, 65]. It has also been shown that GIPLs play a role in attachment of the parasite to the luminal midgut of the vector *Rhodnius prolixus* [55]. *T. cruzi* modulates this interaction by altering GIPL expression levels during its life cycle, as epimastigotes have much higher expression of GIPLs than trypomastigotes [55, 65, 66].

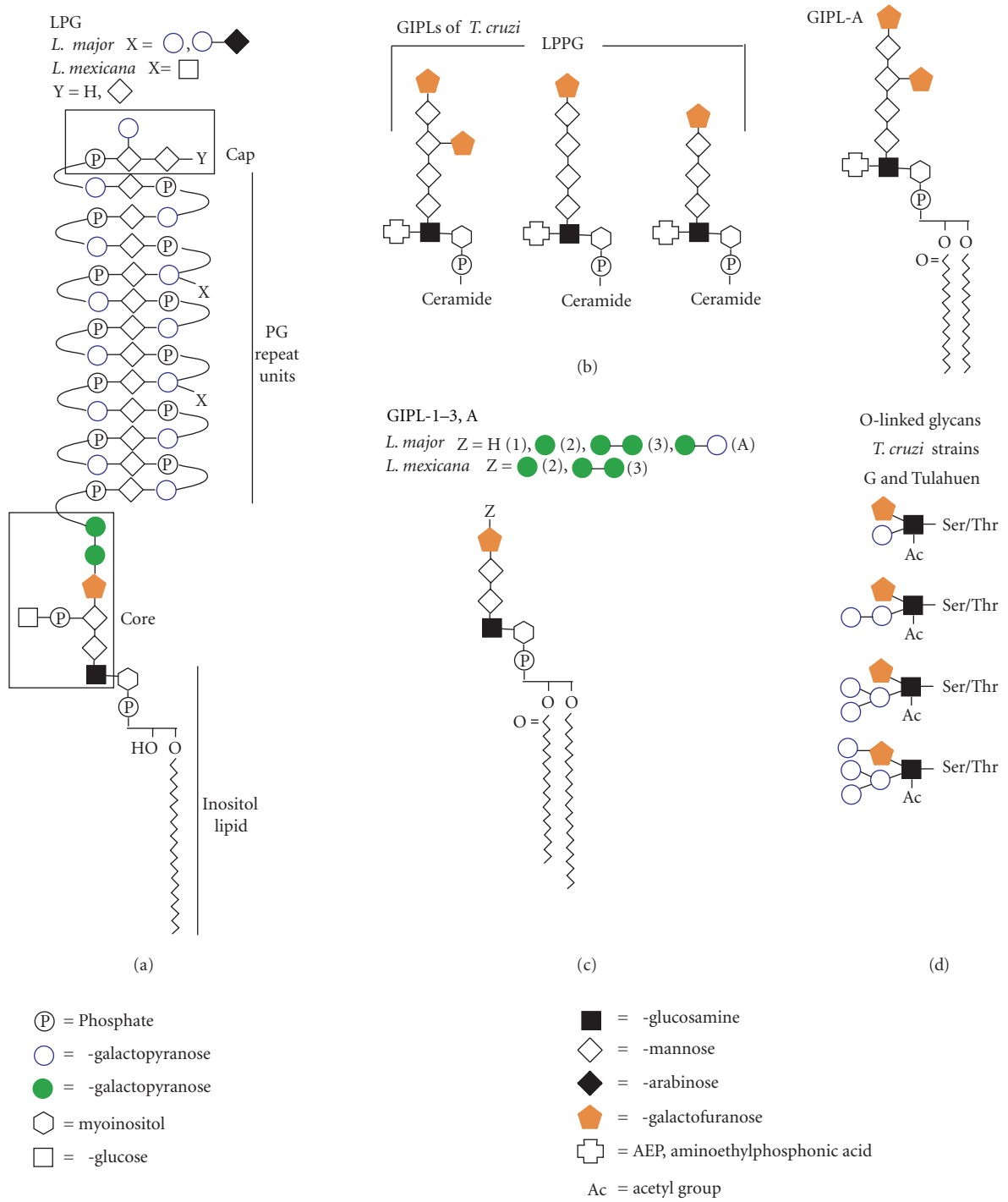

Figure 4: Structures of Gal*f*-containing glycans of *Leishmania* spp. and *T. cruzi*. (a) Structure of LPG from *Leishmania* spp. (b) Structures of GIPLs from *T. cruzi*, including the previously annotated LPPG and GIPL-A (c) Structures of GIPL-1-3, (A) from *L. major* and *L. mexicana*. (d) Selected subset of structures of O-linked glycans found in both *T. cruzi* strains G and Tuhulan.

2.1.3. N-Linked Glycans. β-Gal*f* is found in mannose N-linked oligosaccharides in several species of trypanosomatid flagellates including *T. cruzi*, *Leptomonas samueli*, *Herpetomonas samuelpessoai*, *Crithidia fasciculate*, and *Crithidia harmosa* [36, 67–70]. The glycan structures have been solved for *L. samueli*, *C. fasciculate*, and *C. harmosa* and are shown to be species dependent [67, 69]. β-Gal*f* units are found as terminal sugars linked to mannose residues in high mannose type N-linked glycans [67, 69]. The role of N-linked glycans has currently not been significantly explored for *T. cruzi*.

2.1.4. T. cruzi O-Linked Glycans and Mucins. *T. cruzi* mucins are a family of GPI-linked glycoproteins with high levels of O-linked glycosylation [71]. Several studies have been conducted to determine the composition of the oligosaccharides bound to Thr and Ser residues in these glycoproteins [72–76]. In *T. cruzi*, the O-glycans are not linked to N-acetylgalactosamine as in mammals and other organisms; instead, they are linked to N-acetylglucosamine [77]. It has been demonstrated that these glycans vary highly among *T. cruzi* strains, and β-Gal*f* is a component of the glycan structures of *T. cruzi* strains G, Tulahuen, and Dm28c; however, β-Gal*f* is not found in *T. cruzi* strains CL-Brener and Y (Figure 4(e)) [72–74, 78, 79]. These mucins play an important role in parasite-host interaction by both protecting against host defense mechanisms and ensuring targeting of specific cells and tissues [71, 77].

3. Galactofuranose Is a Virulence Factor in Kinetoplastids

It has been shown that incubation of *L. major* or *T. cruzi* with Gal*f*-specific antibodies blocks parasite binding to macrophages or mammalian cells, resulting in a 50–80% decrease in infection rates [60, 66, 80, 81]. It was further shown that the antibody specifically bound to the β-Gal*f* present in GIPLs of *T. cruzi* and GIPL-1 of *L. major* [60, 66]. This suggests that β-Gal*f* and the GIPLs of *T. cruzi* and GIPL-1 of *L. major* play a role in cell adhesion and infection. Furthermore, it was shown that macrophages incubated with *p*-nitrophenol-β-Gal*f* were infected 80% less by *L. major*, while macrophages incubated with *p*-nitrophenol-β-Gal*p* saw no decrease in infectivity [60]. Together, these results confirm that β-Gal*f* plays an important role in parasite-host interaction and suggest that β-Gal*f* biosynthetic enzymes are potential drug targets.

3.1. UDP-Glucose 4′-Epimerase (GalE). In *T. cruzi*, GalE is the first protein required for Gal*f* biosynthesis [82]. GalE is classified as a short-chain dehydrogenase/reductase (SDR) with a conserved Tyr-X-X-X-Lys motif and a characteristic Rossmann fold structure for $NAD(P)^+$ binding [38, 83]. GalE is a homodimer that consists of two domains, an N-terminal domain with the Rossmann fold and a C-terminal domain that binds the substrate, UDP-Glc [84, 85]. The catalytic site is located in the cleft between the two domains [84, 85]. The mechanism is shown to be conserved across species and involves the deprotonation of the Glc O4′ hydroxyl and hydride transfer from the C4 carbon of Gal to NAD^+ [84, 85]. The intermediate 4-keto sugar rotates in the active site and NADH transfers back the hydride to the opposite face forming UDP-Gal [84, 85].

Mutant strains of *T. brucei* and *T. cruzi* with deletion of the *galE* gene have not been obtained suggesting that Gal metabolism is essential for parasite survival [45, 82, 86, 87]. Conditional null mutants were created in *T. brucei* using tetracycline-regulated expression [45, 86]. Studies with this strain showed that removal of tetracycline from the trypomastigote parasite led to cell death and decreased Gal surface-expression levels by 30% [45, 86]. These studies showed that, upon Gal starvation, Gal was eliminated from *T. brucei* variant surface glycoprotein (VSG) and from poly-N-acetyllactosamine-containing glycoproteins causing cell growth to cease and differentiation to a stumpy-like form, ultimately leading to cell death [87].

Single *galE* knockout mutants of *T. cruzi* epimastigotes were also constructed [82]. These cell strains showed several phenotypic differences including shortened flagella and agglutination, which is thought to be the result of a lack of surface mucins [82]. Interestingly, these cell strains show a preference for expressing high levels of Gal*f*-containing GIPLs over Gal*p* mucins, whose expression levels were reduced 6–9-fold, suggesting levels of Gal*f* is preferentially maintained in the glycocalyx over Gal*p* [82]. In *Leishmania* spp., Gal can be obtained from extracellular sources, presumably by a family of hexose transporters [40, 88]. Thus, GalE is not essential in these parasites.

Studies have been undertaken to identify novel inhibitors that specifically target the GalE of *T. brucei* [89, 90]. Using high-throughput screens and computer modeling experiments, inhibitors that showed preference to *T. brucei* GalE over human GalE were identified [89, 90]. However, when these compounds were tested *in vitro* with *T. brucei* and either mammalian CHO cells or liver (MRC5) cells, these compounds either were cytotoxic to both the parasite and mammalian cells or the compound was ineffective against *T. brucei* [89, 90]. These studies suggest that, while GalE remains a potential drug target, there will be many difficulties in designing specific inhibitors for the treatment of these diseases without unwanted side effects.

3.2. UDP-Galactopyranose Mutase (UGM). UGM is a flavo-dependent enzyme that catalyzes the conversion of UDP-Gal*p* to UDP-Gal*f*. UGM was first identified in *Escherichia coli* K-12 in 1996, and since then it has been identified in several other pathogenic microorganisms including *M. tuberculosis*, *L. major*, *T. cruzi*, and *A. fumigatus* [5–8]. Interestingly, while *T. cruzi* produces UGM the related *T. brucei* does not, and as a result, *T. brucei* does not produce Gal*f* [70]. UGM has been found to be the sole biosynthetic source of Gal*f* and since it is not found in mammals is considered an ideal drug target.

Deletion of the UGM gene in *L. major* shows that this enzyme plays an important role in pathogenesis [25]. In the absence of UGM, *L. major* mutants were completely depleted of Gal*f*, lacked LPG PG repeats, and contained truncated forms of GIPLs [25]. Furthermore, mice infection by *L. major* lacking Gal*f* was significantly attenuated [25]. As previously mentioned, deletion of UGM also showed that Gal*f* is a virulence factor in *A. fumigatus* and *Aspergillus nidulans* [23, 95]. These studies show the importance of UGM and validate this enzyme as a drug target in protozoan and other eukaryotic human pathogens.

Although the reaction catalyzed by UGM does not involve a net redox change for the conversion of UDP-Gal*p* to UDP-Gal*f*, the reaction requires the flavin cofactor to be in the reduced form [96, 97]. Structural and mechanistic studies of the prokaryotic UGM have led to two proposals for the ring contraction mechanism (Figure 5). One mechanism

Table 1: UDP-galactopyranose mutases.

Species	Amino acids	% identity[a]	Oligomeric state	Reference
E. coli	367	100	Dimer	[91]
M. tuberculosis	399	44	Dimer	[92]
L. major	491	15	Monomer	[b]
T. cruzi	480	15	Monomer	[b]
A. fumigatus	510	14	Tetramer	[93]

[a] Identity to the *E. coli* enzyme.
[b] Oppenheimer and Sobrado unpublished results.

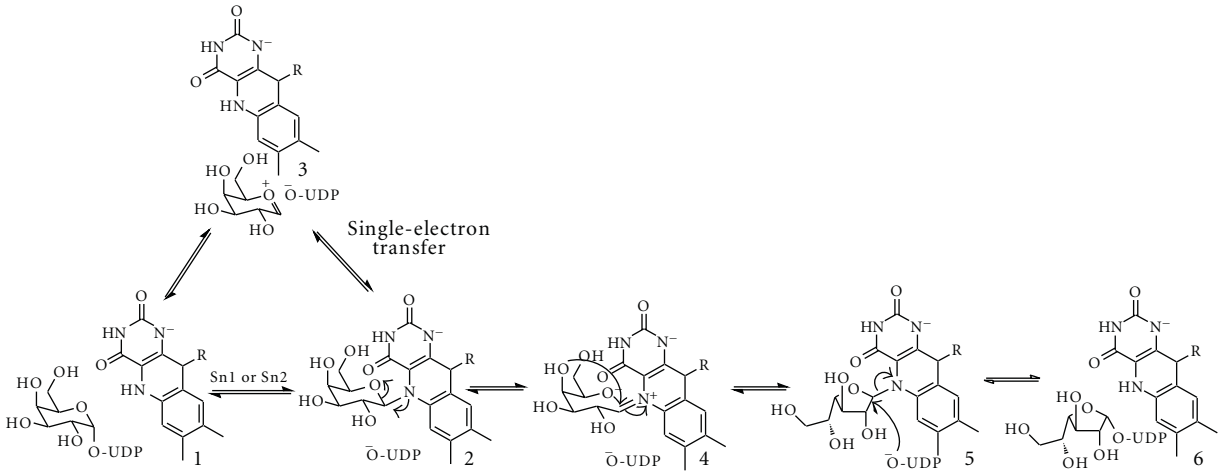

Figure 5: Proposed chemical mechanism for UGMs. Nucleophilic attack by the reduced flavin (1) leads to a flavin-galactose adduct (2). This step can either occur via an Sn1 or Sn2 reaction. Alternatively, the flavin can transfer one electron to a galactose oxocarbenium ion, forming a sugar and flavin radical that can also form the flavin-galactose adduct. Formation of a flavin iminium ion leads to sugar ring opening (4). Sugar ring contraction occurs by attack of the C4 hydroxyl to the C1-carbon (5). The final step is the bond formation to UDP (6).

depicts the reduced flavin acting as a nucleophile, attacking the anomeric carbon (C1) of Gal to form a flavin N5-C1 Gal adduct [98]. This adduct has been isolated and characterized in the prokaryotic UGM from *K. pneumoniae* [98, 99]. The other proposed mechanism involves a single-electron transfer from the reduced flavin to Gal, which then forms the sugar-flavin adduct [100].

Several structures have been solved for prokaryotic UGMs, in both oxidized and reduced states with and without substrate bound, providing excellent groundwork for the development of specific inhibitors [92, 99, 101]. The structure of prokaryotic UGMs show that it is a homodimer and a mixed α/β class protein with 3 domains: an FAD-binding domain with a typical Rossmann fold, a 5-helix bundle, and a 6-stranded antiparallel β-sheet [91, 101]. The structures of the reduced protein with substrate bound show that Gal is properly positioned for interaction with the flavin [99, 101].

Much less is known about the mechanism and structure of eukaryotic UGMs. These enzymes share low sequence identity, and the presence of inserts in the primary structure predicts significant structural differences (Figure 6). In fact, comparison of the oligomeric states between prokaryotic and eukaryotic UGMs indicates that quaternary structures vary among species (Table 1) [93]. Furthermore, our group, as well as others, has demonstrated that known inhibitors of eukaryotic UGM are not effective or have decreased potency against *L. major*, *A. fumigatus*, and *T. cruzi* UGMs [7] (Qi and Sobrado unpublished results). Therefore, mechanistic and structural work is urgently needed on the eukaryotic enzymes.

3.3. UDP-Galactofuranose Transferases. UDP-α-Gal*f* is synthesized in the cytosol by UGM and is transported into the Golgi where it is attached to the LPG and GIPLs by galactofuranosyl transferases (GalfTs) [102]. Currently, all known linkages of Gal*f* in *T. cruzi* and *Leishmania* spp. are in the β anomer conformation. The most extensively studied GalfT is LPG-1 from *L. major* and *L. donovani*. Studies on LPG-1 have revealed that it is localized to the Golgi apparatus, where it adds the β-Gal*f* to the core LPG structure [102, 103]. LPG-1 is a metal glycosyltranferase with typical conserved motifs including a cytoplasmic tail, a transmembrane domain, and a DXD metal-binding motif [104]. LPG-1 has been shown to only be responsible for the addition of Gal*f* to LPG and to not play a role in the addition of Gal*f* in the GIPLs [103, 105]. Mutants with the deletion of *lpg-1* gene in both *L. major* and *L. donovani* show LPG-1 to be important for LPG formation. Due to the lack of LPG, the mutant strains with *lpg-1* gene deleted in *L. major* display

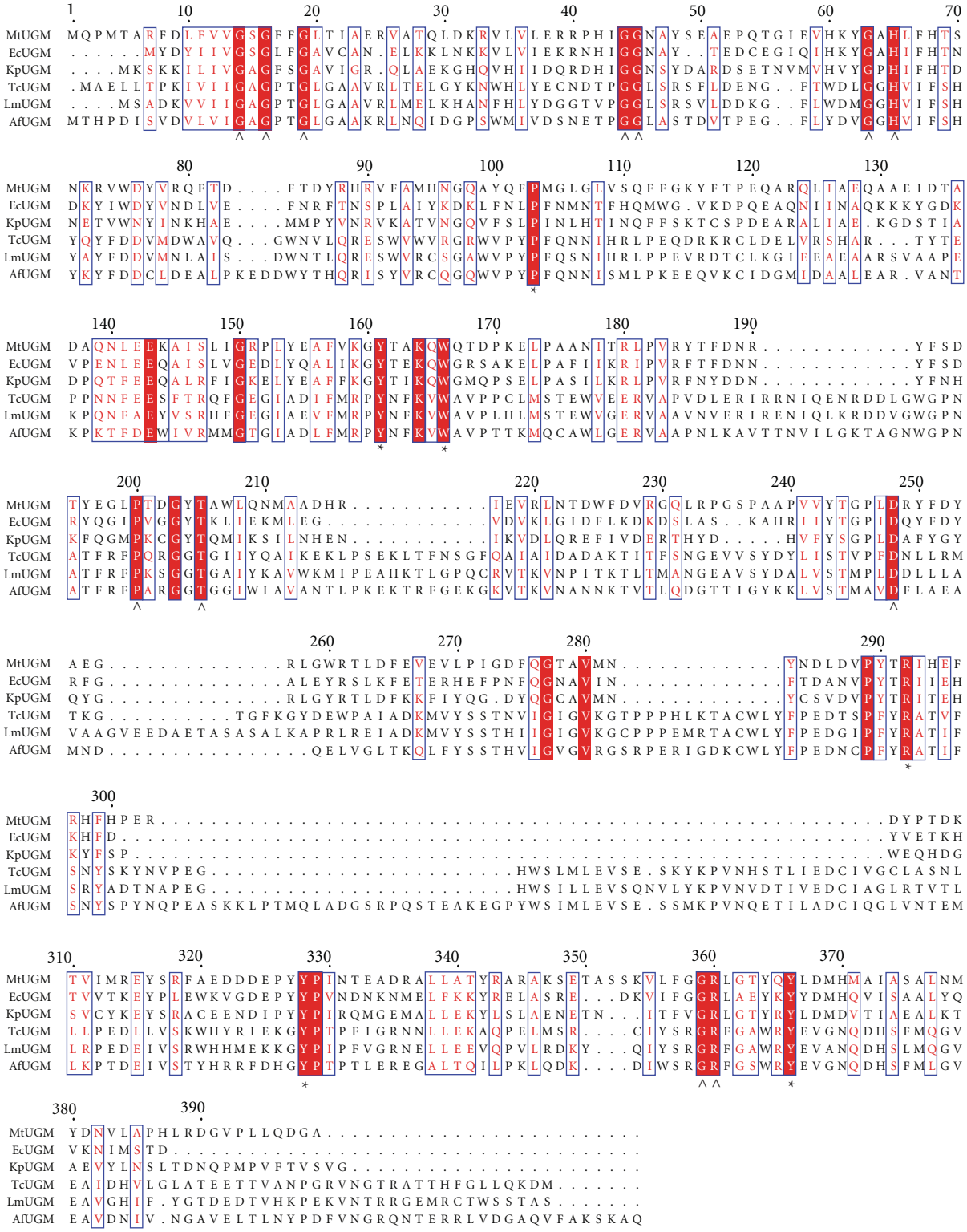

Figure 6: Multiple sequence alignment of UDP-galactopyranose mutases. Conserved amino acids found in the active site of bacterial UGM are marked with a star, and those involved in flavin binding are marked with arrowheads. Mt: *M. tuberculosis*, Ec: *E. coli*; Kp, *K. pneumoniae*; Tc: *T. cruzi*; Lm: *L. major*; Af: *A. fumigatus*. The program ClustalW was used to generate the alignment and Espript 2.2 to create the figure [94].

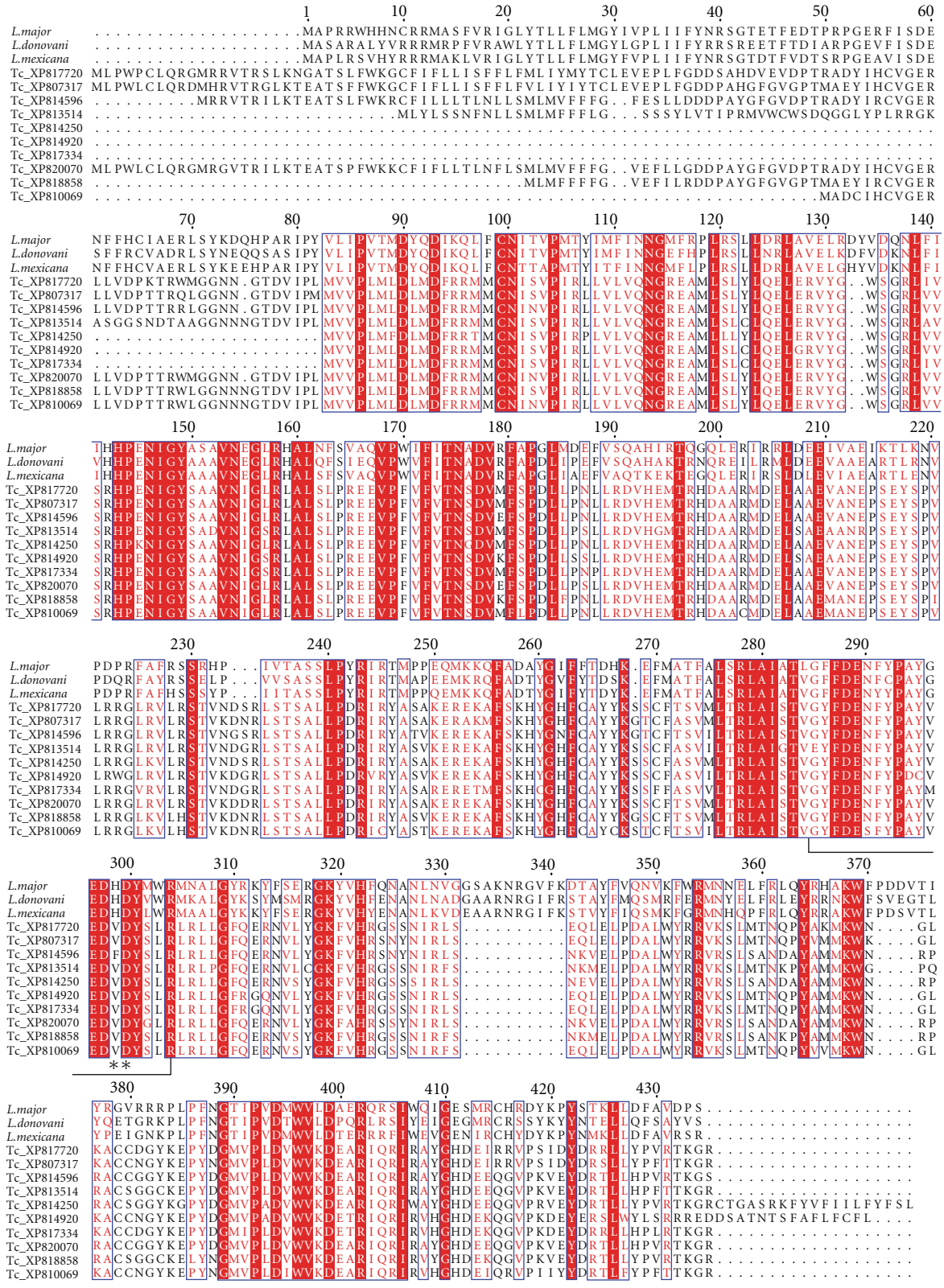

Figure 7: Alignment of *L. major* LPG-1 (XP001683753), *L. donovani* LPG-1 (ADG26596), *L. mexicana* LPG-1 (CAB6682), and ten putative *T. cruzi* GalfTs. Putative *T. cruzi* GalfTs were identified by BLAST search using *L. major* LPG-1 as the probe. The active site residues are shown in brackets, and the metal binding motif is represented with asterisks. The alignment was created as indicated in Figure 6.

attenuated virulence [103, 105]. These studies showed that LPG-1 could serve as a drug target in *L. major*.

There are no published studies on the GalfT from *T. cruzi*. In order to identify GalfTs in *T. cruzi*, a BLAST search was conducted using LPG-1 from *L. major* as a template, and more than 30 putative proteins annotated as β-GalfTs in the *T. cruzi* genome were identified [106, 107]. The top 10 putative GalfT sequences from the *T. cruzi* BLAST search were aligned with the *L. major* and *L. donovani* LPG-1 showing high sequence identity between these sequences (Figure 7). These sequences all contain the proposed catalytic site and demonstrate redundancy of the genes [104]. Redundancy of GalfTs is common in many different species, as often different transferases are used for each linkage type based on anomericity, bond linkage, and the substrate acceptors for Gal*f* [108]. Due to the high number of GalfTs within *T. cruzi*, targeting GalfTs for drug design most likely would not be effective.

4. Concluding Remarks

To cause infection, protozoan parasites must recognize the mammalian host environment, bind and infect the target cells, and evade the immune system. Undoubtedly, the cell surface of these pathogens plays important roles in these processes. Current drugs are able to kill most of the parasites during treatment; however, these treatments do not eliminate all the parasites, presumably because they can "hide" in the intracellular forms. Modification of the cell surface sugar composition will alter the mechanism of infection. Enzymes involved in the biosynthesis of Gal*f* have been shown to play a role in parasite growth and pathogenesis. GalE is essential for growth in *T. cruzi* and *T. brucei*, while UGM, and LPG-1 are important virulence factors in *L. major* [25, 82, 103]. Due to the presence of a GalE homolog in humans, compounds that inhibit this enzyme have toxic side effects. Furthermore, this enzyme is not important for virulence in *Leishmania* spp. UGM plays a central role in Gal*f* biosynthesis and is the only source of UDP-Gal*f*, which is the substrate for all the GalfT that attach Gal*f* to the final sugar-acceptor molecules. Consequently, UGM emerges as an attractive drug candidate, as no homolog is found in humans [109]. The unique chemical structure of UGM suggests that specific inhibitors can be identified. Targeting UGM in *T. cruzi* and *L. major* will affect their virulence in humans and perhaps allow the immune system to effectively clear the parasite. Alternatively, inhibition of UGM will enhance the activity of other antiparasitic drugs. Such combination therapy might be necessary to combat these complex eukaryotic human pathogens.

Acknowledgments

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Research Article

Identification of a Functional Type IA Topoisomerase, *Ld*TopIIIβ, from Kinetoplastid Parasite *Leishmania donovani*

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DNA topoisomerases of kinetoplastids represent a family of DNA processing enzymes that essentially solve the topological problems not only in nuclear DNA but also in kinetoplast DNA. We have, for the first time, identified a *Leishmania donovani* homologue of bacterial and eukaryotic IA type of topoisomerase III protein and termed as *Ld*TopIIIβ. Complementation study of wild-type and mutant *Ld*TopIIIβ with slow-growing topoisomerase III mutant yeast *S. cerevisiae* revealed the functional conservation of the leishmanial counterpart of topoisomerase IIIβ protein, the 327 tyrosine being the active site amino acid. A C-terminal deletion construct of *Ld*TopIIIβ could not suppress the slow-growth phenotype of mutant yeast, indicating the requirement of C-terminal region for the enzyme function *in vivo*. *Ld*TopIIIβ localized inside the nucleus and kinetoplast of the parasite. Taken together, our study indicates functional conservation and possible role of *Ld*TopIIIβ in parasite DNA processing.

1. Introduction

DNA topoisomerases are ubiquitous enzymes found in all prokaryotic and eukaryotic cells and in some viruses. They are involved in all aspects of DNA metabolism such as replication, transcription, recombination, and chromosome segregation [1, 2]. These reactions are based on sequential breakage and rejoining of the DNA phosphodiester backbone [2–4]. Type I DNA topoisomerases catalyze the cleavage of one strand of DNA, whereas type II DNA topoisomerases catalyze the cleavage of a double-stranded DNA, requiring ATP as a cofactor [4].

Type I DNA topoisomerases are further classified in two subfamilies, IA and IB, based on differences in amino acid sequence and reaction mechanisms [5]. The type IA enzymes link covalently to cleaved DNA through the 5′-phosphate. They are represented by bacterial topoisomerase I and III and the eukaryotic topoisomerase III enzymes. Type IB topoisomerases, exemplified by eukaryotic topoisomerase I, in contrast, become attached to 3′-phosphate end of the cleaved strand of the DNA [4]. Type IA topoisomerases are highly conserved from bacteria to humans.

While the function of topoisomerase II and I are quite well established, the role of topoisomerase III in DNA metabolism is still being defined. Genes encoding topoisomerase III enzymes are highly conserved in evolution from bacteria to human, and the phenotypic consequences of loss of topoisomerase III function are generally quite severe. It has been shown to possess a weak, ATP-independent relaxation activity towards negatively supercoiled DNA only and strict dependence on magnesium [6].

The *E. coli* chromosome encodes two type IA topoisomerase, DNA topoisomerase I [7] and topoisomerase III [8, 9]. Loss of topoisomerase III in *E. coli* results in an increase in deletions arising from recombination events between direct repeats [10, 11]. Yeast cells express a single type IA topoisomerase, topoisomerase III encoded by the *Top3* gene. In *S. pombe*, *top3* is essential for viability and plays a role in chromosome segregation [12]. It has been shown that *top3*-ts mutant *S. pombe* cells are sensitive to the DNA damaging agents UV and MMS (methyl methanesulfonate) at the restrictive temperature revealing that topoisomerase III is involved in DNA damage survival [13]. In *S. cerevisiae*, *top3*Δ mutants are viable, but very slow-growing and have

defects in S phase responses to DNA damage and in both mitotic and meiotic recombination [14, 15]. In vertebrates, there are two isoforms of topoisomerase III enzymes termed α and β [16–19]. Deletion of mouse topoisomerase IIIα gene led to embryonic lethality [20]. Deletion of mouse topoisomerase IIIβ gene displayed shortened lifespan and infertility [21, 22].

DNA topoisomerases of kinetoplastids represent a family of DNA processing enzymes that essentially solve the topological problems not only in the nuclear DNA but also in the kinetoplastid DNA. The IB type of bi-subunit topoisomerase I and topoisomerase II of the parasites which maintain vital cellular processes, are also proven target for clinically useful antitumor drugs [23]. Apart from this IB type of topoisomerase I, three type IA topoisomerases are there in the parasite genome, termed as topoisomerase IA, and two topoisomerase III. Topoisomerase IA of *T. brucei* has been reported and shown to be mitochondrial and essential for late theta structure resolution [24]. Very recently, a Topoisomerase IIIα from *T. brucei* has been shown to play a critical role in antigenic switching [25]. In the present study, for the first time, we have identified functionally active DNA topoisomerase IIIβ from kinetoplastid parasite *L. donovani*, which localized both inside the nucleus and kinetoplast of the parasite and rescued the topoisomerase III mutant yeast from slow-growth phenotype.

2. Materials and Methods

2.1. Parasite Culture and Maintenance. L. donovani strain AG 83 promastigotes were grown at 22°C in M199 liquid media supplemented 10% heat inactivated fetal calf serum. Transfected cells were maintained under the same conditions with 100 μg/mL G418.

2.2. Strains, Media, and Growth Conditions. The *Escherichia coli* strains used were DH5α and BL21 (DE3) pLysS. If required, ampicillin and chloramphenicol were used at 100 and 34 μg/mL final concentrations, respectively. The yeast strains used in the studies were W5909-3B (MAT alpha trp1-1 his3-11, 15 leu2-3, 112 ura3-1 RAD5 LYS2 MET15 ADE2) and W2633-4C (a/alpha top3:: TRP1/+) (kindly gifted by Dr. R Rothstein). The yeast cells were grown at 25°C on YEPD medium containing 1% peptone, 2% yeast extract, 2% dextrose and 1.5% agar or synthetic minimal media as required.

2.3. Cloning of Topoisomerase IIIβ Gene from Leishmania donovani. LdTopIIIβ gene was PCR amplified from the genomic DNA of *L. donovani* parasites using the sense primer 5′-GGAAATTCCATATGGGCCGCA ATGTGTTGATG-3′ and antisense primer 5′-CGGGATCCTCACCTGCGATC-CTCGCGGTTGCC-3′ and was cloned in bacterial expression vector pET16b in *Nde*1 and *Bam*H1 restriction sites, termed as *LdTopIIIβ-pET16b*.

2.4. Structural Analysis and Homology Modeling. Multiple sequence alignment of *Ld*TopIIIβ sequences from various species was carried out using CLUSTAL W (http://expasy.org/tools). Three-dimensional models of *Ld*TopIIIβ based on the crystal structure of *E. coli* topoisomerase III were generated using Swiss Prot (http://expasy.org/sprot). The generated files were opened in RasMol (http://www.rasmol.org/). The protein sequences were represented in ribbon format and the active site residues were represented in ball and stick format over the ribbon structure.

2.5. Construction of Expression Vectors and Transfection in Leishmania. LdTOPIIIβ genes was PCR amplified using *LdTopIIIβ-pET16b* as templates and was subcloned using the sense primer 5′-CGGGATCCATGGGCCGCA ATG-TGTTGATG-3′ and antisense primer 5′-GATATCCCTGCG-ATCCTCGCGGTTGCC-3′ in *Bam*H1 and *Eco*RV sites of *Leishmania* transfection vector pXG-B2863 (a kind gift from Dr. S. M. Beverley), to produce C-terminal-GFP-tagged full-length *Ld*TopIIIβ protein and termed as *LdTopIIIβ*-GFP. The constructs and empty vector pXG-B2863 were transfected into *L. donovani* promastigotes separately by electroporation as described earlier [26]. Briefly, late log-phase promastigotes were harvested and washed twice in OPTI-MEM (GIBCO). Cells were finally suspended at a density of 1×10^8/mL and 0.4 mL was taken into a 0.2 mm ice-chilled electroporation cuvette. Thirty microgram of plasmid DNA was taken in 100 μL of electroporation buffer and added to the cells. After 10 min on ice, the cells were electroporated with a single pulse by Bio-Rad Gene Pulsar apparatus using 450 V and 550 μF capacitance. The cells were incubated on ice for further 5 min and then added to10 mL of drug-free growth medium. After 24 h of survival 10 μg/mL G418 was added and kept at 22°C. The transfected cells were monitored visually by microscope and drug concentration was increased gradually. Finally the transfected cells were routinely maintained in medium containing 100 μg/mL G418.

2.6. Fluorescence Microscopy. Localization of C-terminal GFP tagged chimeric *Ld*TopIIIβ-GFP protein was visualized by fluorescence microscopy (Olympus IX81). Cell nucleus and kinetoplast were stained with DAPI. Differential visualization of the fluophores was achieved using a 488 nm excitation filters and 523 nm emission filter for GFP and 258 nm excitation and 361 nm emission filter for DAPI.

2.7. Construction of Mutants. The full-length *LdTopIIIβ* was subcloned in *Xba*I and *Bam*H1 sites into the yeast shuttle vector pVT100U, a kind gift from Dr. Rolf Sternglanz [27] and termed as *LdTopIIIβ-pVT* using the sense primer 5′-GCTCTAGAATGGGCCGCAATGTGTTGATG-3′ and antisense primer 5′-CGGGATCCTCACCTGCGATCCTC-GCGGTT-3′. For construction of C-terminal deletion construct of *LdTopIIIβ*, regions corresponding to amino acids 1-608 was PCR amplified using the primers 5′-GCTCTAGAATGGGCCGCAATGTGTTGATG-3′ (sense) and 5′-CGGGATCCGGCGGCGGAGATGGCGGAGAA-3′ (antisense) and was cloned in *Xba*1 and *Bam*H1 sites of pVT100U vector.

2.8. Site-Directed Mutagenesis. Single mutations were introduced in *LdTOPIIIβ* at position Tyr 327 (Y327). Mutagenesis was performed by using the QuikChangeXL site-directed kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. To carry out the desired mutations, *LdTopIIIβ-pVT* was used as templates for all mutagenesis experiments. For each mutation, the wild-type nucleotide was replaced using a specific pair of mutagenic primers. The following sense primer, along with the antisense counterparts (with codons in boldface and substitutions underlined), were used; for Y327 of *LdTopIIIβ*, sense primer was 5′-CCGCGGCTATATTTCG**TT**C**C**CCTCGTACCCGAATCC-3′ and antisense primer was 5′-GGATTCGGTACGAGG**GA**C**A**CGAAATATAGCCGCGG-3′.

2.9. Complementation Assay. The *top3* mutant yeast strain W2633-4C (a/alpha top3:: TRP1/+) (a kind gift from Dr. R Rothstein) was used for transformation with recombinant topo III proteins from *L. donovani* by the lithium acetate and polyethylene glycol method [28]. The transformants were cultured on solid synthetic minimal medium at 30°C for 2 days. Colonies were picked and cultured in tubes with 2 mL of synthetic minimal media at 30°C overnight.

2.10. Expression of Recombinant LdTopIIIβ Using the Expressway Cell-Free E. coli Expression System (Invitrogen). In vitro transcription and translation of *Ld*TopIII*β* proteins were carried out according to the manufacturer's protocol. *LdTopIIIβ-pET16b* plasmids were used as DNA templates for synthesis of the protein. After the reaction is over, the crude bacterial lysate containing the newly synthesized protein was tested for activity.

2.11. DNA Relaxation Activity by LdTopIIIβ. The type IA DNA topoisomerases were assayed by decreased mobility of the relaxed isomers of supercoiled pBS (SK+) [pBluescript (SK+)] DNA in an agarose gel. Relaxation assay was carried out with the crude lysates containing the *in vitro* transcribed and translated *Ld*TopIII*β*. Supercoiled pBS DNA (85%–95% were negatively supercoiled with the remaining being nicked circles) was used as substrate in the relaxation buffer (25 mM Tris-HCl, pH 7.5, 5% glycerol, 0.5 mM DTT, 2 mM $MgCl_2$, 50 μg/mL BSA). The amount of supercoiled monomer DNA band fluorescence after EtBr (0.5 μg/mL) staining was visualized using Gel Doc 2000 under UV illumination (Bio-Rad Quality one Software).

3. Results

3.1. Type IA Topoisomerase Genes in Leishmania. A search of the *Leishmania major* genome database yielded three type IA topoisomerases. One is on chromosome 21, annotated as topoisomerase IA (LmjF21.0125) with an ORF of 2453 bp. Two other type IA topoisomerases are present on chromosome 28 and 36, respectively, both of which are annotated as topoisomerase III (LmjF28.1780 and LmjF36.3200, resp.).

3.2. Identification of Topoisomerase III Genes in Leishmania donovani. One of the two topoisomerase III genes present in *L. major* geneDB is 2601 bp (LmjF28.1780) and encodes a 95 kDa predicted protein. The other topoisomerase III ORF (LmjF36.3200) is 2844 bp, and encodes a 104 kDa predicted protein. Topoisomerase III gene with 2601 bp was PCR amplified from the genomic DNA of *L. donovani*, cloned and sequenced (GeneBank accession number GQ499197). Blast analysis of the sequence confirmed the topoisomerase III lineage of the protein and henceforth referred as *LdTopIIIβ*. The alignment of *Ld*TopIII*β* with *S. cerevisiae* and *S. pombe* topoisomerase III and human topoisomerase III is shown in Figure 1. The active site tyrosine is located at the 327 position within a highly conserved GYISYPRTES sequence. The protein has 46.22% identity and 76.09% similarity with human topoisomerase III*β*. It contains seven CXXC sequences instead of eight found in other topoisomerase III*β* proteins. The intervening spacers are also highly conserved. Glycine (G) and arginine (R) rich clusters at the C-terminus end, which is another hallmark of topoisomerase III*β*, are also present. It has a continuous stretch of 19 G and R residues in the C-terminus. Three-dimensional structure generated by Swiss Prot has been shown in Figure 2(a). Figure 2(b) shows the magnified view of the active site. The conserved amino acid residues are represented in ball and stick format and have been labeled. Homology comparisons of *Ld*TopIII*β* with other IA type of topoisomerases have been provided in Table 1, which strongly indicates its topoisomerase III lineage.

3.3. Localization Study of LdTopIIIβ-GFP. In silico search was carried out to determine possible localization of *Ld*TopIII*β* protein. A 0.244 probability of mitochondrial transport was predicted by Mitoprot (http://expasy.org/tools) analysis and 73.9% cytoplasmic and 17.4% nuclear distribution was revealed by PSORT II analysis (http://expasy.org/tools). To determine the precise localization of the protein, full-length *Ld*TopIII*β* (865 aa) was cloned in *Leishmania* expression vector as a C-terminal fusion protein with GFP, termed as *Ld*TopIII*β*-GFP, and the construct was transfected in *L. donovani* parasites. Localization of *Ld*TopIII*β*-GFP was viewed under fluorescence microscopy (Figure 3(a)). Nucleus and kinetoplast DNA was stained with DAPI (Figure 3(b)). Comparison of DAPI and GFP fluorescence and merged images (Figure 3(c)) revealed that *Ld*TopIII*β* protein localized both inside the nucleus and kinetoplast of the parasites. Figures 3(d) and 3(e) show cytoplasmic distribution of control GFP protein in *L. donovani* parasites.

3.4. LdTopIIIβ Suppresses the Yeast top3Δ Slow-Growth Phenotype. Mutation of the *S. cerevisiae top3* gene is known to result in several phenotypes, including a growth rate which is only 50% that of wild-type [14]. In order to assess whether the *Ld*TopIII*β* possesses functional similarity to the yeast topoisomerase III, we have used a functional complementation assay of *Ld*TopIII*β* protein to rescue *top3* mutant *S. cerevisiae* strain from slow-growing phenotype. We have cloned the *LdTOPIIIβ* gene in a shuttle vector pVT100U

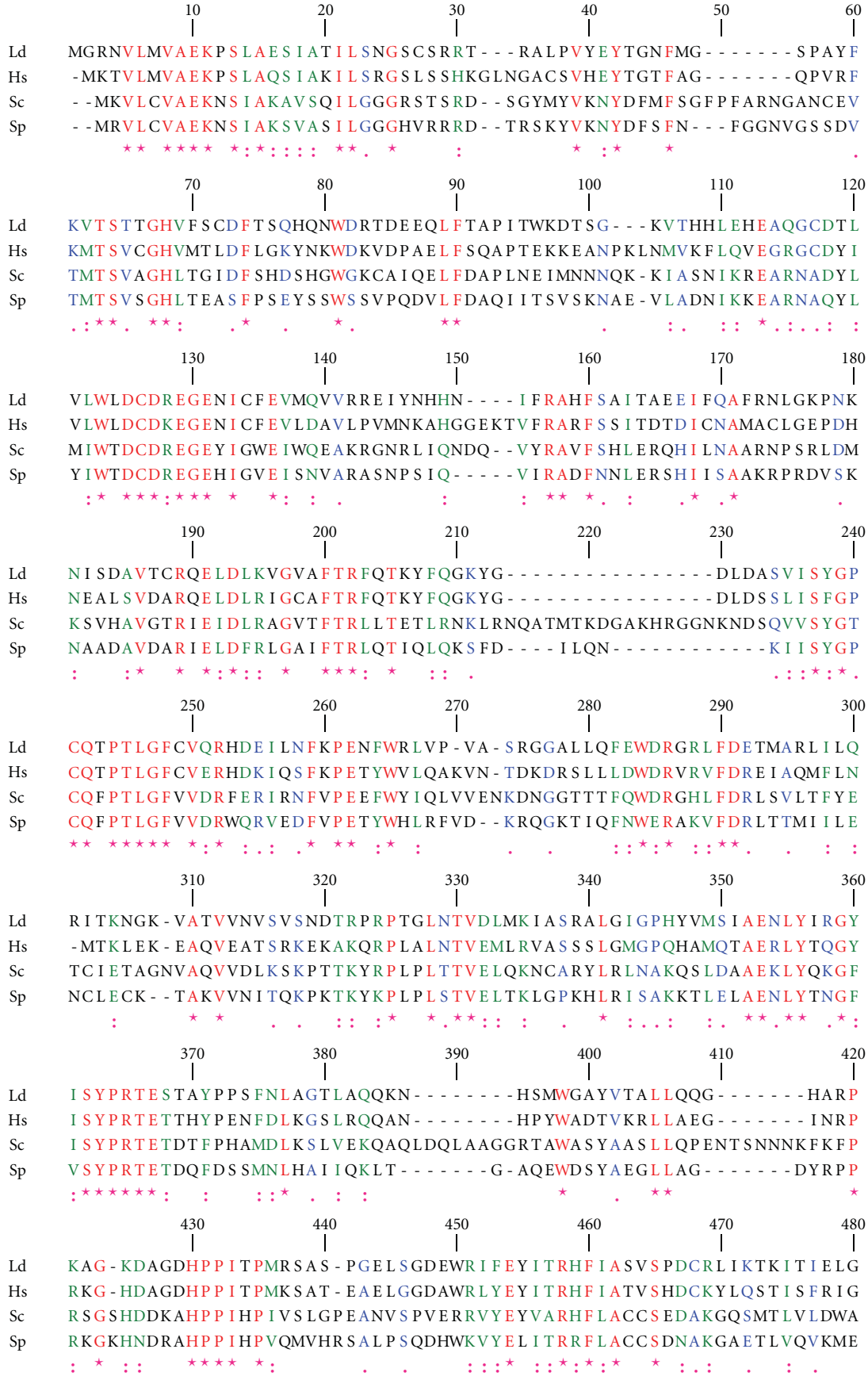


Figure 1: Continued.

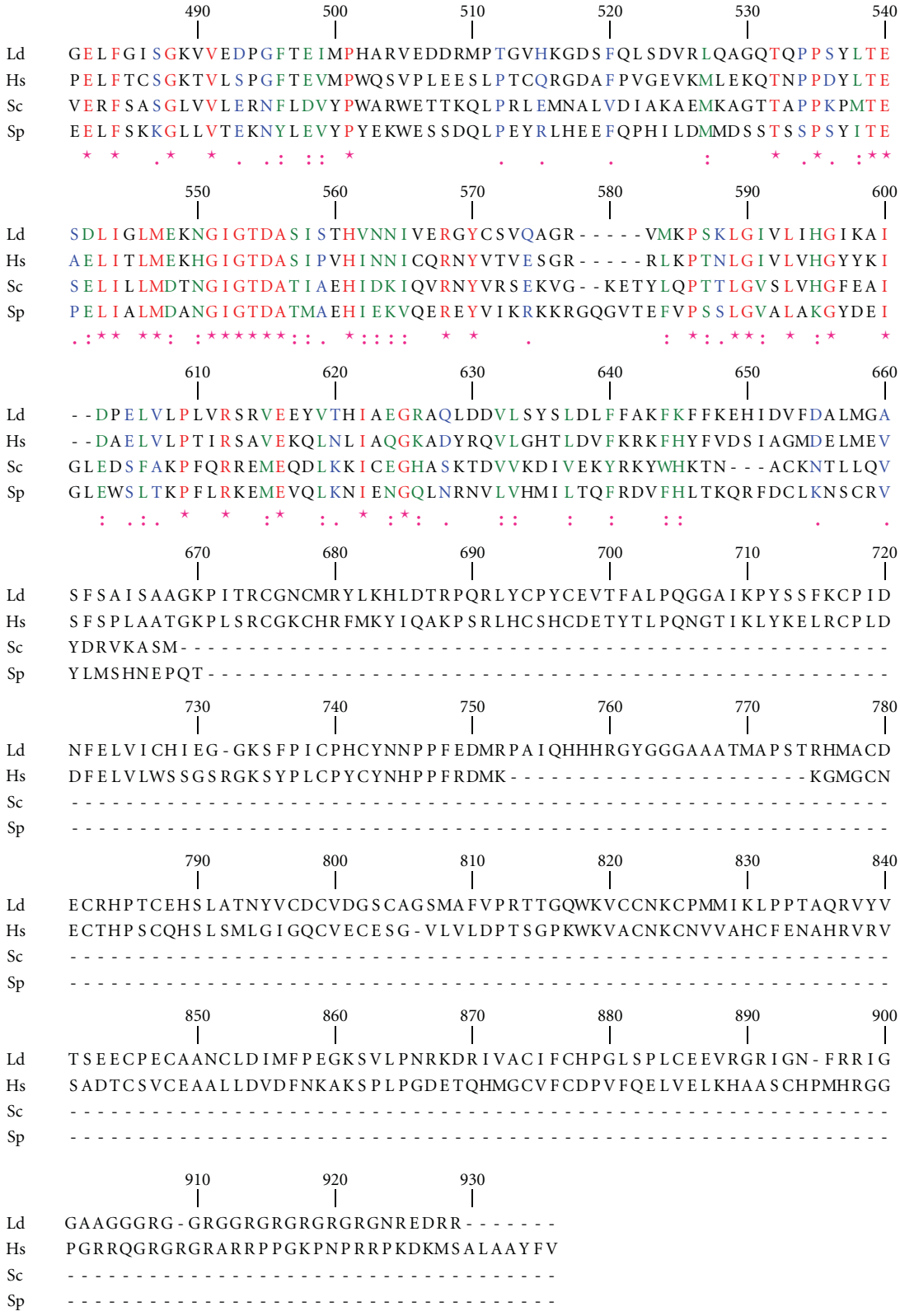
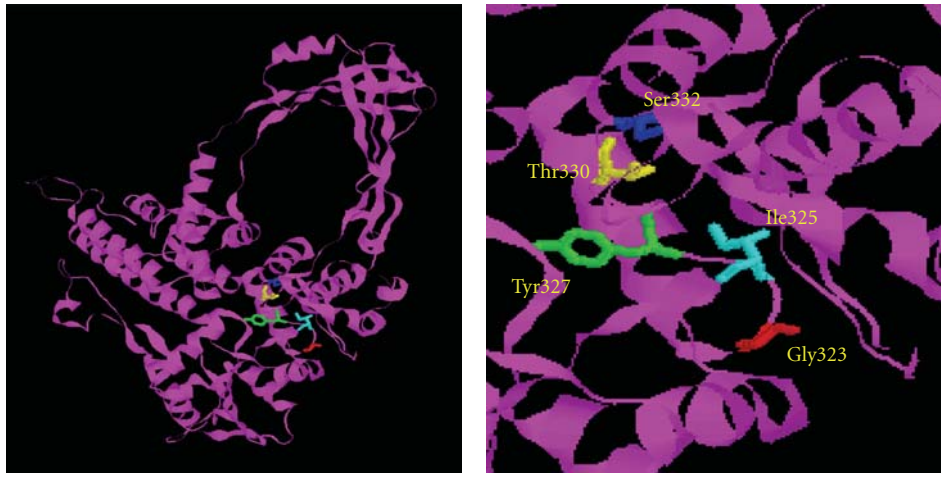


Figure 1: Amino acid sequence alignment. Sequence of *Ld*TopIIIβ (Ld) was aligned with the amino acid sequences of *H. sapiens* topoisomerase IIIβ (Hs), topoisomerase III from *S. cerevisiae* (Sc) and *S. pombe* (Sp) using CLUSTAL W. The amino acids are numbered on the top of the sequences. Active site motifs and other important conserved and identical residues are depicted in red. Green and blue indicate strongly similar and weakly similar amino acids, respectively.



(a) (b)

Figure 2: (a) Three-dimensional structure of *Ld*TopIII*β*. A ribbon structure representation of *Ld*TopIII*β* generated based on the crystal structure of *E. coli* topo isomerase III. Catalytically conserved residues are represented in ball and stick format over the ribbon structure. (b) Close up view of *Ld*TopIII*β* in which the amino acid residues that are vital for enzyme action are labeled and represented in ball and stick format. The positions of the amino acids are also mentioned.

Table 1: Amino acids homology comparisons between *Leishmania donovani* topoisomerase III*β* (866 amino acids) and other members of typeIA topoisomerases.

Type IA topoisomerases	Identity (%)	Similarity (%)	Size (amino acids)
*Ld*TopIII*α*	28.54	59.66	947
*Ld*TopIA	13.22	48.56	812
Human TopIII*α*	27.14	55.47	1001
Human TopIII*β*	46.22	76.09	862
S. cerevisiae TopIII	22.96	47.33	656
S. pombe TopIII	22.56	49.32	622

to generate *LdTopIIIβ-pVT* and transformed in *top3*Δ yeast *S. cerevisiae* and Ura+ colonies were selected. Yeast cells transformed with vector pVT100U served as control in the complementation assay. The *Ld*TopIII*β*-pVT partially complemented the slow-growth of *top3*Δ yeast (Figure 4(a)). The improved growth rate was not observed in case of the vector control (Figure 4(a)). This observation suggests that *Ld*TopIII*β* can be functionally expressed in yeast and shares functional similarity with *S. cerevisiae top3* gene, which is consistent with earlier observations made with *Drosophila* and human topisomerase III*β* proteins [19, 29]. To observe this complementation of *Ld*TopIII*β* in liquid medium a yeast growth curve analysis was carried out (Figure 4(b)). Equal amounts of the wild-type, topoisomerase 3 mutant yeast cells, topoisomerase 3 mutant yeast cells containing empty vector (pVT100U) and topoisomerase 3 mutant yeast cells containing *Ld*TopIII*β* (grown overnight at 30°C) were inoculated in fresh minimal medium and grown at 30°C. At every 2 hr interval up to 12 hrs, the growth was monitored and plotted.

3.5. Effects of Active Site Mutation of LdTopIIIβ on Complementation Ability. Tyrosine 327 of *Ld*TopIII*β* was predicted to be the active site amino acid residue from sequence alignment analysis. In order to determine that *Ld*TopIII*β* functionally complements the *top3* mutant yeast and the growth recovery was not due to any compensatory mechanism induced by *Ld*TopIII*β* we carried out site directed mutagenesis. We have mutated the active site residue of *Ld*TopIII*β* to phenylalanine (Y327F) by site directed mutagenesis and transformed in *top3* mutant yeast. Transformed cells were grown on plate, as well as in liquid minimal media. It was observed that the active site mutant construct could not suppress the slow-growth of *top3* mutant *S. cerevisiae* (Figures 5(a) and 5(b)) confirming role of active site tyrosine 327 in functional conservation of *Ld*TopIII*β* inside mutant yeast cells.

3.6. The C-Terminal Domain of LdTopIIIβ Is Essential for In Vivo Complementation. The *Leishmania* enzyme has a C-terminal segment of amino acids with no counterpart in yeast protein. The leishmanial protein contains Zn-binding motif at its C-terminus, which is absent in the topoisomerase III proteins of *E. coli* and yeast. The C-terminus residues of *E. coli* topoisomerase III have been previously shown to be involved in DNA binding [30]. To determine the role of the C-terminal stretch of *Ld*TopIII*β* in functional complementation, we have made a C-terminal deletion construct (*Ld*TopIIICΔ258) removing the 258 amino acids and transformed in topoisomerase III mutant yeast. The transformants were grown in plates and it was observed that the C-terminal deletion construct failed to rescue the mutant yeast from slow-growth (Figure 5(a)), suggesting essentiality of the C-terminal segment for functional complementation *in vivo*. To validate this observation in liquid medium we inoculated overnight grown cultures at 30°C in fresh minimal medium

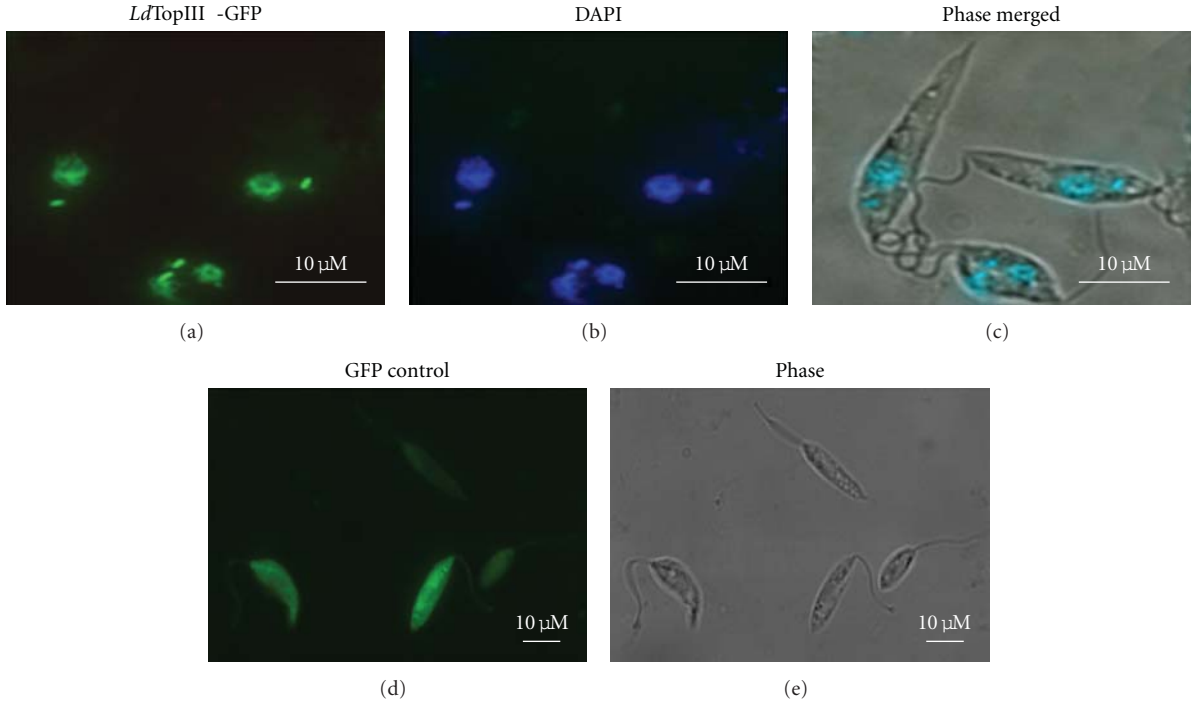


Figure 3: Localization *Ld*TopIIIβ. Wild-type construct of *Ld*TopIIIβ was transfected in *L. donovani* parasites as C-terminally fused GFP proteins and viewed under fluorescence microscope (100x). Nucleus and kinetoplast DNA are visualized by DAPI staining.

and monitored their growth at 3 hr intervals. The growth curve (Figure 5(b)) clearly indicates that *Ld*TopIIICΔ258 could not functionally complement the slow-growing topoisomerase III mutant yeast. This indicates that the conserved C-terminal region between amino acid residues 608–866 contains important residues that are required for *in vivo* function of *Ld*TopIIICΔ258. To get a better insight into the functional characteristics of the enzyme, we next sought to obtain recombinant *Ld*TopIIIβ protein *in vitro*.

3.7. In Vitro Activity of Recombinant LdTopIII Protein. *Ld*TopIIIβ was cloned in bacterial expression vector pET-16b and overexpressed in BL21 (DE3)-pLysS strain and induced with IPTG. But the overexpressed protein went to inclusion body and were found in the pellet as insoluble protein which could not be recovered in the soluble fraction in active state. However, to test the activity of the recombinant protein, we have used *in vitro* transcription-translation kit, which is specially designed for *in vitro* transcription and translation of target DNA to protein in a single reaction. The crude lysate containing the newly synthesized proteins were used for DNA relaxation assay. Figure 6(a) shows DNA relaxation by increasing amount of recombinant *Ld*TopIIIβ (lanes 2–8). Lane 1 is the DNA control. The results clearly show that the recombinant protein containing lysates were able to relax the negatively supercoiled DNA. To test that the activity was not coming from the lysate itself, we have carried out DNA relaxation activity with the empty vector containing lysate which contained insignificant amount of activity, shown in Figure 6(b) (lane 3). Lane 2 shows DNA relaxation activity by recombinant *Ld*TopIIIβ.

4. Discussion

The type IA topoisomerases are among the most conserved proteins in nature, and their presence in all organisms is supported by extensive biochemical and genomic sequence data [2, 4]. This universal presence suggests that the type IA DNA topoisomerases play an indispensable role in one or more fundamental processes involving DNA, plausibly in the removal of double Holliday junctions [2]. Topoisomerases IIIα and IIIβ of kinetoplastid parasites seem to be orthologues of same kind of enzymes in other eukaryotes, notable for branching early within their respective groups. In the present study, for the first time we have identified functionally active DNA topoisomerase IIIβ from *L. donovani*. Blast sequence alignments suggested topoisomerase IIIβ from *Leishmania* has high homology with human and drosophila topoisomerase IIIβ. It shares many features, which are typical for other topoisomerase IIIβ proteins including the CXXC type of motifs and a long stretch of G and R residues at its C-terminus. GFP-fused *Ld*TopIIIβ localized both inside the nucleus and the kinetoplast of *L. donovani* parasites indicating the involvement of *Ld*TopIIIβ in DNA processing inside both the parasite organelle. Our results show for the first time the presence of an IA type of topoisomerase in the nucleus, as well as in the kinetoplast of *Leishmania* parasites. Previously, a IA type of topoisomerase from bacterial origin has been reported to be mitochondrial in *T. brucei* [24].

*Ld*TopIIIβ could suppress the slow-growth phenotype of the mutant yeast indicating the functional conservation of topoisomerase III activity. The result is consistent with the

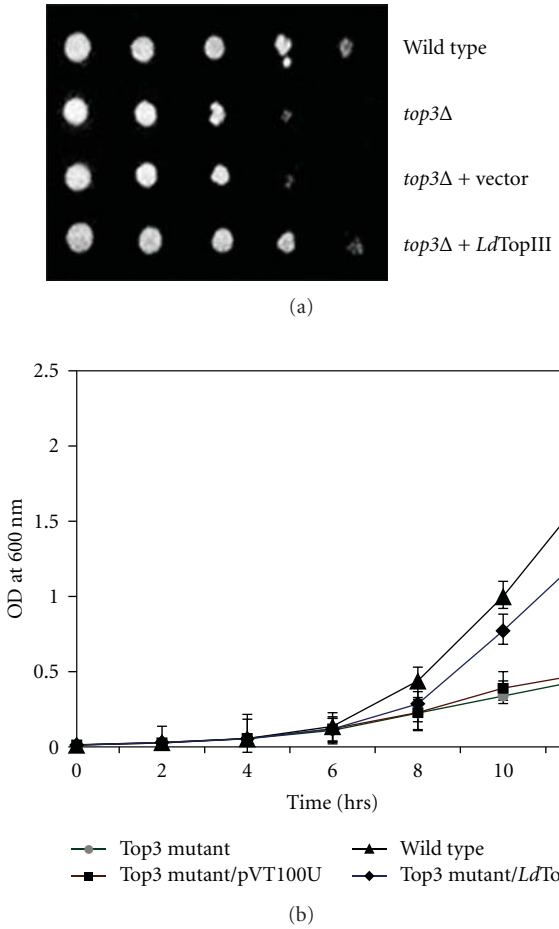


Figure 4: Functional complementation of *Ld*TopIII*β*. (a) *S. cerevisiae top3*Δ strain was transformed with a vector pVT100U and the vector carrying wild-type *Ld*TopIII*β*. Transformed cells were streaked on solid synthetic minimal media and incubated at 28°C. Ten-fold serial dilutions of exponentially growing wild-type strain, *top3*Δ strain, *top3*Δ strain harboring an empty vector, or *top3*Δ strain harboring plasmid encoded *Ld*TopIII*β*, as indicated on the right, grown on the plate. (b) Growth rate of the above-described strains were measured in the liquid synthetic medium and OD_{600} was plotted against time. Results represent the means ± standard errors of three independent experiments.

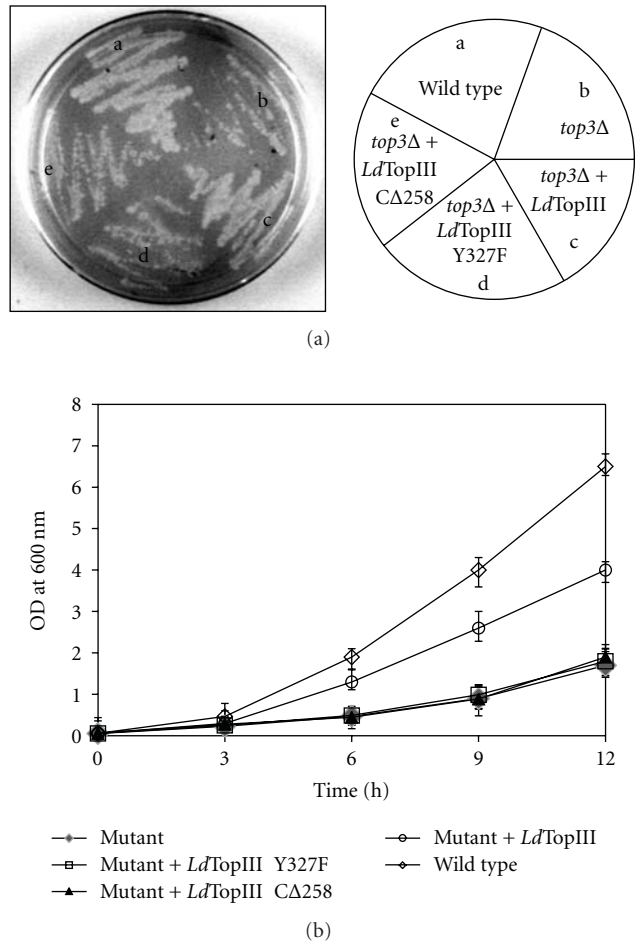


Figure 5: Complementation assay with mutant *Ld*TopIII*β*. (a) Topoisomerase III mutant yeast strain was transformed with the plasmid containing wild-type (c), active site mutant (d) and C-terminal deletion construct (e) of *Ld*TopIII*β*, separately. Transformed cells were streaked on solid synthetic minimal media and incubated at 30°C. (b) Complementation assay as described above carried out in liquid synthetic medium and OD_{600} plotted against time. Results represent the means ± standard errors of three independent experiments.

earlier observations made with human and *Drosophila* topoisomerase III*β* enzymes. The C-terminal deletion construct of *Ld*TopIII*β* lacking its Zn binding domain was unable to rescue the topoisomerase III mutant yeast from slow-growing phenotype revealing that the C-terminal 258 amino acids were indispensable for functional complementation of *Ld*TopIII*β* *in vivo*. Previous report reveals the requirement of the C-terminus region of bacterial topoisomerase III in substrate specificity [30]. It is possible that C-terminal end of the leishmanial topoisomerase III*β* protein is essential for DNA binding which requires further investigations. Site directed mutagenesis study revealed that tyrosine at 327 position within the conserved amino acid stretch is the active site tyrosine of *Ld*TopIII*β* and when this tyrosine is mutated to phenylalanine, the protein failed to complement the slow-growing mutant yeasts. The result indicates towards involvement of the functionally active *Ld*TopIII*β* in rescue of the mutant yeast from slow-growth. Our attempts to purify recombinant *Ld*TopIII*β* enzymes in active state from bacteria were unsuccessful as the proteins consistently went to inclusion body. But we were able to study, for the first time, the *in vitro* DNA relaxation activity the recombinant topoisomerase III protein from the kinetoplastid parasite *Leishmania*, when synthesized using cell free *in vitro* transcription-translation kit. Altogether, this is the first report of functionally active topoisomerase III*β* protein from unicellular kinetoplastid parasite *Leishmania*.

The biological functions of eukaryotic topoisomerse III proteins are intriguing. Important nonoverlapping function

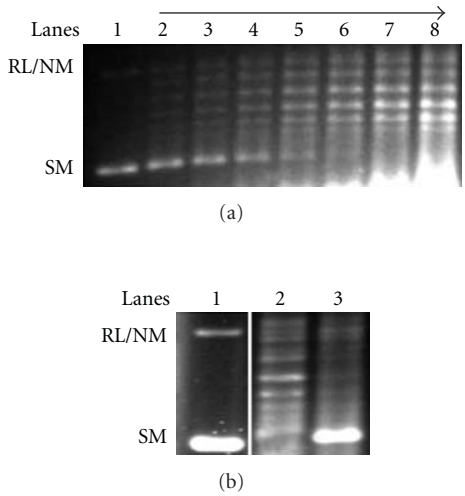


Figure 6: DNA relaxation assay by recombinant *Ld*TopIIIβ. (a) Negatively super coiled DNA was incubated with 1, 2, 3, 4, 5, 7 and 10 μL of recombinant *Ld*TopIIIβ containing lysate for 30 min (lanes 2–8). Lane 1 is the DNA control. (b) DNA relaxation assay carried out with recombinant *Ld*TopIIIβ (lane 2), and empty vector containing lysate (lane 3). Lane 1 is the DNA control.

of the two isozymes of topoisomerse III has been revealed by previous studies. The mouse-knockout experiments suggests, the α form is essential for embryonic development, whereas the β form is critical for life span [20, 21]. Genetic experiments in yeast have demonstrated that *TOP3* plays a role in suppressing mitotic recombination and in resolving recombined homologous chromosomes during meiosis I [14, 31]. Preferential cleavage of plasmid-based R- and D-loops, has been reported by *Drosophila* topoisomerase IIIβ [32]. Furthermore, the combined action of either yeast or bacterial topoisomerse III and the DNA helicase RecQ can promote the formation of DNA catenanes [33]. The unwinding action of a RecQ type helicase appears to generate a DNA structure that can be recognized by a topoisomerase III. RecQ helicases are also conserved in kinetoplastide parasites. The only report of functionally significant topoisomerase IIIα from kinetoplastide parasite came very recently, which describes that topoisomerse IIIα from *Trypanosoma brucei* influences antigenic variation by monitoring expression-site-associated *VSG* switching [25]. Existence of functionally active topoisomerase III protein in *Leishmania* indicates towards its role in DNA metabolism in the parasites, which requires further studies and might emerge as a new therapeutic target that can be exploited against the deadly parasites.

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Review Article

The Sphingolipid Biosynthetic Pathway Is a Potential Target for Chemotherapy against Chagas Disease

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The protozoan parasite *Trypanosoma cruzi* is the causative agent of human Chagas disease, for which there currently is no cure. The life cycle of *T. cruzi* is complex, including an extracellular phase in the triatomine insect vector and an obligatory intracellular stage inside the vertebrate host. These phases depend on a variety of surface glycosylphosphatidylinositol-(GPI-) anchored glycoconjugates that are synthesized by the parasite. Therefore, the surface expression of GPI-anchored components and the biosynthetic pathways of GPI anchors are attractive targets for new therapies for Chagas disease. We identified new drug targets for chemotherapy by taking the available genome sequence information and searching for differences in the sphingolipid biosynthetic pathways (SBPs) of mammals and *T. cruzi*. In this paper, we discuss the major steps of the SBP in mammals, yeast and *T. cruzi*, focusing on the IPC synthase and ceramide remodeling of *T. cruzi* as potential therapeutic targets for Chagas disease.

1. Introduction

Sphingolipids (SLs) belong to a diverse group of amphipathic lipids that have essential functions in eukaryotes. They are constituents of cellular membrane compartments and participate in a diverse array of signal transduction processes [1, 2]. The final products of the sphingolipid biosynthetic pathways (SBPs) are different in mammals, fungi, plants and protozoa. Thus, certain steps of this pathway are potential targets for chemotherapy against fungal [3] and protozoal infections [4–6]. Sphingomyelin (SM) is the primary phosphosphingolipid that is produced by mammalian cells, including in humans [7]. This molecule is formed by the transfer of the choline-phosphate head group from phosphatidylcholine (PC) to ceramide, a reaction catalyzed by SM synthase [8]. In contrast, plants, fungi, and some protozoa synthesize inositolphosphorylceramide (IPC) as their primary phosphosphingolipid [9]. In this pathway, the IPC synthase enzyme [10] catalyzes the transfer of inositol phosphate to ceramide. IPC makes up a relatively low proportion of fungal phospholipids. Nonetheless, it is essential, as IPC synthase-null mutants are not viable [11] and inhibitors of this enzyme kill fungal cells [12, 13].

Numerous proteins and glycolipids are attached to membranes by a glycosylphosphatidylinositol (GPI) anchor. This posttranslational modification is conserved among yeast, protozoa, plants and animals [14]. All of these groups except animals have GPI anchors containing IPC as an attached lipid. GPI biosynthesis is essential for mammalian embryonic development and the growth of yeasts and trypanosomes [15–17]. The biosynthesis and maturation of GPI anchors occurs during the ER-to-Golgi transit, beginning with the sequential addition of sugars and ethanolamine phosphates to phosphatidylinositol (PI). Subsequent structural remodeling reactions can happen during biosynthesis or after attachment to proteins. Most of these steps have been studied at the biochemical and molecular levels [18, 19]. Recently, it has been shown that GPI lipid remodeling reactions are important for maintaining the correct fate of GPI-anchored glycoconjugates and their proper association with microdomains in certain cellular processes [20, 21].

Several neglected tropical diseases worldwide are caused by a group of trypanosomatid protozoan parasites (also known as Tritryps), including the following: (i) African trypanosomes (*Trypanosoma brucei* subspecies), which cause sleeping sickness, (ii) multiple *Leishmania* species, which cause cutaneous and visceral forms of leishmaniases, and (iii) *Trypanosoma cruzi*, which causes Chagas disease [22–26]. Recent studies have shown that all of these parasites are capable of synthesizing IPC and that the expression of IPC is regulated during development.

The genome sequences of these pathogenic microrganisms have recently been published, allowing us to search for differences between the SL and GPI structures of mammals and Tritryps to identify novel drug targets. Here, we will discuss the major steps of the SBP in mammals, yeast and Tritryps. We will focus on the IPC synthase and ceramide remodeling of *T. cruzi* as potential therapeutic targets for Chagas disease.

2. Initial Steps in the *De Novo* Synthesis of a Sphingoid Long-Chain Base

In all eukaryotes, *de novo* SL biosynthesis starts with the condensation of L-serine and palmitoyl-CoA into 3-ketodi-hydrosphingosine (3-KDS), as shown in Figure 1 and Table 1. A pyridoxal 5′-phosphate-dependent enzyme called serine palmitoyltransferase (SPT) catalyzes this reaction. The SPT enzyme (Figure 1 and Table 1, Step 1) is a complex of two subunits, SPT1 and SPT2 [27]. In yeast, the small peptide TSC3 significantly enhances SPT activity [28]. Two open reading frames (ORFs) with homology to yeast *LCB1* and *LCB2* can be found in the Tritryp genome database. Although SPT1p and SPT2p function as a heterodimer, all experimental data indicate that the SPT2 subunit contains the catalytic site [28]. For this reason, most of the studies on Tritryps (mainly *L. major* and *T. brucei*) have focused on SPT2.

The expression of the *LmSPT2* gene (also called *LmLCB2*) is developmentally regulated. LmSPT2p is undetectable in the late stationary growth phase of promastigotes, as well as in metacyclic trypomastigotes and intramacrophage amastigotes [29, 30]. Deletion of the *SPT2* gene in *L. major* results in a complete loss of IPC and ceramide, whereas other alkyl/acyl and acyl/acyl phospholipids remain unchanged [29]. Although *spt2*$^-$ mutant promastigotes are viable and grow during log phase, they fail to efficiently differentiate into infective metacyclic trypomastigotes and die rapidly at this stage. This phenotype can be rescued either by the addition of sphingoid bases (3-ketodihydrosphinsosine or 3-KDS, dihydrosphingosine, sphingosine, and phytosphingosine), ethanolamine (EtN) or EtN-phosphate to the medium, or by complementation with the original *LmLCB2* gene [29–33]. However, neither ceramide nor SM can rescue the stationary phase defects or restore IPC synthesis [29]. Similar observations have previously been made in yeast and mammalian *SPT*-deficient mutants [34, 35]. Denny and Smith [30] showed that exocytic trafficking is compromised in *spt2*$^-$ mutants, but Zhang and colleagues [29, 32] observed little negative effect on vesicular trafficking. However, both groups found that *spt2*$^-$ parasites retain their ability to form membrane microdomains and lipid rafts [29, 32, 33]. It has been suggested that *Leishmania* can compensate for the loss of SLs by increasing its overall level of lipid synthesis, for example, by increasing ergosterol [31] or GIPL [33] production. These *spt2*$^-$ mutants are still able to establish infection in a mouse model, although with some delay [33], confirming that the first step in the *de novo* SBPs is unnecessary for either the survival of *Leishmania* within host macrophages or the resulting pathogenesis.

In contrast, the first step of the SBPs, which is catalyzed by SPT, is essential in *T. brucei*. This conclusion was based on pharmacological experiments with myriocin (Figure 1, Step 1), an inhibitor of SPT [36], and genetic experiments [37, 38]. These perturbations most profoundly affect viability, cellular proliferation and cytokinesis, with marginal effects on secretory trafficking and lipid raft formation. SL depletion can be rescued by the addition of 3-KDS, the immediate downstream intermediate in the SBPs, but not by the addition of ceramide or EtN [37, 38]. These results indicate that *T. brucei* absolutely requires *de novo* synthesis of SLs.

In the second step of SL biosynthesis (Figure 1 and Table 1, Step 2), the product 3-KDS is rapidly converted into dihydrosphingosine (DHS; sphinganine) in a NADPH-dependent manner by the 3-ketodihydrosphingosine reductase (KDSR) encoded by *TSC10* (temperaturesensitive suppressor of *cgs2*Δ) in *S. cerevisiae* [39] and by FVT-1 (follicular lymphoma variant translocation-1) in mammals [40]. Although KDSR activity has not been measured in parasites, *TSC10* homologues can be found in the genomes of Tritryps (Table 1). A predicted *TcKDRS* can be found in the GeneDB database in two genomic fragments (*Tc00.1047053510997.10* and *Tc00.1047053506959.64*) whose nucleotide and amino acid sequences are 98% identical. These probably correspond to the two haplotypes present in the hybrid CL-Brener strain [41].

3. Ceramide Synthase: The Central Axis of the SBPs

The next step in the SBPs, the synthesis of ceramide, is a key component of the pathway (Figure 1 and Table 1, Step 3). Ceramide is critical for cell growth and functions in several different cellular events, including apoptosis, growth arrest, endocytosis and stress responses [42–44]. Ceramide can be degraded by a ceramidase, or the sphingoid bases can be phosphorylated to produce DHS-1-P/SPH-1-P signaling molecules (Figure 1). Ceramide is synthesized mainly from the reaction of a fatty acyl-CoA with a sphingoid base catalyzed by an acyl-CoA:sphingosine *N*-acyltransferase or ceramide synthase (CerS) [45, 46]. An acyl-CoA-independent CerS activity has been described [47, 48] although it probably represents a reversal of ceramidase action. In yeasts, the long-chain base (LCB) DHS can be hydroxylated at C-4 by *SUR2/SYR2* [49] to form the sphingoid base phytosphingosine, which is later *N*-acylated by either of the two CerSs, encoded by *LAG1* (longevity assurance gene) or *LAC1* [50, 51], to yield phytoceramide.

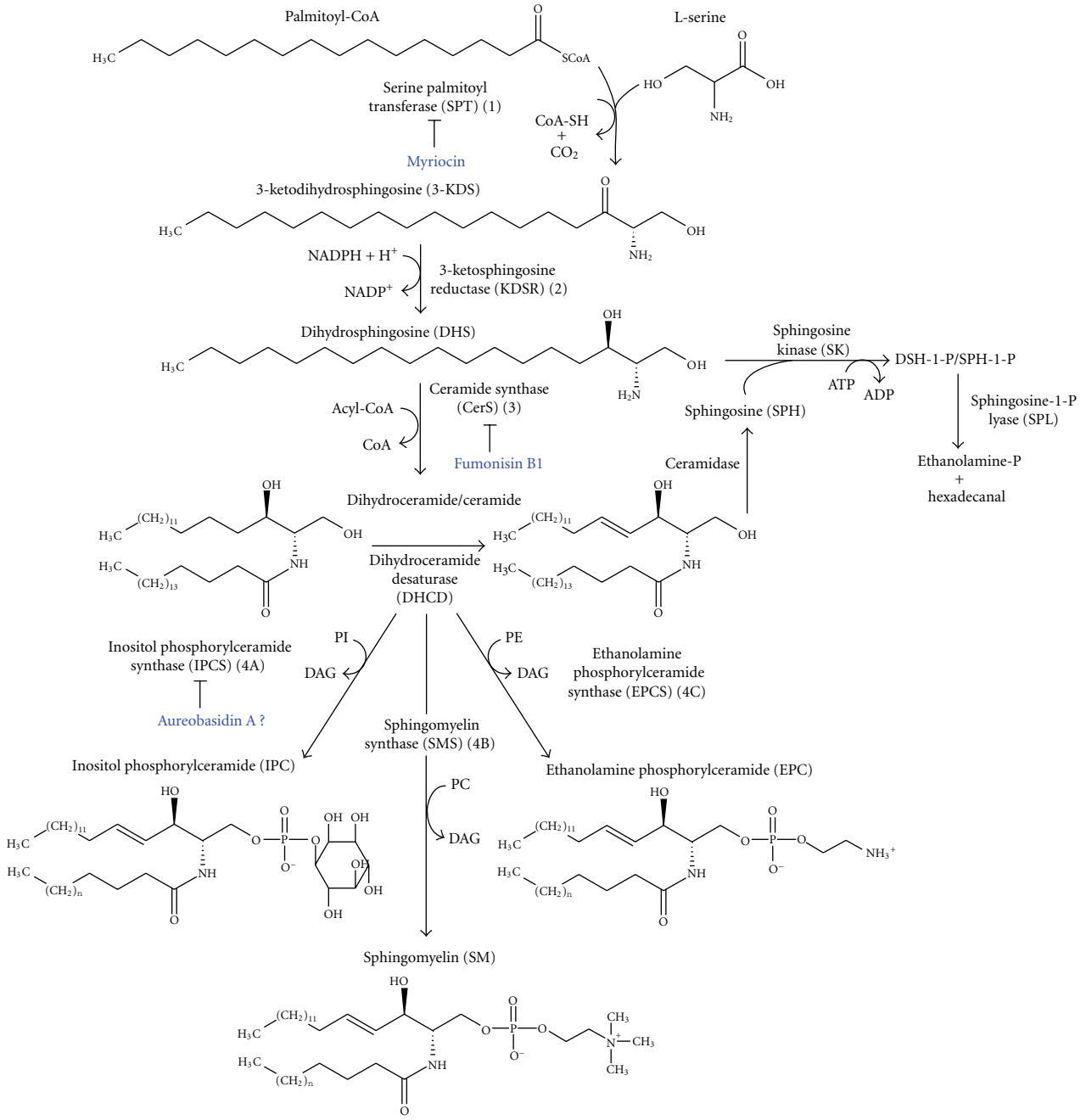

Figure 1: General scheme of the SBP in Tritryps. Substrates, products, enzymes and effective/potential (?) inhibitors of the four (1–4) initial SBP steps are indicated as described in the text and in Table 1.

In mammals, DHS can be directly *N*-acylated by a family of CerSs encoded by *CERS1-6* genes [52]. In addition, another essential component of the yeast CerS, known as Lip1p, was described recently. It forms a heteromeric complex with Lac1p and Lag1p and is required for CerS activity in yeast [53]. No orthologue of Lip1 has yet been found in nonfungal species.

In Trypanosomatids, ceramide can be found as a lipid component of phospholipids like SM, in *T. brucei,* and IPC, which is expressed in all Tritryps [54–62]. CerS activity has been identified [54] and characterized at the biochemical and molecular levels only in *T. cruzi* ([63], submitted). The TcCerS was initially identified by the incorporation of [^{3}H]palmitic acid into ceramides, which were chemically degraded to radiolabeled dihydrosphingosine and fatty acid [54]. More recently, TcCerS activity has been detected in a cell-free system using the microsomal fraction of epimastigote forms of *T. cruzi*. In this system, the enzyme was shown to employ both sphingoid long-chain bases (DHS and SPH) ([63], submitted). This activity requires acyl-CoAs,

Table 1: Genes required for sphingolipid biosynthesis and lipid remodeling steps in mammals, *Saccharomyces cerevisiae*, *Trypanosoma brucei*, *Leishmania major*, and *Trypanosoma cruzi*.

Step	Activity	Mammals	*S. cerevisiae*	*T. brucei*	*L. major*	*T. cruzi*
1	SPT	*LCB1* *LCB2*	*LCB1/LCB2* *TSC3*	*TbSPT1* *TbSPT2*	*LmSPT1* *LmSPT2*	*TcSPT1* *TcSPT2*
2	KDSR	*FVT-1*	*TSC10*	*Tb927.10.4040*[a]	*LmjF.35.0330*	*Tc00.1047053510997.10* *Tc00.1047053506959.64*[a]
3	CERS	*CERS1-6*	*LAG1/LAC1* *LIP1*	*Tb927.8.7730* *Tb927.4.4740*	*LmjF.31.1780*	*TcCERS1*
4	SLS	*SMS1* *SMS2*	*AUR1* *KEI1*	*TbSLS1-4*	*LmIPCS*	*TcIPCS1* *TcIPCS2*
5	ID	*PGAP1*	*BST1*	*GPIdeAc2*	—[b]	*Tc00.1047053508153.1040*[a]
6	GPIPLA2	*PGAP3*	*PER1*	—	—	—
7	LGPIAT-I	*PGAP2*	*GUP1*	*TbGUP1*	*LmjF.19.1000/LmjF.19.1320* *LmjF.19.1340/LmjF.19.1345* LmjF.19.1347[a]	*TcGUP1*[c]
8	CR	—	*CWH43*	—	*LmjF27.1770*[a]	*Tc00.1047053504153.120*[a]

[a] Corresponding homologues of each yeast gene were found by BLAST at NCBI and GeneDB (http://www.genedb.org/).
[b] Not found.
[c] *TcGUP* can be *Tc00.1047053508943.4*, *Tc00.1047053511355.59*, or *Tc00.1047053503809.90*.

with palmitoyl-CoA being preferred. In addition, Fumonisin B_1, a broadly active and well-known acyl-CoA-dependent CerS inhibitor (Figure 1, Step 3), blocks parasite CerS activity ([63], submitted). However, unlike what has been observed in fungi, the CerS inhibitors Australifungin [64] and Fumonisin B_1 [65] do not affect the proliferation of epimastigotes in culture ([63], submitted). Orthologues of the conserved Lag1-domain from yeast CerS, *LAG1*, were identified in a search of the Tritryp genome sequences (Table 1). The *T. cruzi* candidate gene (*TcCERS1*), which was hypothesized to encode the parasite's CerS orthologue, can functionally complement the lethality of a *lag1*Δ *lac1*Δ double-deletion yeast mutant that has no detectable acyl-CoA-dependent CerS ([63], submitted).

Glycoinositolphospholipids (GIPLs) are abundant surface glycoconjugates of *T. cruzi* and are involved in the pathogenesis of Chagas disease [66, 67]. GIPLs contain an IPC-lipid anchor that is formed by dihydroceramide *N*-acylated with palmitic or lignoceric acids [68–72]. TcCerS uses only palmitoyl-CoA as a substrate donor ([63], submitted); it is not known how the parasite incorporates C24:0 into ceramides. Recently, a novel fatty acid synthesis system was identified in the Tritryps [73]. In this system, synthesis is mediated by elongases that prime a butyryl-CoA molecule with malonyl-CoA units as the donor substrate and promote fatty acid extension to a length of 18 carbons or longer. Therefore, it is possible that this system elongates shorter fatty acids to C24:0 so that they can then be incorporated into ceramides. Alternatively, the substrate could be another fatty acid, like arachidonate (C20:4 from extracellular sources), which could be elongated and desaturated further to generate very-long-chain fatty acids [73]. Finally, IPC acyl-hydrolase and IPC acyl-transferase activities have been detected in membranes of *T. cruzi* [74, 75] and could be involved in the remodeling of the endogenous ceramide C16:0 fatty acids by an extracellular fatty acid (see below).

4. IPC Synthase Activity

The synthesis of IPC (Figure 1 and Table 1, Step 4A) occurs by the transfer of inositol phosphate from PI to the C-1 hydroxyl group of ceramide or phytoceramide. This reaction is catalyzed by IPC synthase, which is localized to the Golgi of yeasts. IPC synthase is encoded by *AUR1* (also called *IPC1*) [11]. As already mentioned, IPC represents a relatively low proportion of fungal phospholipids, but it is essential. IPC synthase-null mutants are not viable [11], and fungal cells are killed by the IPC synthase inhibitors Aureobasidin A (AbA) [12] and Rustmicin [13]. Recently, a critical protein interaction partner for yeast IPC synthase was identified and named Kei1p. It was shown that Kei1p is essential for both yeast IPC synthase activity and for its sensitivity to AbA [76]. As shown in Figure 1 (Step 4B), mammals cannot synthesize IPC; instead, they produce SM using two major SM synthases [8] encoded by *SM1* and *SM2* (Table 1, Step 4).

In yeasts, IPC is found as a lipid in complex SLs [9] or in mature GPI-anchored surface proteins. It is composed of a sphingoid-base with *N*-acylated C18:0–C26:0 fatty acids [18, 77, 78]. In *T. cruzi*, IPC is found in the majority of GIPLs (in epimastigotes) [67–70], the GPI anchors of Ssp4 antigen (in amastigotes) [54], *trans*-sialidase and Tc-85 glycoprotein (in trypomastigotes) [79, 80], mucins and 1G7-Ag (in metacyclic forms) [71, 81, 82]. In replacement of IPC, GPI-anchored proteins contain only 1-*O*-hexadecylglycerol-based PIs [71, 72, 81–84]. The lipid moiety of GIPLs also includes a small amount (2–8%) of 1-*O*-hexadecyl-2-acyl-PIs [72, 85]. Thus, there is a developmentally regulated expression [54] and distribution of ceramide in *T. cruzi* GPI-anchored components. In *T. brucei* bloodstream forms, GPI-protein anchors contain dimyristoylglycerol, whereas in *Leishmania,* these anchors are mainly composed of *sn*-1-alkyl-2-acyl-PI or *sn*-1-alkyl-2-*lyso*-PI [14]. In *Leishmania*, IPC is present together with other SLs and sterols in organized lipid rafts

[86] but it is never found attached to any GPI-anchored protein or GIPL [14]. In *T. brucei*, IPC has been found in insect-stage procyclic forms (PCFs), but its role remains unclear [38, 60, 87].

Another SL that is produced by *T. brucei* is SM. This lipid has been detected using a combination of methods, including metabolic labeling, enzyme treatments and high-resolution mass spectrometry, in both insect and mammalian stages of the parasite [61, 88]. The relative amount of SM in PCF cells is significantly lower than that in blood-stream forms (BSFs) [87], probably because the ceramide in BSFs is used in conjunction with PC to form SM, whereas in PCFs ceramide is also used to form IPC from PI [38, 61]. In BSF parasites of *T. brucei*, the unusual phosphosphingolipid ethanolamine phosphorylceramide (EPC, Figure 1, Step 4C) was detected for the first time by Sutterwala and colleagues [61]. Its presence was later confirmed by a lipidomic analysis [87].

The IPC synthases of *L. major* (*LmIPCS*) and *T. cruzi* (*TcIPC1* and *TcIPC2*) and the SL synthase (SLS) family of *T. brucei* (*TbSLS1-4*) were initially identified in the GeneDB database based on sequence similarity [4]. These are shown in Table 1 (Step 4). The *TbSLS* genes are organized in a unique linear tandem array. All Tritryp sequences are predicted to have six *trans*-membrane (TM) domains and two luminal motifs that likely constitute the catalytic domain. Each Tritryp sequence contains histidine and aspartate residues that mediate nucleophilic attack on the lipid phosphate ester bonds. This predicted topology more closely resembles that of mammalian SMSs, which also have the signature motifs (D1-4), than the fungal IPC synthase, which contains only the D3-4 motifs and is encoded by *AUR1/KEI1* genes [4, 8, 11, 61, 62].

All the *TbSLSs* genes are constitutively expressed in both stages of the life-cycle (PCF and BSF). Simultaneous knockdown of the four *TbSLS* genes using a pan-specific RNAi showed that the *TbSLS* gene products are required for cell viability [61]. The activity of each of the *TbSLS* gene products has been validated with genetic and biochemical analyses, as well as with a recently developed cell-free system for the synthesis of active polytopic membrane proteins. TbSLS1p is an IPC synthase and is expressed in PCFs, whereas TbSLS2p is an EPC synthase, and TbSLS3p and TbSLS4p are bifunctional SM/EPC synthases [61, 62]. Sequence alignments and site-specific mutagenesis indicate that the specific phospholipid head group donor depends on subtle differences in active site residues [62]. Taken together, the existing data support the ability of *T. brucei* to synthesize IPC (Figure 1, Step 4A), SM (Figure 1, Step 4B) and EPC (Figure 1, Step 4C).

The Lederkremer's group was the first to identify IPC in *T. cruzi* epimastigotes [57], trypomastigotes [58] and amastigotes [54, 75]. IPC synthase activity was initially found in the microsomal membranes of all life-cycle stages of *T. cruzi* [59]. The TcIPC synthase activity is consistent with the proposed reaction scheme for IPC synthase in fungi and plants, though there are differences in the optimal pH conditions, metal requirements and detergent preferences [59]. The classical inhibitors of fungal IPC synthase, rustmicin and AbA, do not inhibit *T. cruzi* IPC synthase *in vitro* (over the range of 0.9–7 μM) and do not affect the proliferation of epimastigotes in culture (>40 μM). However, AbA inhibits both the proliferation of amastigotes inside macrophages and the release of trypomastigotes from these cells in a dose-dependent manner [59]. The reduction in intracellular proliferation can be partially attributed to the effect of this drug on macrophage function, diminishing phagocytic capacity and nitric oxide production [59].

Similar results have been obtained with AbA in *T. brucei* SL synthesis [62], suggesting that the IPC synthase enzyme is not the main target of AbA in parasites. Nonetheless, TbSLSs remain potential chemotherapeutic targets, as *T. brucei* is critically dependent on *de novo* synthesis of sphingolipids to survive. Mass spectrometry of lipids extracted from AbA-treated *L. major* promastigotes has shown that there is no effect on IPC synthesis, unless very high concentrations of AbA are administered (>5.0 μM) [29].

5. The Lipid Remodeling Reactions

More than 20 genes involved in GPI biosynthesis and protein attachment have been identified. In most cases, these genes are conserved from yeast to mammalian cells [18, 89]. As in mammals, yeasts, *T. brucei* and *Leishmania*, the first steps of GPI anchor biosynthesis in *T. cruzi* do not include a ceramide precursor [90, 91]. Thus, ceramide is probably added during a later remodeling step in *T. cruzi* [75, 90, 91], as in yeasts [78, 92, 93]. Although there are differences in the ceramide composition, remodeling in yeast happens after the attachment of the GPI anchor to the proteins, whereas in *T. cruzi,* remodeling may occur on the GPI protein anchors and/or GIPLs.

Lipid remodeling of GPI-anchored proteins has been studied in fungi, mammals and *T. brucei* [20, 21]. The four most important enzymes and the genes involved in this process are listed in Table 1 (Steps 5–8); the lipid remodeling reactions are depicted in a simplified scheme in Figure 2.

In mammals, the first reaction is a deacylation to remove the fatty acid linked to position C-2 of the GPI anchor inositol ring (Table 1 and Figure 2, Step 5). This reaction is catalyzed by PGAP1p (postGPI attachment to protein 1) and occurs before the GPI-attached proteins leave the ER, as it is critical for efficient transit of GPI anchored proteins to the Golgi [94]. The second reaction is the removal of the unsaturated acyl chain from the *sn*-2 position of the alkyl-acyl-glycerolipid to form a *lyso*-GPI (Table 1 and Figure 2, Step 6). This reaction is catalyzed by PGAP3p [95]. The final reaction in mammals is the transfer of a saturated acyl chain (C18:0) to the *sn*-2 position of the *lyso*-GPI species (Table 1 and Figure 2, Step 7). PGAP2p is one protein involved in this process [96], but it is probably not an acyl-transferase because it has no homology to acyltransferases [21].

Mature GPI-anchored proteins in yeasts contain two different types of lipid moieties [77, 78, 92]. The first is a diacylglycerol with a C26:0 fatty acid at the *sn*-2 position. The second is a ceramide containing mainly C18:0 phytosphingosine and a C26:0 fatty acid [78]. In both cases, the C26:0 fatty acid may be 2-hydroxylated. As mentioned above

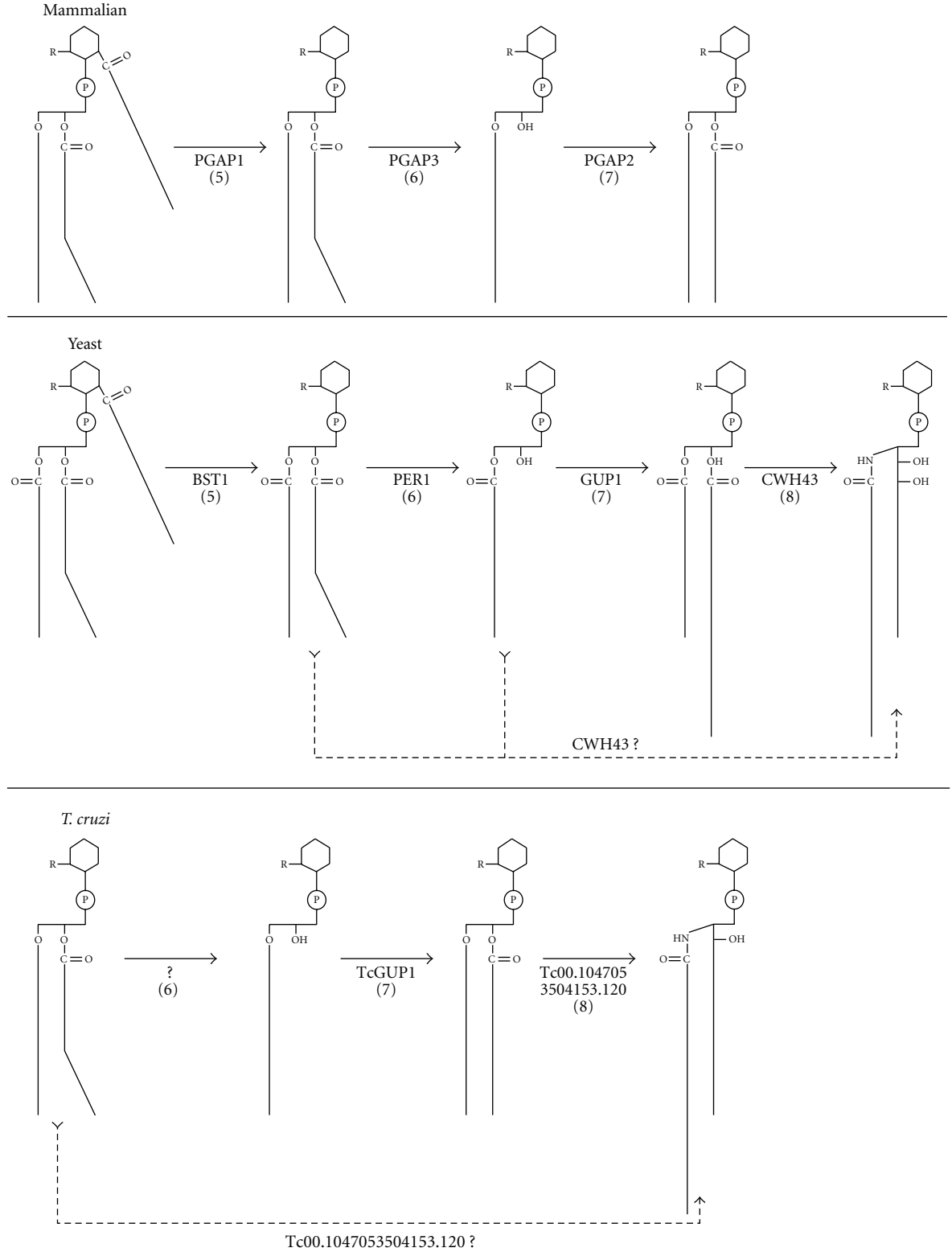

Figure 2: Lipid remodeling pathways in mammals, yeast and *T. cruzi*. "R" represents the entire glycan structure of each GPI anchor precursor linked to a protein. Although this assumption has been validated in mammals (top panel) and yeast (middle panel), no such experimental data are available for *T. cruzi* (bottom panel). Each step (in parentheses) has corresponding entries in Table 1.

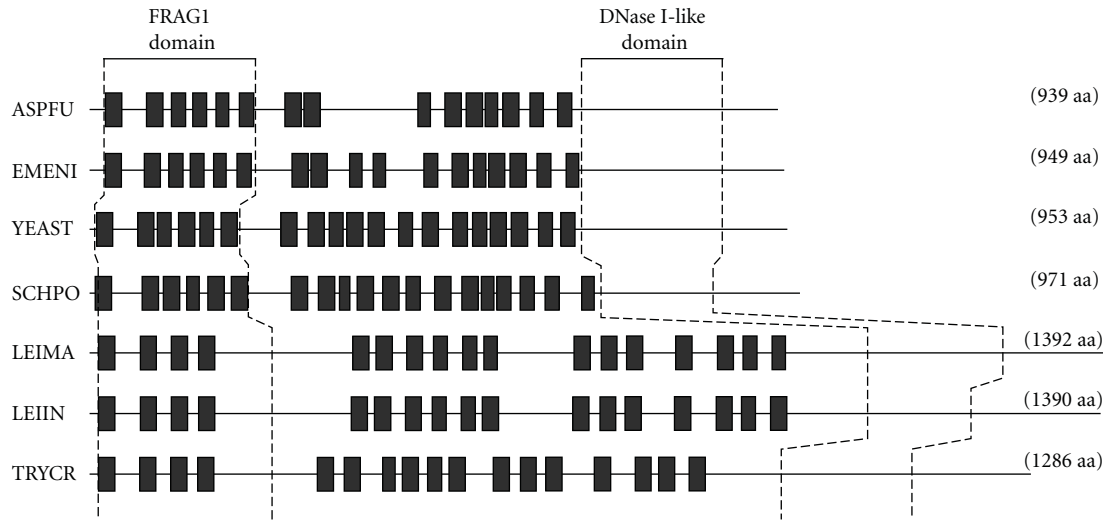

Figure 3: Comparison of the CWH43 proteins from fungi and Tritryps. The amino acid sequences of CWH43 proteins from *Aspergillus fumigatus* (ASPFU), *Emericella nidulans* (EMENI), *Saccharomyces cerevisiae* (YEAST) and *Schizosaccharomyces pombe* (SCHPO) were compared with the putative CWH43p candidates from *Leishmania major* (LEIMA), *Leishmania infantum* (LEIIN) and *Trypanosoma cruzi* (TRYCR). Thick horizontal bars indicate the relative positions of the membrane-spanning domains as predicted by the TMHMM Server. Dashed lines delineate the relative positions of the FRAG1 and DNase I-like domains, which are located at the N- and C-terminus, respectively.

for mammals, lipid remodeling of the yeast GPI-anchored proteins starts in the ER with the removal of the fatty acid linked to the C-2 position of the GPI anchor inositol ring (Table 1 and Figure 2, Step 5). This reaction is catalyzed by the PGAP1p orthologue BST1p [94]. Unlike mammals, which have a *sn*-1-alkyl-2-acyl-glycerolipid attached to the GPI, yeasts have a diacyl-glycerolipid (Figure 2). The next step is the removal of the C18:1 fatty acid at the *sn*-2 position of diacylglycerol to form a *lyso*-GPI (Figure 2, Step 6). This reaction is performed by GPI phospholipase A_2 (GPI-PLA_2), which requires PER1p (Table 1, Step 6) for its activity [94, 95]. Next, the free *sn*-2 position is filled with a C26:0 fatty acid (Figure 2, Step 7) by an acyltransferase called GUP1p [97]. Finally, the diacylglycerol lipid moiety is replaced by a ceramide (Figure 2, Step 8) with C18:0 phytosphingosine and a hydroxy-C26:0 fatty acid [78]. It has been reported recently that CWH43p (Table 1, Step 8) is responsible for this replacement [98, 99]. Indeed, the exchange reaction requires the C-terminus of CWH43p, and the association of the CWH43p-N with the CWH43p-C enhances the lipid remodeling reaction [99]. The alignment of CWH43p to its homologues in fission yeast (*Shizosaccharomyces pombe*), filamentous fungi, mice and humans has identified conserved residues that are important for the lipid remodeling function, including H802, D862 and R882, and protein stability (G57) [97, 99]. The N-terminal region of yeast CWH43 has a FRAG1 domain (Figure 3), which is also present in PGAP2p and is thought to act as a protein interaction motif that enhances stability under conditions of replicative stress [21]. The C-terminal region of CWH43 also has a DNase I-like motif (Figure 3) that is found in Isc1p, Inp51p, Inp52p, Inp53p and Inp54p. Isc1p is an inositol phosphosphingolipid phospholipase C [20], and the Inp51/52/53/54 proteins are phosphoinositol phosphatases [21]. This motif may be involved in the recognition of inositol phosphate, in which case the DNase I-like region in the C-terminal domain of CWH43p could be important for the recognition of PI on the GPI anchor.

The sequential remodeling reactions mentioned above comprise one of the three possible pathways for lipid remodeling in yeast (Figure 2, compare full with dashed lines). Another may be a divergent pathway, in which the *lyso*-GPI generated by PER1p is a direct substrate for the ceramide remodeling activity of CWH43p [99]. CWH43p could be involved in the direct exchange of glycerolipids containing an unsaturated fatty acid for the ceramide moiety. This third alternative is proposed to function as a backup if the first one, which is mediated by PER1p and GUP1p, is defective [94, 98, 99].

Acyl exchange also occurs in *T. brucei*, but in this organism, the remodeling happens before (during GPI anchor biosynthesis) and after the GPI anchor precursor is transferred onto the protein [100]. *T. brucei* contains two different GPI deacylation/reacylation pathways. One pathway (termed lipid remodeling) acts on lipid A' (a GPI anchor biosynthetic precursor containing a *sn*-2-heterogenous fatty acid) to generate lipid A (containing *sn*-1, 2-dimyristoylglycerol), creating the intermediates lipid θ (*lyso*-GPI) and lipid A'' (*sn*-1-stearoyl-2-myristoylglycerol). The other pathway (termed lipid exchange) acts on both GPI proteins and lipid A and exchanges the original myristate for another myristate [100, 101]. The acyl transferase GUP1p has homologues encoded in the genomes of *T. brucei* and *T. cruzi* (Table 1, Step 7). In *T. brucei*, TbGUP1p is required for the acylation of lipid θ (*lyso*-PI) to generate lipid A'' (*sn*-1-stearoyl-2-myristoylglycerol) in the remodeling of GPI lipids [100, 101].

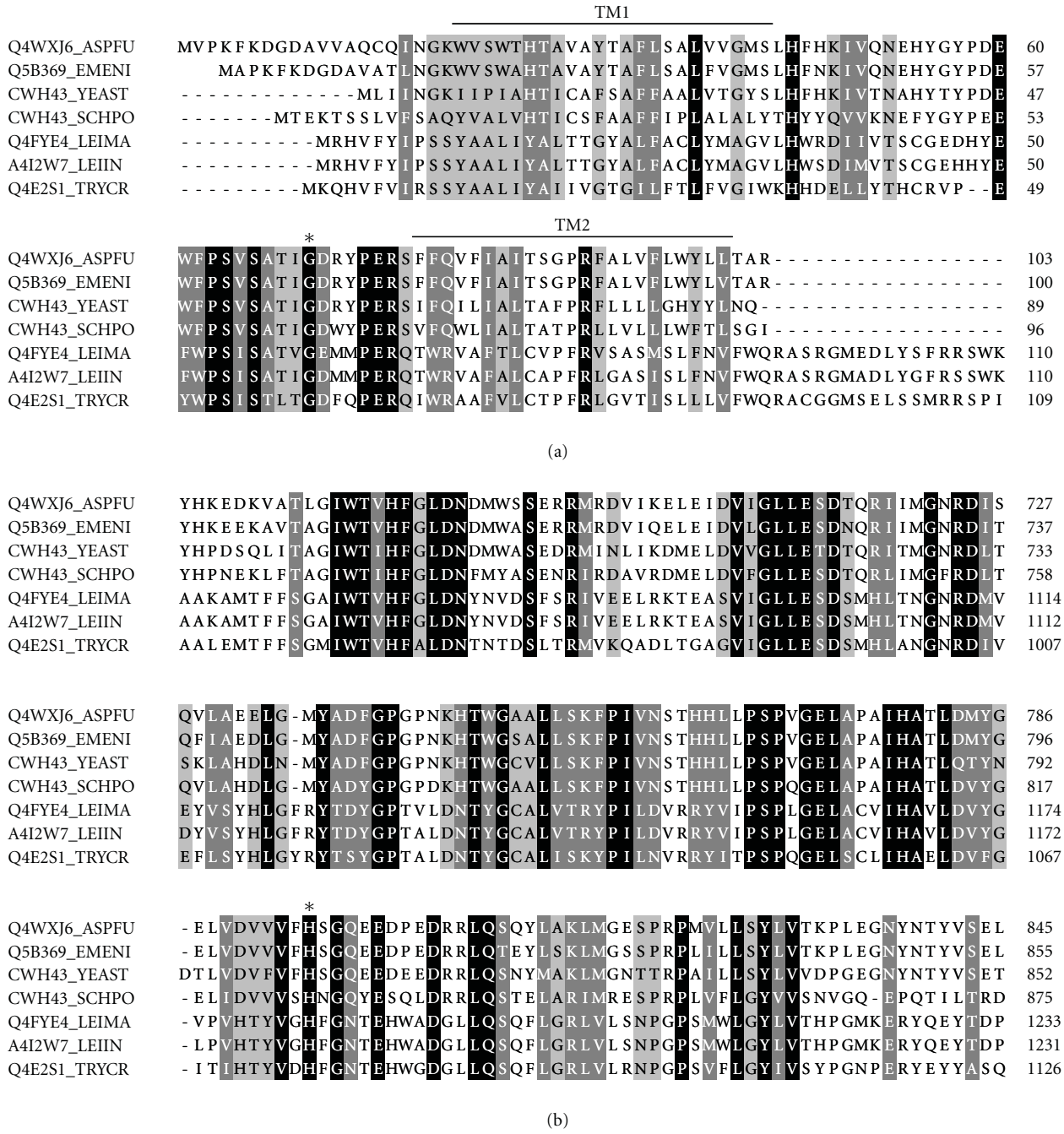

Figure 4: Alignment of the amino acid sequences of CWH43p homologues. Amino acid sequences of *Aspergillus fumigatus* (Q4WXJ6_ASPFU), *Emericella nidulans* (Q5B369_EMENI), *Saccharomyces cerevisiae* (CWH43_YEAST) and *Schizosaccharomyces pombe* (CWH43_SCHPO) proteins were aligned with the sequences of putative CWH43p candidates in *Leishmania major* (Q4FYE4_LEIMA), *Leishmania infantum* (A4I2W7_LEIIN) and *Trypanosoma cruzi* (Q4B2S1_TRYCR) using ClustalW. Identical amino acids are in reverse type, and conserved residues are shaded accordingly. The FRAG1 domain is shown in (a) the DNase I-like domain is shown in (b) Asterisks (∗) indicate the relative positions of amino acid residues that are essential for ceramide remodeling catalysis. TM, transmembrane stretches.

Although the remodeling of GPI anchors is important in several species to firmly anchor GPI proteins onto lipid bilayers and direct them to the correct cellular compartments and membrane domains [95], GPI lipid remodeling is not important for the stability and surface expression of the essential variant surface glycoprotein (VSG) [101]. The lack of GPI remodeling could be compensated *in vivo* by the myristate exchange pathway [100].

Like TbGUP1p, the *T. cruzi* GUP1p (Table 1, Step 7) can reacylate *lyso*-GPI anchors (Figure 2, Step 7), indicating that a similar pathway mediated by GUP1p is present in both yeasts and protozoa [97, 101]. Although several putative

GUP1 orthologues have been found in *Leishmania* (Table 1, Step 7), there is no experimental evidence for the existence of this kind of lipid remodeling in these parasites. Additionally, IPC has not been found to be linked to GPI anchors or GIPLs [14]. Therefore, targeted deletion studies in *Leishmania* could be used to determine the participation and function of these SBPs genes [29].

In *T. cruzi*, lipid remodeling occurs most likely as depicted in Figure 2. Unlike what has been described for mammals and yeasts, in this organism, the GPI anchor precursor molecule that is attached to the proteins is not acylated at position C-2 of the inositol ring [90]. Nonetheless, a putative TbGPIdeAc orthologue can be found in the *T. cruzi* genome database (Table 1, Step 5). In *T. cruzi*, intermediate GPI anchor precursors combine acylated and nonacylated inositol, as in *T. brucei* [90]. Thus, the putative orthologue (*Tc00.1047053508153.1040*) could be involved in deacylation during GPI biosynthesis. Although they are not acylated at the inositol ring, the GPI anchor precursors (either before or after attachment to proteins) contain a *sn*-1-alkyl-2-acyl-glycerolipid moiety [90] (Figure 2). However, Lederkremer's group has also detected a GPI-anchor precursor in *T. cruzi* that contains a mono-acyl-glycerol moiety [91]. There are no orthologues for the mammalian PGAP3p or yeast PER1p in the Tritryp genomes (Table 1, Step 6). It has been shown that *T. cruzi* lysates contain a PLA_2 activity that uses PI as a substrate [74], but it is not known whether this activity can also act on GPI-containing substrates. As mentioned above, TcGUP1p (Figure 2 and Table 1, Step 7) can reacylate *lyso*-GPI substrates [99, 101], but it is not clear which of the putative homologues encoded in the genome was used in those studies (Table 1, Step 7).

A putative orthologue of yeast CWH43p has been identified in the genomes of *T. cruzi* (*Tc00.1047053504153.120*), *L. major* (*LmjF27.1770*) (Table 1, Step 8) and *L. infantum* (*LinJ27_V3.1670*). This protein could have the ceramide remodeling activity that is supposed to exchange the *sn*-1-alkyl-2-acyl-glycerolipid of the GPI for a ceramide moiety in *T. cruzi* (Figure 2, Step 8 continuous or dashed lines). As already mentioned, no information is available on the function of these putative orthologues in *Leishmania* since ceramide is not found in their GPI anchors.

To learn more about the CWH43p orthologues in Tritryps, a multiple sequence alignment was prepared to compare these sequences with "bonafide" fungal CWH43p sequences. The results are presented in Figures 3 and 4. As shown in Figure 3, the Tritryp orthologues contain as many TM domains as the fungal CWH43p sequences. However, these domains have a greater distribution along the length of the sequences of *T. cruzi* and *Leishmania*. In addition, all the sequences have a highly conserved FRAG1 domain at the *N*-terminus and a DNase I-like domain at the C-terminus (Figure 3). A closer view of each of these conserved domains is shown in Figure 4. The FRAG1 domain has a high degree of identity across all sequences. The conserved residue G59 (G57 in yeast), which is essential for catalysis [99, 101], is located between the first and second TM domains (Figure 4(a), "∗"). A high degree of identity is also apparent in the DNase I-like domain at the C-terminus. The catalytically important residue H1076 (H802 in yeast) is conserved across all sequences (Figure 4(b), "∗"). Taken together, these data indicate that *T. cruzi* encodes a putative ceramide remodeling enzyme, which is essential in fungi and has no homologues in mammals.

6. Concluding Remarks

In *T. cruzi*, GPI-anchored glycoconjugates such as mucins, *trans*-sialidases, gp82/90 glycoproteins and GIPLs may extensively coat the plasma membrane of the parasite. These glycoconjugates are involved in many aspects of the host-parasite interaction, such as adhesion and invasion of host cells, modulation and evasion from the host immune response, and pathogenesis [66, 67, 83]. In addition, the GPI anchors, or certain parts of them, seem to act as strong proinflammatory molecules during the immune response against this parasite [66]. Therefore, mechanisms that interfere with the surface expression of GPI-anchored proteins and GIPLs or with the biosynthesis of GPI anchors are very attractive targets for new therapies against Chagas disease. Here, we discussed two novel targets in the SBPs of *T. cruzi*: the IPC synthase and ceramide remodeling. Because fungicidal inhibitors of IPC synthase activity do not affect the trypanosomal enzyme, the identification of novel inhibitors of this enzyme should be a goal of future research. This research direction could require the development of novel HTPS methods, such as the plate-based assay for screening *Leishmania* IPC synthase inhibitors that was recently developed by Mina and colleagues [102]. Unfortunately, recombinant parasite IPC synthase is prepared by overexpression in a fungal heterologous system, which has completely different optimal enzyme conditions and extra cofactors that would affect inhibition by novel candidates. Biochemical enzymatic assays have not been developed for ceramide remodeling, but recent advances have been made in monitoring the *in vitro* incorporation of ceramides into GPI-anchored proteins in *S. cerevisiae* [103]. These methods could be developed for use in *T. cruzi*.

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Research Article

The Involvement of Glutamate Metabolism in the Resistance to Thermal, Nutritional, and Oxidative Stress in *Trypanosoma cruzi*

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The inhibition of some glutamate metabolic pathways could lead to diminished parasite survival. In this study, the effects of L-methionine sulfoximine (MS), DL-methionine sulfone (MSO), and DL-methionine sulfoxide (MSE), three glutamate analogs, on several biological processes were evaluated. We found that these analogs inhibited the growth of epimastigotes cells and showed a synergistic effect with stress conditions such as temperature, nutritional starvation, and oxidative stress. The specific activity for the reductive amination of α-ketoglutaric acid, catalyzed by the $NADP^+$-linked glutamate dehydrogenase, showed an increase in the $NADP^+$ levels, when MS, MSE, and MSO were added. It suggests an eventual conversion of the compounds tested by the *T. cruzi* cells. The fact that trypomastigote bursting was not significantly inhibited when infected cells were treated with these compounds, remarks the existence of relevant metabolic differences among the different life-cycle stages. It must be considered when proposing a new therapeutic drug.

1. Introduction

Chagas' disease, also known as American trypanosomiasis, is a parasitic illness caused by the hemoflagellate *Trypanosoma cruzi*, which is transmitted to humans by blood-sucking triatomine vectors. The parasite is prevalent in South and Central America and infects 12–15 million people in the region, with approximately 40,000 new cases per year, and approximately 12,500 deaths per year due to cardiac disease (WHO, http://www.who.int/tdr/). In addition, more than 100,000 chronically infected individuals currently reside in the United States, Canada, and Europe due to migration from endemic countries [1]. At present, no clinically approved drug exists for the satisfactory treatment of chronic stage of the infection. Benznidazole and nifurtimox are the only available drugs prescribed for *T. cruzi* and are 60–80% effective in the treatment of acute infection, but the use of these drugs to treat the chronic phase remains controversial due to their low effectiveness and high toxicity. As a consequence of the adverse reactions, oral treatments frequently have to be discontinued [2]. The limitations of existing therapies encourage the search for alternative new drugs for both the acute and chronic treatment of Chagas' disease.

T. cruzi has a complex life cycle, alternating between the insect vector and the mammalian vertebrate host, which may include humans. The parasite presents different stages during the cycle: epimastigotes (replicative and noninfective forms in the insect vector), bloodstream and metacyclic trypomastigotes (nonreplicative and infective forms to vertebrate host cells), and amastigotes (intracellular and replicative forms of the vertebrate host) [3–5]. These different forms of the parasite must adapt to the changes of the particular environment inside the insect vector and vertebrate host cells, such as temperature, pH, and nutrient availability.

Amino acids are crucial nutrients during the *T. cruzi* life cycle, because they can be used as carbon and energy sources [6] and can participate in several biological processes that help the parasite adjust to these environmental changes.

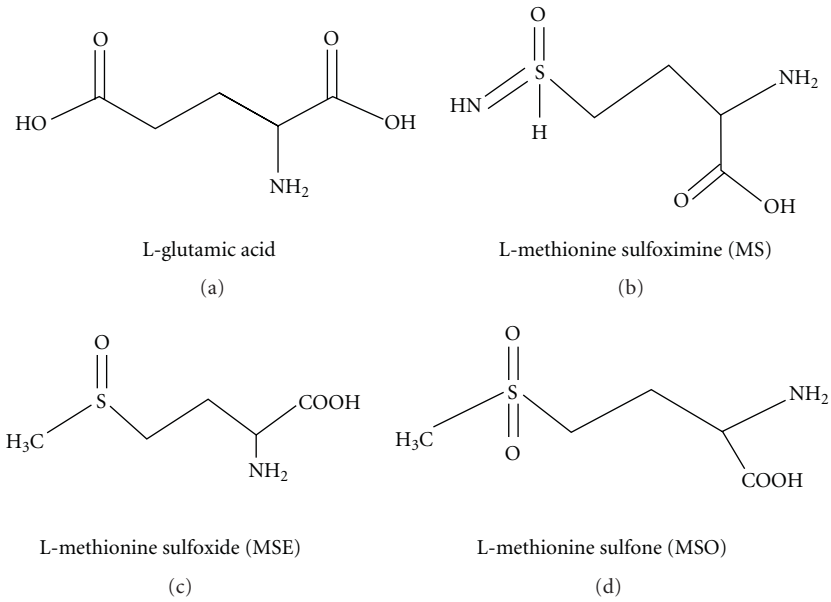


Figure 1: Chemical structures of the compounds used in this study.

For example, arginine metabolism is coupled to *T. cruzi* growth [7] and is involved in the management of cell energy under conditions of pH and nutritional stress [8]. Proline, glutamate, and aspartate are essentials in the differentiation process from epimastigote to metacyclic trypomastigote (metacyclogenesis) [9, 10]. More specifically, proline, an amino acid metabolically related to glutamate, seems to have a broad variety of functions: it fulfills the energy requirements for host cell invasion [11] and growth in low-glucose environments such as the host-cell cytoplasm [12], it is involved in the differentiation from the intracellular transient epimastigote-like stage to trypomastigote forms [13], and recently, it has been shown to be involved in mechanisms of resistance to oxidative stress [14]. The amino acid glutamate is directly involved in osmoregulation and cell volume control [15, 16]. Taking into account the important biological functions of amino acids in *T. cruzi*, it has been hypothesized that some components of their metabolic pathways could serve as possible therapeutic targets against Chagas' disease [6].

The uptake of glutamate occurs in epimastigotes and trypomastigotes cells through a single kinetic transport system, which is driven by the cytoplasmic membrane H^+ concentration gradient [17]. Inside the cell, it could be converted into α-ketoglutarate by the glutamate dehydrogenase and processed via the Krebs cycle to obtain energy [18–20]. Both isoforms of this enzyme also participate in the biosynthesis of glutamate by their ability to incorporate NH_3 into α-ketoglutarate [21]. According to nutritional requirements, *T. cruzi* can incorporate L-glutamate into proteins, or transfer the carbon chain to the metabolism of arginine or proline via Δ^1-pyrroline-5-carboxylate dehydrogenase [6]. Interestingly, L-glutamate can also serve as a substrate for glutathione synthesis via γ-glutamylcysteine synthetase and glutathione synthetase [22]. Glutathione can, in turn, act as a substrate for the synthesis of trypanothione, a milestone metabolite in the regulation of the redox state of the intracellular medium in trypanosomas [23]. Furthermore, glutamine can be synthesized from glutamate and ammonia in the presence of glutamine synthetase [21]. As this reaction is reversible, it is possible for glutamine to be oxidized through the glutamate pathway. All these glutamate pathways could be targets of inhibition by structural or functional analogs of this amino acid, the common substrate of most of the key enzymes related to glutamate metabolism.

The effects of several glutamate analogs have been analyzed in prokaryotic and eukaryotic organisms, showing inhibitory effects on some enzymatic functions. L-methionine sulfoximine (MS), L-methionine sulfone (MSO), and DL-methionine sulfoxide (MSE) (Figure 1) were found to be potent inhibitors of both enzymes, glutamate synthetase [24] and glutamine synthetase [25–27] in bacteria. In the present work, we analyze the effects of three glutamate analogs MS, MSE, and MSO on the growth and survival of *T. cruzi* epimastigotes and on trypomastigote bursting from infected host cells. We also investigated the synergism or antagonism of these analogs with the thermal, oxidative, and nutritional stress, conditions that *T. cruzi* is subjected to along its natural life cycle. The effect of these compounds on the specific activity for three glutamate-related enzymatic reactions was also evaluated.

2. Results and Discussion

The possible trypanocidal or trypanostatic effects of the three glutamate analogs, MS, MSE, and MSO, were investigated in *T. cruzi* epimastigotes. The growth of epimastigotes on LIT medium with or without 200 μM rotenone and 0.5 μM antymicin [28] was used as a control. The epimastigotes

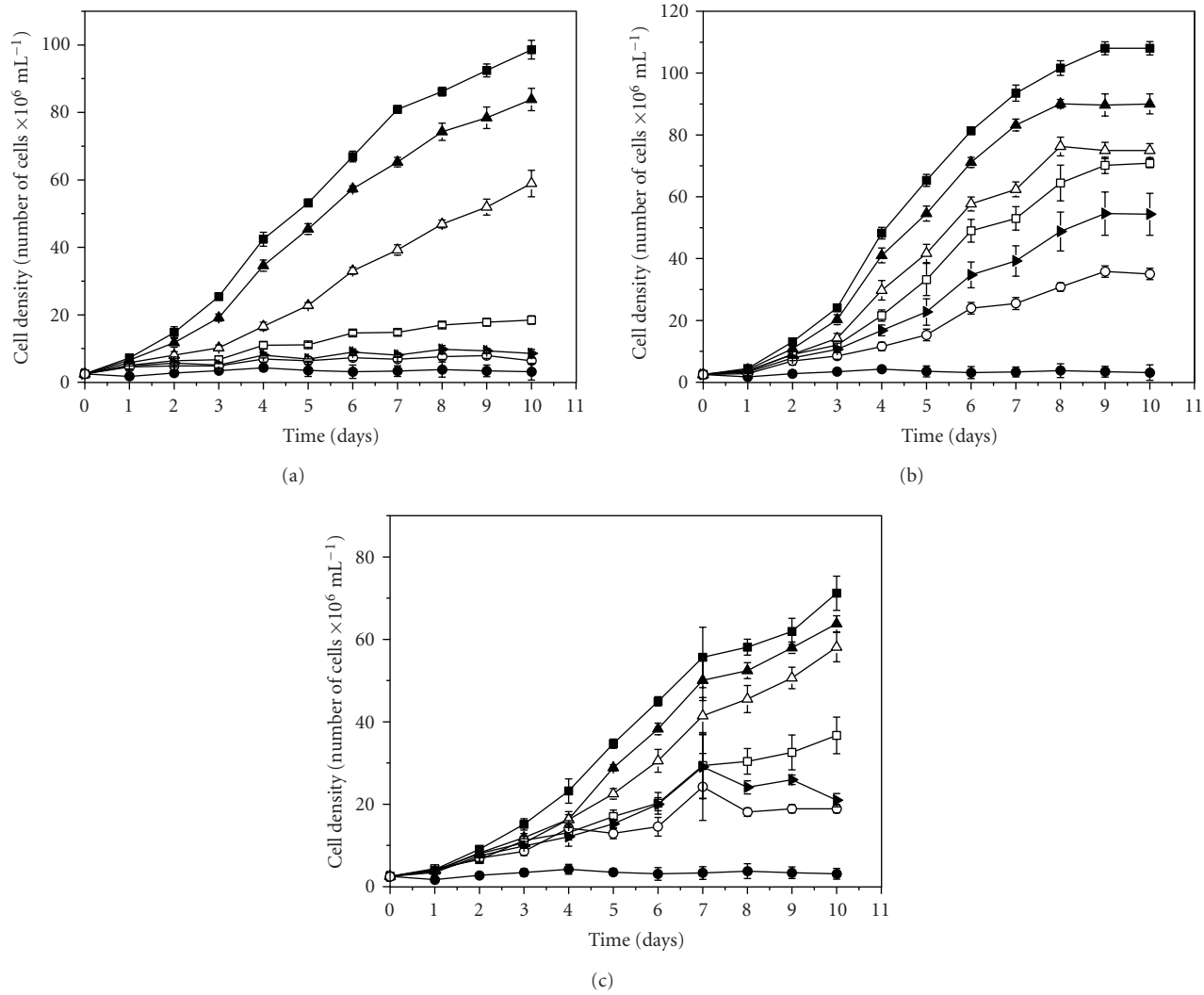


Figure 2: Growth curve of epimastigotes of *Trypanosoma cruzi* treated with MS (a), MSE (b) or MSO (c) at 28°C and pH 7.5: ■ 0 mM, ▲ MS = 10 mM, MSE = 20 mM, MSO = 15 mM; △ MS = 20 mM, MSE = 40 mM, MSO = 30 mM; □ MS = 30 mM, MSE = 60 mM, MSO = 45 mM; ▶ MS = 40 mM, MSE = 80 mM, MSO = 60 mM; ◯ MS = 50 mM, MSE = 100 mM, MSO = 75 mM. Positive control (•) was used with 0.5 μM antimycin and 200 μM rotenone.

reached the maximum cell density, at values between 71 ± 4×10^6 cells mL^{-1} and 108 ± 2×10^6 cells mL^{-1}, at the ninth or tenth day of growth in LIT (Figure 2). All three glutamate analogs showed a dose-dependent inhibition on epimastigote growth with significant differences between the cells treated with drugs and controls ($P < .01$). The IC_{50} values estimated at the fifth day of growth were higher for MSO and MSE (52.6 ± 4.3 mM and 58.8 ± 3.3 mM, resp.) than for MS (17.0 ± 0.3 mM). MS and MSO are well characterized as inhibitors of prokaryotic and eukaryotic glutamine synthetase [25, 26, 29, 30], whereas MSE inhibits the enzyme glutamate synthetase [24]. Since the IC_{50} values are high (more than 50-times) when compared with the *K*m reported for the glutamate transporter (0.3 mM) [17], it seems that these compounds are not acting as efficient glutamate uptake inhibitors. On the other hand, since measured enzyme activities are not abolished, the fact that these compounds could be acting with low efficiency on several targets cannot be ruled out. Further experiments of glutamate and glutamine-uptake, in the presence of MS, MSE and MSO as competitors, will be done in order to determine if these transport systems are inhibited.

Until now, no glutamate synthetase activity or putative genes coding for this enzyme had been identified in *T. cruzi*. However, the *T.cruzi* NADP$^+$-linked glutamate dehydrogenase is a very well characterized enzyme, both at molecular and biochemical level [18, 20, 31]. Previous data have demonstrated the presence of the genes coding (accession numbers: Tc00.1047053505843.10 and Tc00.1047053508111.30 http://www.tritrypdb.org/) for the NADP$^+$-linked glutamate dehydrogenase on *T. cruzi* [32]. The first of these alleles is the only one with published data on the expression level along the *T. cruzi* life cycle. It was found that the corresponding mRNA are

downregulated in the trypomastigote stage and upregulated in the epimastigote stage. No significant variations were reported for amastigotes or metacyclic trypomastigote stages [33]. This enzyme catalyses reversibly the formation of glutamate on *T. cruzi*. Adittionally, we have identified two putative sequences for glutamine synthetase (accession numbers: Tc00.1047053508175.370 and Tc00.1047053503405.10, http://www.tritrypdb.org/). The transcriptome analysis shows that the glutamine synthase mRNA is down-regulated in the amastigote stage and upregulated in the metacyclic trypomastigote stage with respect to the epimastigote or trypomastigotes stages [33]. Both essentiability and druggability parameters were investigated in The TDR Targets Database v4 (http://www.tdrtargets.org/) for both gene products: glutamate dehydrogenase and glutamine synthase. The only sequence for which the data were available was that corresponding to the glutamate dehydrogenase allele (Tc.00.1047053507875.20). This sequence was defined as a non-essential gene. In addition, no druggability data were found.

The first assumption of the functionality of a GS was previously reported [21]. We confirmed the functionality of this biosynthetic step by measuring the GS specific activity in crude extracts from epimastigotes forms (32 ± 3 U/mg protein, Figure 7(c)). The cloning of this genes and its biochemical characterization are necessary to extrapolate these data with the measurements in the parasite. However, no significant differences on the specific activity for GS were observed in the presence of MS, MSE or MSO. The fact that the IC_{50} values for both MSE and MSO are similar gives support to the conversion of MSE into MSO [34], both acting directly or indirectly on the same metabolic target. It was also remarkable that the IC_{50} obtained for *T. cruzi* was higher than that obtained for other organisms [24, 25, 30]. In view of this, it seems likely that these two compounds are metabolized to molecules that are less toxic to the parasite, or that are converted to methionine as described for some bacteria, yeast, and mammalian organisms [35].

MS, which was proposed as a drug candidate against tuberculosis [26, 27] and was successfully evaluated in cancer therapy [36], showed the highest toxicity for *T. cruzi* epimastigotes (17.0 ± 0.3 mM). MS is a well-characterized inhibitor of glutamine synthetase in mammalians [26, 27, 37, 38] that could cause an abnormal increase of glutamate in astroglia, producing seizures and toxicity in animals [39]. However, no significant inhibitory effect was observed on the GS activity, from *T. cruzi*, when MS or other one analog (MSE, MSO) were tested. In spite of requiring high doses to inhibit *T. cruzi* growth, several factors affecting MS selectivity could be optimized based on several strategies. It should be taken into account that the evaluated MS in this study was the racemic form (S/R), which is the only one commercially available. Usually, only one of the enantiomers has biological activity. From previous studies, It is likely that the S-enantiomer is active against glutamine synthetase [40]. A stereospecific preparation of this drug will probably result in a 2- to 5-fold reduction of the IC_{50}, as described for *Mycobacterium tuberculosis* [26]. These data suggest that the inhibitory effect on *T. cruzi* demonstrated in this study could be largely optimized by using the appropriate enantiomer. Additionally, a quantitative determination of the glutamate content would help to determine if the parasite is converting the analogs in consumable substrates.

Table 1: The effects of the temperature on IC_{50} values. The IC_{50} values were obtained by adjusting the data to a dose-response sigmoid equation for each glutamate analog at the three culture temperatures.

Temperature	MS	MSE	MSO
28°C	17.0 ± 0.3	58.8 ± 3.3	52.6 ± 4.3
33°C	20.2 ± 1.7	47.1 ± 7.9	32.4 ± 3.2
37°C	9.4 ± 1.5	37.2 ± 2.6	11.1 ± 0.7

When *T. cruzi* invades the mammalian host cell, it is exposed to different environmental changes. Among these changes are the acidic medium of the parasitophorous vacuole, the reactive oxygen species (ROS) produced by the host cells, changes in the nutrients availability, and high temperature of mammalian host. All these factors acting simultaneously with drugs could reduce parasite viability, revealing the role of their targets on the resistance to these stress conditions. We evaluated the viability of epimastigotes under the interaction of analogs with thermal, nutritional and oxidative stress. For thermal stress, we chose three temperatures: 28°C, the optimal temperature of growth for the insect vector stages, 37°C, the temperature of the mammalian host, and 33°C, the optimal temperature for the progression of *in vitro* infection of mammalian cells [13]. As previously reported [14], the maximum cell density (between $99 \pm 4 \times 10^6$ cells mL^{-1} and $141 \pm 4 \times 10^6$ cells mL^{-1}) was reached when cultures were maintained at 33°C in spite of the fact that Asin and Catala observed an optimum temperature of 28°C [41], a discrepancy that can likely be attributed to differences in the *T. cruzi* strain (Figure 2). When the parasites were cultured at 37°C (nearly the human temperature), the maximum cell density ($28 \pm 2 \times 10^6$ cells $\times$ mL^{-1}) diminished with respect to the standard epimastigote temperature ($91 \pm 3 \times 10^6$ cells $\times$ mL^{-1}) [14]. The IC_{50} was obtained for each analog and temperature. Results indicated that the sensitivity of parasites to the drugs was significantly increased with temperature (Table 1) being that the synergistic interaction between these factors was significant at 37°C ($P < .01$) (Figure 3). Interestingly, the highest inhibitory interaction between a specific analog and temperature was observed with MSO at 37°C, which produced an almost 5-fold reduction in the IC_{50} compared to that obtained at 28°C (Table 1).

To analyze the possible effect of drugs on nutritionally stressed parasites, three different carbon sources were added separately to each culture (glucose, glutamate, and proline). The parasite can metabolize glutamate and proline to obtain energy as well as glucose [6]. Then, the epimastigotes were starved for 72 h in PBS (PBS) or in 3 mM glucose (GLC), 3 mM L-glutamate (GLU), or 3 mM L-proline (PRO), separately. The cultures were treated with the analogs at concentrations near the IC_{50} obtained for parasite growth.

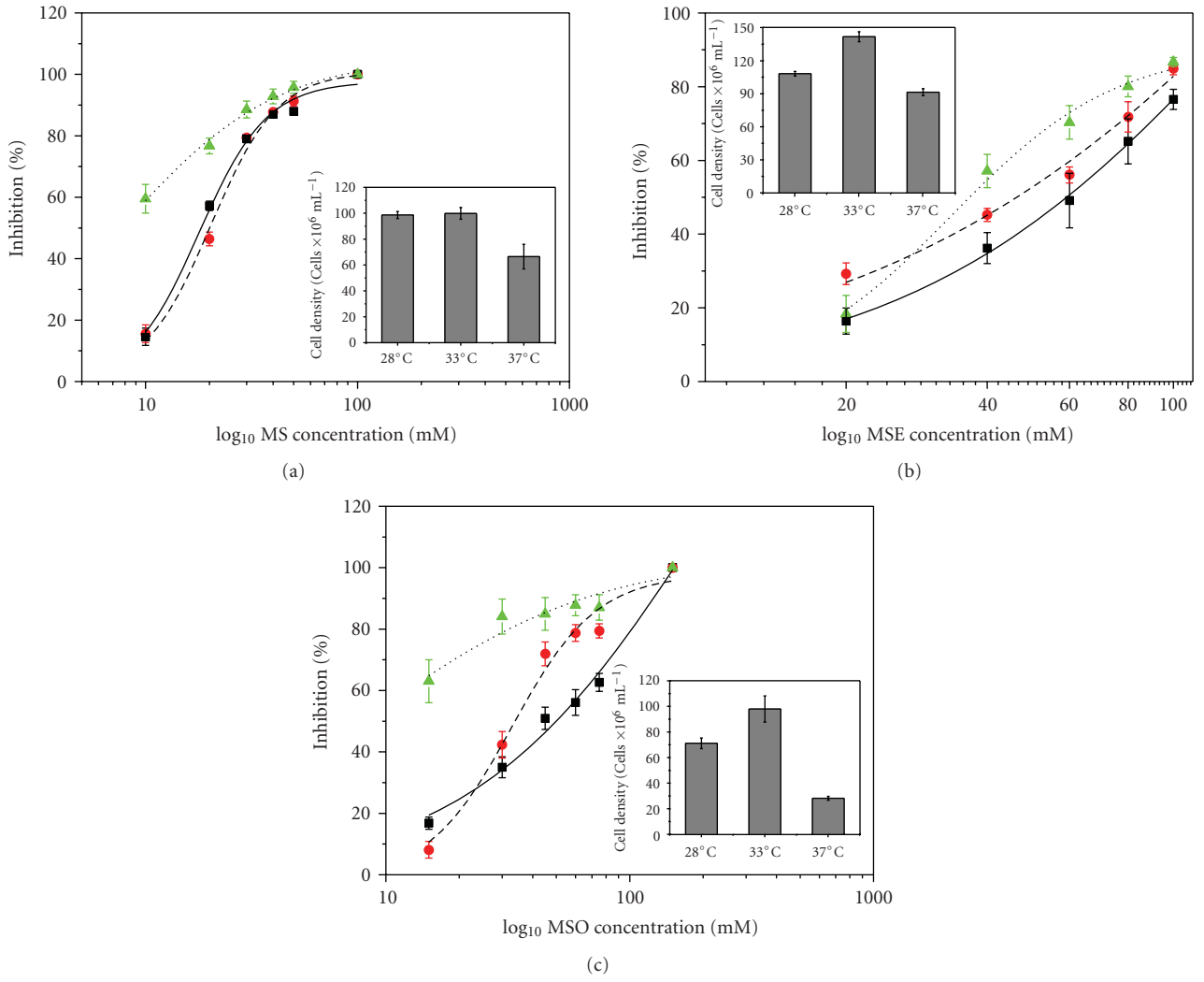


FIGURE 3: Dose-response curves of epimastigotes cultured at different temperatures and concentrations of MS (a), MSE (b), and MSO (c) treatment. The parasite growth inhibition was measured on the fifth day of growth, submitted to thermal stress (• 33°C, and ▲ 37°C) or not (■ 28°C) and drug treatments. The curves correspond to the adjusting to sigmoidal dose-response equation at 28°C (—), 33°C (- - -), and 37°C (· · ·). Insets: the maximum cell growth obtained in the control (no drug treatment) for each temperature is shown.

Untreated cells were used as controls. As expected, the presence of the three different carbon sources contributed to maintain parasite viability when compared with those starved in just PBS (Figure 4). Each culture treated with inhibitors showed significant differences ($P < .01$) when compared to controls, except for those incubated with PBS and MSE. Furthermore, MS and MSO produced greater inhibition than MSE (Table 2). Again, these results indicate that MSE may not be an effective inhibitor against *T. cruzi*. As evidenced in Figure 4 and Table 2, MS reversed the effect of glutamate on viability, confirming that this analog interferes with glutamate metabolism.

To analyze the effect of each drug on parasites subjected to oxidative stress, the cultures were incubated for 90 min with PBS supplemented with or without 120 μM H_2O_2 and 10 mM MS, 30 mM MSE, and 25 mM MSO. The combined treatment with H_2O_2 and MSO or MSE showed a statistically significant synergistic effect ($P < .01$), whereas MS did not show synergism (Figure 5). Following a previously presented rationale [14], it could be proposed that the augmented effect of the analogs in the presence of H_2O_2 was due to the fact that these reagents affected glutamate- or glutamine-dependent

TABLE 2: The effect of the nutritional state of the parasites on treatment with glutamate analogs. The inhibitory percentages (%) were calculated from the viability of the epimastigotes obtained in each starved condition with or without the addition of drug (MS = 20 mM, MSE = 60 mM, and MSO = 50 mM).

Nutrients	MS	MSE	MSO
PBS (no nutrients)	87	18	51
GLC	55	38	84
L-GLU	90	44	77
L-PRO	56	43	68

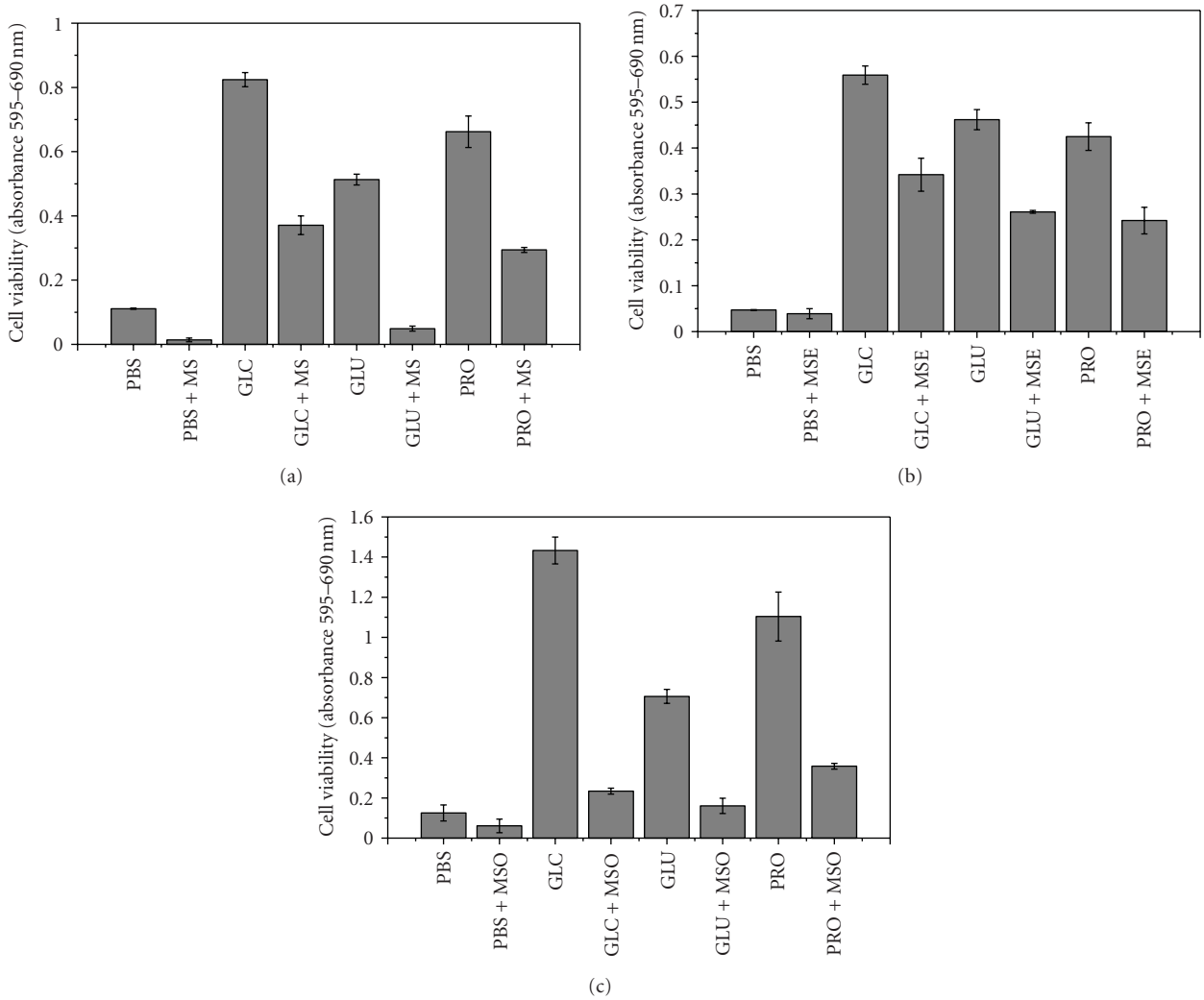


Figure 4: Response of epimastigotes to nutritional stress and MS (a), MSE (b), or MSO (c) treatment. Parasites were incubated for 72 h in PBS, 3 mM glucose (GLC), 3 mM glutamate (GLU), or 3 mM proline (PRO), and treated with 20 mM MS, 60 mM MSE, or 50 mM MSO. The cell viability was evaluated using the MTT assay.

oxidative stress resistance mechanisms. Alternatively, it could be argued that the enhancement of the effects of the analogs seen under oxidative stress conditions was due to the fact that these compounds made the cells more fragile by partially arresting glutamate or glutamine metabolism.

To better understand both the involvement of glutamate/glutamine metabolism in resistance to oxidative and metabolic stresses as well as the interaction between these factors and MSE, MSO, or MS treatments, a three-variable experiment was performed: cell viability was evaluated as a function of oxidative and metabolic stress challenges and MSE, MSO, or MS treatment. The parasites were maintained for 48 h at the conditions previously described for the nutritional stress experiment, then washed with PBS and incubated for 3 h with or without 80 μM H_2O_2. After incubation, cell viability was evaluated. All three drugs significantly diminished ($P < .05$) the epimastigote viability after the 3-h incubation (with or without oxidative stress) with respect to those not subjected to preincubation (named To, see Figure 6). The absence of treatment with analogs resulted in a diminished effect of oxidative stress ($P < .01$). The protection observed in parasites maintained in 3 mM L-proline against oxidative stress had been previously reported for several organisms [42, 43] and for *T. cruzi* [14]. In the same way, the glutamate analogs could act as possible coadjuvants for therapeutic drugs by enhancing the parasite sensitivity to ROS generated by the treatment with nifurtimox or benznidazole [22]. The interaction between the analogs and H_2O_2 was only synergistic ($P < .05$) when the parasites were previously incubated with glucose and MS or MSE, glutamate and MSO, or proline and MSO (Figure 6). As expected, these results confirmed that the nutritional state of the parasites is a main factor to be taken into account in the interpretation of these assays. Most importantly, these data showed the existence of at least three oxidative-stress resistance mechanisms involving glutamate-related metabolic pathways: (1) MS and MSE interfere with a glucose-dependant mechanism, (2) MSO, as expected,

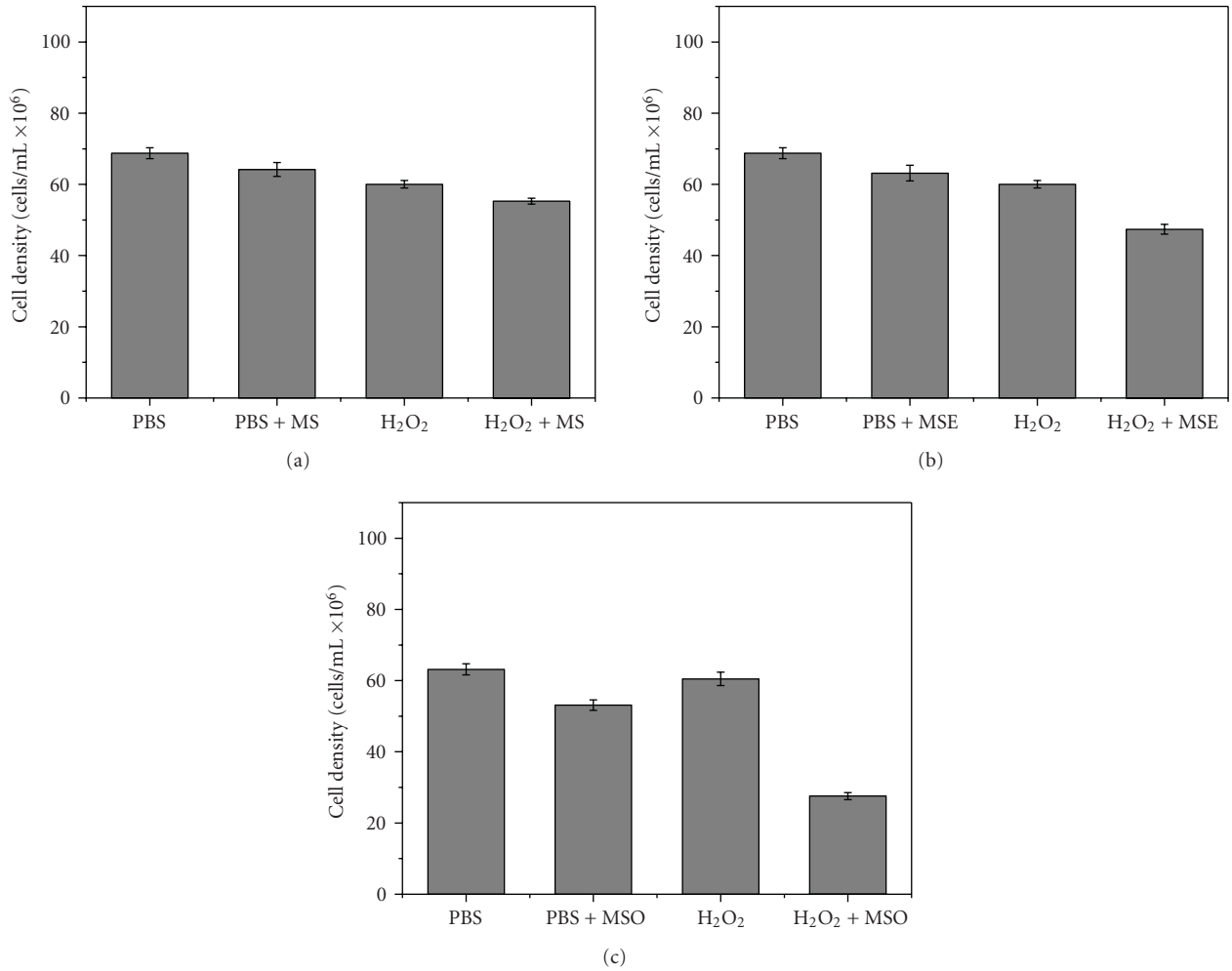


Figure 5: Response of epimastigotes to oxidative stress and MS (a), MSE (b), or MSO (c) treatment. Exponential growth culture parasites (control samples and samples treated with 10 mM MS, 30 mM MSE, or 25 mM MSO) were resuspended in PBS and subjected either to oxidative stress for 3 h by addition of 120 μM H_2O_2 or to water as the control. After washings, cells were cultured in LIT and counted after 5 days to evaluate their recovery after treatments.

interferes with a glutamate-dependant mechanism, and (3) MSO also interferes with a proline-dependant mechanism. Other protection mechanisms that could be altered by the tested analogs cannot be discarded. For example, it is known that ROSs can oxidize the surface-exposed methionine residues of proteins to methionine sulfoxides and this damage could be repaired by two methionine sulfoxide reductases [44, 45], a mechanism that occurs throughout the aerobic world, from bacteria [35] to mammals [45, 46]. The interconversion of methionine and methionine sulfoxide can function as a natural process to regulate the biological activity of proteins [47]. Mechanisms that protect cells against oxidative-stress are mainly important in the *in vivo* infection establishment, or when parasites are exposed to drugs used in the treatment of Chagas' disease [22].

The effect of these three glutamate analogs on the host-cell viability (see Figure S1 in Supplementary Material available online at doi: 10.4061/2011/486928) and on the host cell infection by trypomastigotes (Figure S2, Supplementary Material) were also evaluated at three concentrations of each drug (MS = 10–20 mM, MSO = 30–50 mM, and MSE = 30–50 mM). No significant inhibition on the trypomastigote burst was observed (data not shown). This fact, together with recent findings showing the inability of trypomastigotes to recover the ATP levels after starvation on the basis of glutamate as the sole carbon source [11], strongly supports the idea that, for these forms, glutamate-based metabolism is not relevant as glucose- or proline-based metabolism. However, it should be taken into account that the mode of action of nifurtimox and benznidazole includes free radicals (nitroradicals) and the generation of electrophilic metabolites, both of which are toxic for all forms of parasites. The nitroradicals enter redox recycling with molecular oxygen, producing partial oxygen reduction favoring the appearance of ROSs. [22]. Due to the synergistic interaction between the drugs utilized in this study and oxidative stress conditions, it may be hypothesized that the use of these glutamate analogs could help to decrease the clinical dose of currently used

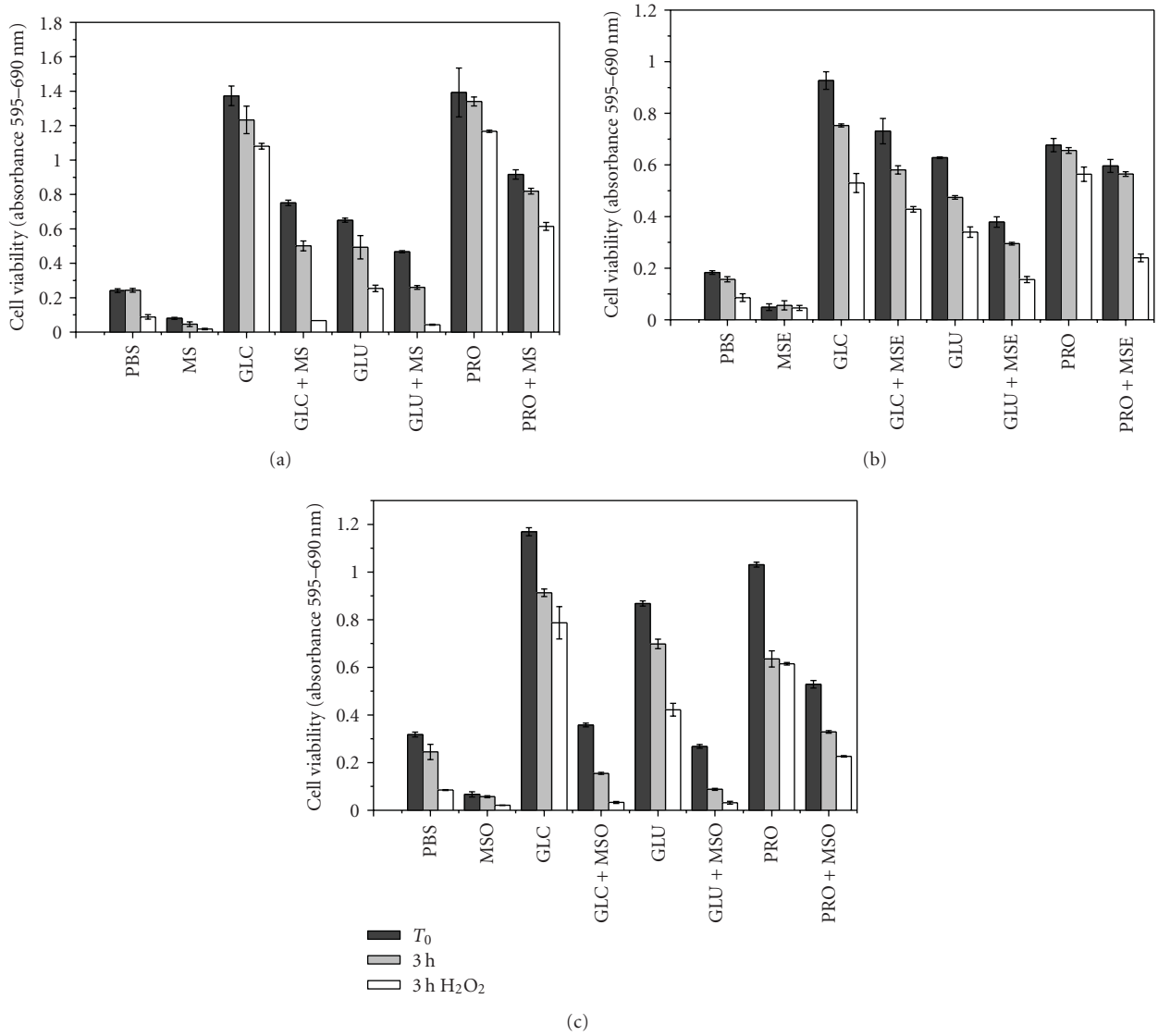
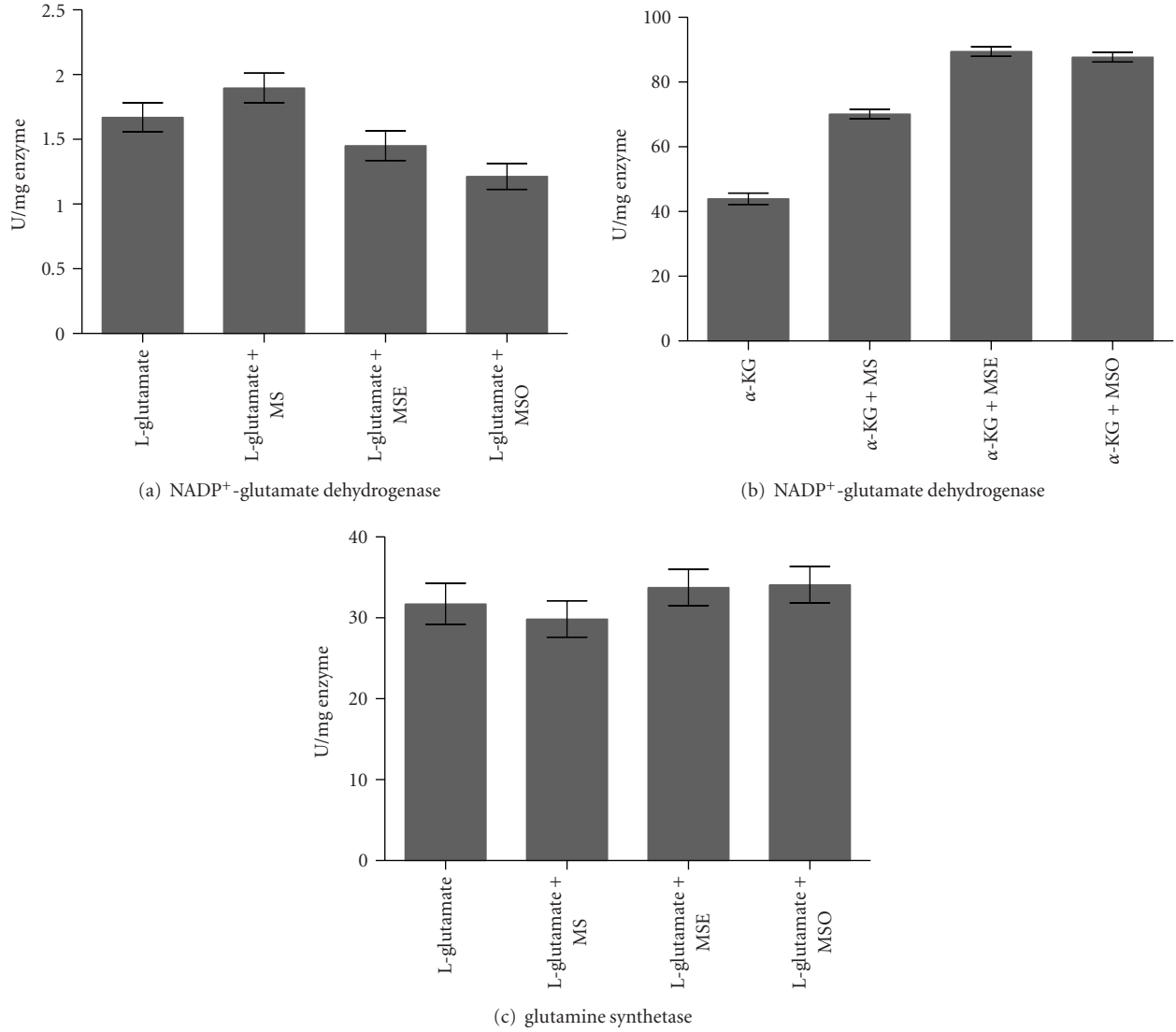


Figure 6: The combined effect of starvation, oxidative stress, and analogues treatments on epimastigotes. Parasites previously incubated in PBS (PBS), 3 mM L-proline in PBS (PRO), or 3 mM glucose in PBS (GLC), and subjected either to oxidative stress for 3 h by addition of 80 μM H_2O_2 or to water, were treated with or without (control) 20 mM MS (a), 60 mM MSE (b), or 50 mM MSO (c). After washing, the parasites were incubated for 3 h in PBS and stressed by the addition of 80 μM H_2O_2. The cell viability was evaluated by MTT assay.

drugs, thereby diminishing the side effects or the length of therapy. Our enzymatic data for the reaction catalyzed by the $NADP^+$-linked glutamate dehydrogenase revealed a higher rate in the direction of the reductive amination of α-ketoglutaric acid producing L-glutamate ($NADPH^+$-glutamate dehydrogenase) than the oxidative deamination of L-glutamate ($NADP^+$-glutamate dehydrogenase) (Figures 7(a) and 7(b)). For this later reaction, no significant differences were observed in the presence of L-glutamate and the analogs tested (Figure 7(a)). The data obtained for the glutamate dehydrogenase activities (1.56 ± 0.23 U/mg for $NADP^+$ GDH and 42.22 ± 3.52 U/mg for $NADPH^+$ GDH) suggest a preference for the glutamate synthesis in the epimastigote stage of *T. cruzi* (Figures 7(a)-7(b)) [18–20]. Indeed, we also have showed the enzymatic activity levels increased when the glutamate analogs are added (Figure 7(b)). This result shows that *T. cruzi*, in the conditions above described, would convert these compounds producing oxidized equivalents which are detected by the method herein used. However, further analytical methods are necessary to elucidate the intermediates produced under these conditions. The presence and enzymatic activity for GS was confirmed in *T. cruzi* epimastigotes, suggesting a nutritional requirement for glutamine. This process is ATP-dependent and the glutamine produced would be transaminated (EC: 2.6.1.16) releasing D-glucosamine-6-phosphate. This product is an intermediate of the amino sugars metabolism necessary for the glycoproteins surface synthesis [48]. The specific activities for GS did not show differences in the presence of any of the glutamate analogs tested (Figure 7(c)). All these data



(a) $NADP^+$-glutamate dehydrogenase

(b) $NADP^+$-glutamate dehydrogenase

(c) glutamine synthetase

Figure 7: Specific activities for three enzymatic reactions of the glutamate metabolism. Epimastigotes cells (5.0×10^8 cells) were used to prepare the crude extracts as enzyme source. The proteins were quantified by the Bradford's method and the activities for $NADP^+$-glutamate dehydrogenase and glutamine synthetase (GS) were measured. Due to reversibility of the glutamate dehydrogenase reaction, the enzymatic activity was measured in both ways, the reductive amination of α-ketoglutaric acid ($NADPH^+$-GDH) and the oxidative deamination of L-glutamate ($NADP^+$-GDH). The bars represent the specific activities in the presence of the natural substrate, as control, and 15 mM of each glutamate analog tested. The graphic was the resultant of three replicates done separately.

support the hypothesis that *T. cruzi* capability to metabolize these compounds interferes in the trypanocidal effects herein evaluated. The higher values of IC_{50} for these compounds are in accordance with the enzymatic profiles exhibited, even in concentrations of 15 mM. for MS, MSE, and MSO.

3. Materials and Methods

3.1. Reagents. L-glutamate, L-proline, L-methionine sulfoximine, DL-methionine sulfoxide, DL-methionine sulfone, rotenone, antimycin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (Saint Louis, MO, USA). RPMI culture medium and fetal calf serum were purchased from Cultilab (Campinas, SP, Brazil). All other reagents were purchased from Amresco.

3.2. Cells and Parasites. The Chinese hamster ovary cell line, CHO-K_1, was routinely cultivated in RPMI medium (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.15% (w/v) $NaHCO_3$ at 37°C in a humid atmosphere containing 5% CO_2 as previously described [14]. Epimastigotes of *T. cruzi*, CL strain, clone 14 [49] were maintained at the exponential growth phase by subculturing every 48 h in liver infusion-tryptose (LIT) medium supplemented with 10% FCS at 28°C [50]. Trypomastigotes were

obtained by infecting CHO-K_1 cells with trypomastigotes, as previously described [13, 51]. Trypomastigotes were collected in the extracellular medium from the sixth day on.

3.3. Inhibition Growth Assays. Epimastigotes in the exponential growth phase (approximately 5.0×10^7 cells mL^{-1}) were centrifuged and resuspended in fresh LIT medium. Then, an inoculum of 2.5×10^6 epimastigotes mL^{-1} was used. The assays were carried out using 200 μL of LIT medium, supplemented with different drug concentrations or without drug treatment (control), on 96 well plates, and maintained at 28°C. Cell growth was estimated by absorbance readings at 620 nm every day for a total of 10 days. The optical densities (absorbance) were transformed into cell density values (cells mL^{-1}) using a calibration linear equation previously obtained from the same conditions ($R^2 = 0.9981$). The concentration of analogs that inhibited 50% of parasite growth (IC_{50}) was determined at the exponential-phase of growth (fifth day) by adjusting the effect (growth inhibition values) as a function of drug concentration to a classical sigmoidal equation. As a control for cell growth inhibition, growth curves were run in parallel for all the experiments in medium containing 200 μM rotenone and 0.5 μM antimycin [52].

3.4. The Effect of Glutamate Analogs on Growth Inhibition in Stress Conditions. To analyze the combined effect of glutamate analogs at different temperatures, the growth curves were performed as described above and by incubating the cultures at 33°C and 37°C. To evaluate the combined effect of drugs and nutritional stress, 2.0×10^7 epimastigotes mL^{-1} were washed twice and resuspended in PBS or in PBS supplemented with 3 mM L-glutamate, 3 mM L-proline, or 3 mM glucose. Additional cultures were used in the same conditions but with the addition of 20 mM MS, 60 mM MSE, or 50 mM MSO. The parasites were maintained at 28°C for 72 h. Then, cell viability was measured by adding 20 μL of MTT (5 mg mL^{-1}) at 100 μL of cultures in PBS. The cells were maintained at 28°C, and after three hours the reaction was stopped by adding 20 μL of 10% SDS. The viability was estimated by reading the optical density at 595 nm and subtracting background values read at 690 nm [14]. To evaluate the inhibition of epimastigote growth induced by the three analogs under oxidative stress conditions, 5×10^6 parasites mL^{-1} from stationary phase cultures were washed twice with PBS, and incubated for 90 min in PBS with or without (control) 120 μM H_2O_2, and in the presence or not of analogs at 10 mM MS, 30 mM MSE, or 25 mM MSO (half IC_{50} concentrations). Then, the cells were collected by centrifugation and resuspended in LIT medium, and after a 5-day incubation, the number of cells mL^{-1} was determined as previously described [53]. To evaluate the combined effect of starvation and oxidative stress with drugs, 2.0×10^7 epimastigotes mL^{-1} were washed twice and preincubated for 48 h at 28°C in PBS supplemented with or without 3 mM L-glutamate, 3 mM L-proline, or 3 mM glucose. Additional cultures were run in the same conditions but with the addition of 20 mM MS, 60 mM MSE, or 50 mM MSO (concentrations close to the obtained IC_{50}). The cells were then washed twice and resuspended in PBS with or without the addition of 80 μM H_2O_2, and maintained in those conditions at 28°C for 3 h. Then, cell viability was estimated by MTT assay [14].

3.5. Effect of Glutamate Analogs on Mammalian Cell Viability. CHO-K_1 cells (5.0×10^5 cells mL^{-1}) were inoculated in 24 well plates in FCS-supplemented RPMI medium as previously described, in the presence of increasing concentrations of analogs or not (controls). The viability of cells was determined by MTT assay and the IC_{50} was obtained from the adjustment of the data to a typical dose-response sigmoid equation.

3.6. Effect of Glutamate Analogs on the Trypomastigote Bursting from Infected Cells. CHO-K_1 cells were infected with trypomastigotes in RPMI medium supplemented with 10% FCS. After 3 h at 37°C, free trypomastigotes in the medium were removed by washing with PBS and the infected cells were maintained at 33°C in RPMI medium supplemented with 2% FCS in the presence of different concentrations of glutamate analogs, or without analogs in the case of the control. Fresh medium containing the respective drug was added every day during the intracellular cycle of the parasite. The trypomastigotes were collected in the extracellular medium on the seventh day, and counted in a hemocytometer as previously described [13, 14].

3.7. Effect of Glutamate Analogs on Enzymatic Activities. In order to determine if the glutamate analogs used in this study would impair the specific activities involved in the glutamate-metabolism enzymes, we have measured, separately, the enzymatic activity for three reactions. The measurements for the $NADP^+$-linked glutamate dehydrogenase (GDH) [EC 1.4.1.4] were done in both senses [18, 20]. The activities for the reductive amination of α-ketoglutaric acid with NADPH dependence ($NADPH^+$-GDH) and the oxidative deamination of L-glutamate with $NADP^+$ dependence ($NADP^+$-GDH) were determined. Additionally, the glutamine synthetase activity (GS) [EC 6.3.1.2] was also calculated [21]. In the three cases, the measurements were done in the presence or not of the glutamate analogs MS, MSO, and MSE (sigma). Total cell-free extracts were prepared from epimastigotes forms (5.0×10^8 cells) at mid-log phase (3rd day). The cells were harvested by centrifugation at 2000 × g and washed three times with saline phosphate buffer 1 X pH: 7,4. The resultant pellet was resuspended in lysis buffer containing Tris-HCl 20 mM pH: 7.9, Triton X-100 0.1%, sucrose 0.25 M, phenylmethanesulfonylfluoride (PMSF) 1 mM, and 30 μL of protease inhibitor cocktail (sigma). The cells were broken by three cycles of sonication/ice during 20 s at 40% of potency. Soluble material was separated by centrifugation at 12,000 × g (4°C—15 min) and the supernatant collected. This fraction was passed through a desalting column (PD-10) and eluted with KH_2PO_4 50 mM pH: 7.4 for enzymatic activity assays. Total proteins concentration was determined by the Bradford's method [54] using bovine serum albumin as standard.

3.7.1. Glutamate Dehydrogenase ($NADP^+$-GDH). The assay reaction mixture (1.5 mL) for determination of $NADP^+$-GDH activity contained Tris-HCl 50 mM pH: 8.6, β-nicotinamide adenine dinucleotide phosphate sodium salt trihydrate (β-NADP) 25 mM, sodium glutamate 20 mM. The reaction was started by the addition of crude extract (100 μg) and incubated at 30°C for 5 min. The increasing of the absorbance at 340 nm was monitored and a blank without the enzyme addition was used. When necessary, 15 mM of each analog was added to the reaction mix and the corresponding activity was recorded. One activity unit (U) is defined as the amount of enzyme in the crude extract required to catalyze the formation of 1 μmol of α-ketoglutaric acid from L-glutamate per minute at 30°C.

3.7.2. Glutamate Dehydrogenase ($NADPH^+$-GDH). The assay reaction mixture (1.5 mL) for determination of $NADPH^+$-GDH activity contained Tris-HCl 83 mM pH: 8.3, α-ketoglutaric acid (α-KG) 7.5 mM, NH_4Cl 225 mM, and β-nicotinamide adenine dinucleotide phosphate, reduced form, (β-NADPH) 0.25 mM (sigma). The reaction was started by the addition of crude extract (100 μg) and incubated at 30°C for 5 min. The increasing of the absorbance at 340 nm was monitored and a blank without the enzyme addition was used. When necessary, 15 mM of each analogue was added to the reaction mix and the corresponding activity was recorded. One activity unit (U) is defined as the amount of enzyme in the crude extract required to catalyze the formation of 1 μmol of L-glutamic acid from α-ketoglutaric acid per minute at 30°C.

3.7.3. Glutamine Synthetase (GS). A coupled method using the pyruvate kinase/L-lactic dehydrogenase enzymes was necessary for the GS activity determination. The assay reaction mixture (1.5 mL) contained imidazole-HCl buffer 34.1 mM pH: 7.4, sodium glutamate 12 mM, adenosin 5′-triphosphate 8.5 mM, phosphoenolpyruvate (PEP) 1.5 mM, $MgCl_2$ 60 mM, KCl 18.9 mM, NH_4Cl 45 mM, β-nicotinamide adenine dinucleotide 0.25 mM, 28 units of pyruvate kinase and 40 units of L-lactic dehydrogenase (sigma). The reaction was started by the addition of crude extract (100 μg) and incubated at 30°C for 10 min. The increasing of the absorbance at 340 nm was monitored and a blank without the enzyme addition was used. When necessary, 15 mM of each analogue was added to the reaction mix and the corresponding activity was recorded. One activity unit (U) is defined as the amount of enzyme in the crude extract required to catalyze the formation of 1 μmol of L-glutamine from L-glutamic acid per minute at 30°C.

3.8. Statistical Analysis. A one-way analysis of variance (ANOVA) followed by a Dunnet's test was used for statistical analysis. A P value less than.05 was considered statistically significant. To analyze synergism in the interaction between the two independent treatments, a two-way ANOVA was performed as described [55].

Abbreviations

LIT:	Liver-infusion tryptose
FCS:	Fetal calf serum
CHO-K1:	Chinese hamster ovary cells
PBS:	Phosphate-buffered saline
RPMI:	Roswell Park Memorial Institute culture medium
GLC:	Glucose
PRO:	Proline
GLU:	Glutamate
IC50:	50% inhibitory concentration
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MS:	L-methionine sulfoximine
MSO:	DL-methionine sulfone
MSE:	DL-methionine sulfoxide
$NADP^+$-GDH:	$NADP^+$-linked glutamate dehydrogenase
GS:	Glutamine synthetase.

Acknowledgments

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Review Article

Role of Heme and Heme-Proteins in Trypanosomatid Essential Metabolic Pathways

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Around the world, trypanosomatids are known for being etiological agents of several highly disabling and often fatal diseases like Chagas disease (*Trypanosoma cruzi*), leishmaniasis (*Leishmania* spp.), and African trypanosomiasis (*Trypanosoma brucei*). Throughout their life cycle, they must cope with diverse environmental conditions, and the mechanisms involved in these processes are crucial for their survival. In this review, we describe the role of heme in several essential metabolic pathways of these protozoans. Notwithstanding trypanosomatids lack of the complete heme biosynthetic pathway, we focus our discussion in the metabolic role played for important heme-proteins, like cytochromes. Although several genes for different types of cytochromes, involved in mitochondrial respiration, polyunsaturated fatty acid metabolism, and sterol biosynthesis, are annotated at the Tritryp Genome Project, the encoded proteins have not yet been deeply studied. We pointed our attention into relevant aspects of these protein functions that are amenable to be considered for rational design of trypanocidal agents.

1. Introduction

Trypanosomes are parasitic protists that cause significant human and animal diseases worldwide [1], among which it is important to highlight the species relevant for human health, such as sleeping sickness or African trypanosomiasis (*Trypanosoma brucei*), Chagas' disease or American trypanosomiasis (*Trypanosoma cruzi*), and leishmaniasis (*Leishmania* spp.). The life cycle of these trypanosomatids is complex, presenting several developmental stages in different hosts. They have developed a digenetic life cycle with one or several vertebrate hosts and a hematophage insect vector that allows the transmission between them. A direct consequence is the environmental changes suffered among their life cycle thus, they have to adapt their metabolism to different nutrient availability [2]. Another feature of these parasites is the presence of nutritional requirements for several essential cofactors where heme is included. They totally or partially lack the heme biosynthetic pathway (revisited by Kořený et al. [3]). Heme plays a fundamental role in many cellular processes. It is an essential cofactor for proteins involved in oxygen transport and storage (hemoglobin and myoglobin), mitochondrial electron transport (Complex II–IV), drug and steroid metabolism (cytochromes), signal transduction (nitric oxide synthases, soluble guanylate cyclases), and transcription and regulation of antioxidant-defense enzymes. Heme is also a regulatory molecule; its cytosolic to nuclear ratio and the absolute amount of its concentration affects gene transcription and translation; thus, the intracellular heme level must be tightly regulated [4, 5]. Hence, these trypanosomatids are dependent on the uptake of this compound from their hosts. After being imported, heme is transported and inserted into target heme-proteins, which are distributed throughout different subcellular compartments. It is not well understood how these organisms acquire heme and how this cofactor is distributed inside the cell. However, they contain heme-proteins-like cytochromes, involved in essential metabolic pathways. This review will be focused in the presence and role of relevant heme-proteins in trypanosomatids.

2. Heme General Features

Heme is an essential molecule for most archaea, bacteria, and eukaryotes. Moreover, since the growth of bacteria and plants rely on the correct formation of tetrapyrroles, their corresponding biosynthetic pathways are attractive targets for antibacterial drug development and herbicide treatment [7–9]. The free living nematode *Caenorhabditis elegans* lacks the complete heme biosynthetic pathway; it feeds on bacteria and thus has easy access to heme [10]. On the other hand, *Plasmodium falciparum,* which has access to the host's abundant heme reservoir, is clearly dependent on its own intrinsic heme biosynthesis [9, 11].

The heme compounds are iron-coordinated porphyrins, specifically protoporphyrin IX (PPIX). The iron at the center of the tetrapyrrol ring can adopt the oxidized ferric (Fe^{+3}) or the reduced ferrous (Fe^{+2}) oxidation states. The majority of the porphyrins contain iron as the central metal ion. The most abundant heme is heme B (or protoheme), and it is found in nearly all the heme-proteins such as hemoglobin, myoglobin, and so forth. The tetrapyrrol structure of heme B contains two propionate, two vinyl and four methyl side chains (Figure 1). The oxidation of the methyl side chain to a formyl group and the substitution of a vinyl side chain with a 17-carbon isoprenoid side chain convert heme B into heme A, the prosthetic group of the mitochondrial cytochrome *c* oxidase and of the several bacterial terminal oxidase. *C*-type hemoproteins, such as cytochrome *c* and *bc1* complex, contain heme *c* in which the two vinyl side chains of the heme B are covalently attached to the protein (Figure 1). For almost all organisms, hemes are essential components of their energy recovering electron transport chains and cofactors for several proteins. Many enzymes like peroxidases, catalases, and the large group of cytochrome P450 also rely on heme as a prosthetic group. Heme-proteins can furthermore serve as sensors for diatomic gases such as O_2, CO, and NO and for CO_2 in signal transduction pathways [4].

2.1. Heme Biosynthesis. The heme biosynthetic pathway is highly conserved through evolution [3, 12]. It is present in most organisms but differs in the synthesis of the first precursor, delta-aminolevulinic acid (ALA). All prokaryotes (with the exception of α-proteobacteria) and photosynthetic eukaryotes synthesize ALA *via* three consecutive enzymatic steps starting with glutamate. The α-proteobacteria and most nonphotosynthetic eukaryotes synthesize ALA by the condensation of glycine with succinyl-CoA using the single ALA-synthase enzyme (ALAS). The remaining seven steps of the pathway (from ALA to heme B) are carried out by the same enzymes in all organisms [12, 13].

Eukaryotes differ also in the intracellular localization of individual enzymatic steps. The photosynthetic eukaryotes synthesize heme exclusively in the chloroplasts, while in most heterotrophic eukaryotes the pathway is split between the mitochondrion and cytosol [12] (Figure 1). An interesting case is the apicomplexan parasite because its heme synthesis starts in the mitochondrion, then ALA is transported to the apicoplast and the subsequent steps take place in this specialized organelle, but the last steps appear to proceed in the mitochondrion [14, 15].

Iron and porphyrins are highly toxic to cells, and heme *per se* is a cytotoxic macrocycle with peroxidase activity. The level of free heme inside the cell is maintained very low, and there is a tight control of its biosynthesis based on cellular requirements. The damaging effect of heme excess is due to iron-induced pro-oxidant effect on DNA, proteins, membrane lipids, and the cytoskeleton. The elevated level of noniron porphyrins has been linked to harmful effects; they accumulate in membranes and can cause cellular damage [5, 16].

2.2. Heme in Trypanosomatids. Most of the eukaryotic organisms are able to synthesize heme and organisms with deficiency in this pathway are not common. Some examples are the anaerobic protists such as *Giardia*, *Trichomonas,* and *Entamoeba*. They possess a rudimentary mitochondria-like organelle called hydrogenosome or mitosome [18]. These protists do not generate energy by oxidative phosphorylation, thus they do not have cytochromes and respiratory chains. Furthermore, heme-proteins involved in oxidative metabolism such as oxidases, peroxidases, catalases, and hydroxylases are not needed in anaerobic conditions and consequently they do not require heme as a cofactor [19]. But there are other organisms that even when they depend on oxidative phosphorylation, are defective in the synthesis of heme. Some examples are a tick [20]; a filarial nematode [21], the free-living nematode *C. elegans* [10] and even most of the kinetoplastid parasites also belong to this category. These organisms can afford their deficiency in heme synthesis due to an easy access to this compound from their environment [21–23].

In a recently published work, Kořený and coworkers [3] discuss from a phylogenetic point of view the absence of a complete heme biosynthetic pathway in Kinetoplastid flagellate organisms. These represent an interesting group of species, where some of them lack of the complete pathway while others possess only the last three biosynthetic steps. The authors propose a scenario in which the ancestor of all trypanosomatids was completely deficient in the heme synthesis. In some trypanosomatids, with the exception of the genus *Trypanosoma*, the pathway was partially rescued by genes encoding enzymes for the last three steps supposedly obtained by horizontal transfer. On the other hand, the trypanosomes have remained fully deficient of heme synthesis and obtain this compound from their hosts ([3] and references cited therein). In particular, the absence of the complete heme biosynthetic pathway in *T. cruzi* has been pointed out by biochemical studies [24, 25]. Later, the absence of the genes for the enzymes involved in heme biosynthesis in the genomes of *T. cruzi* and *T. brucei* was corroborated when the TriTryp genomic sequence project was completed [26, 27] (TriTrypDB, http://tritrypdb.org/tritrypdb/ [28]). The *in vitro* cultivation of the mentioned *Trypanosoma* requires the addition of heme compounds in the form of hemoglobin, hematin, or hemin to the medium [29, 30]. Several other trypanosomatids including *Leishmania* spp. and *Crithidia fasciculata* can grow in media in which hemin

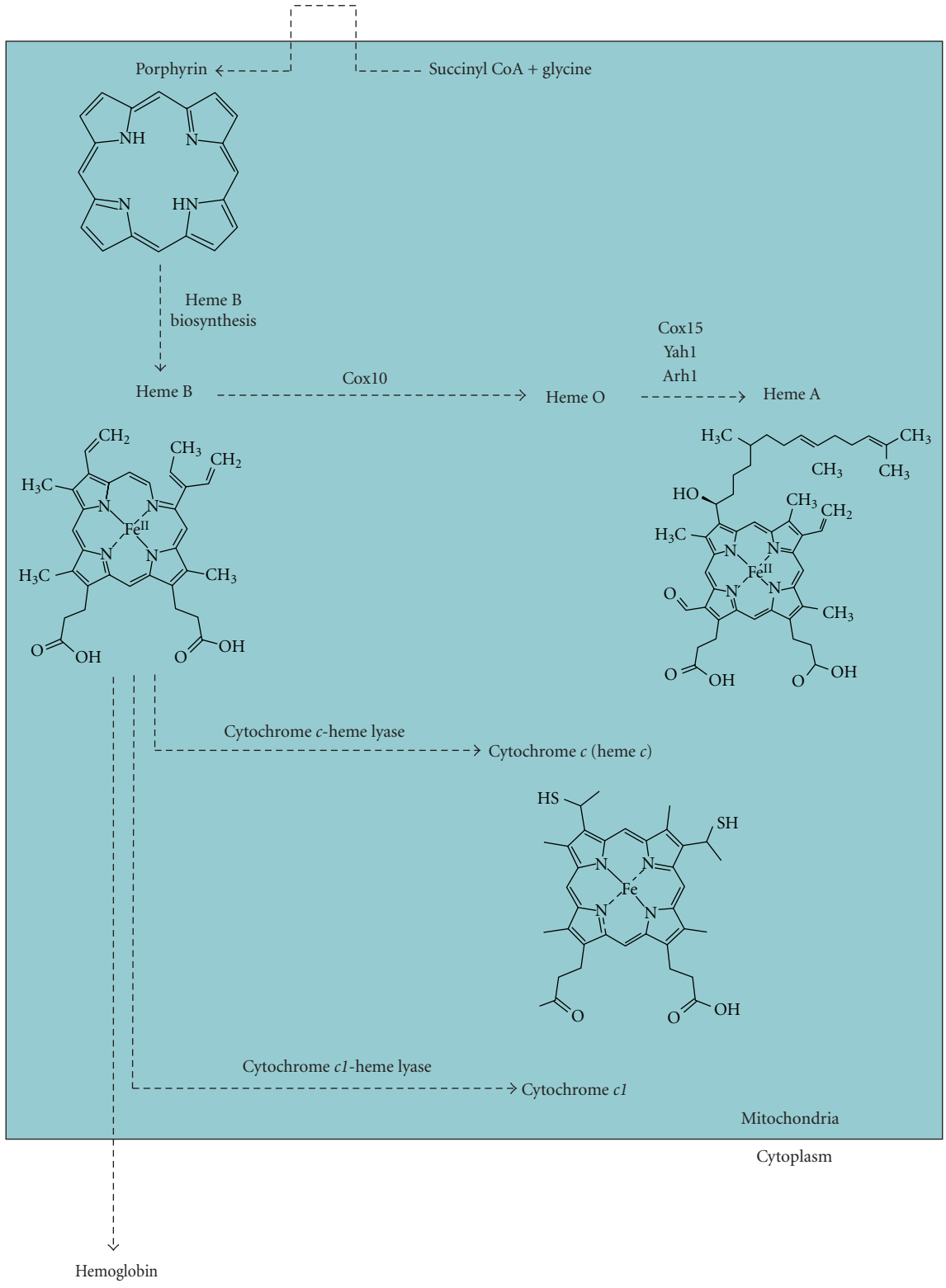

Figure 1: Schematic view of different hemes biosynthesis pathways in *S. cerevisiae*. Adapted from Moraes et al. [6].

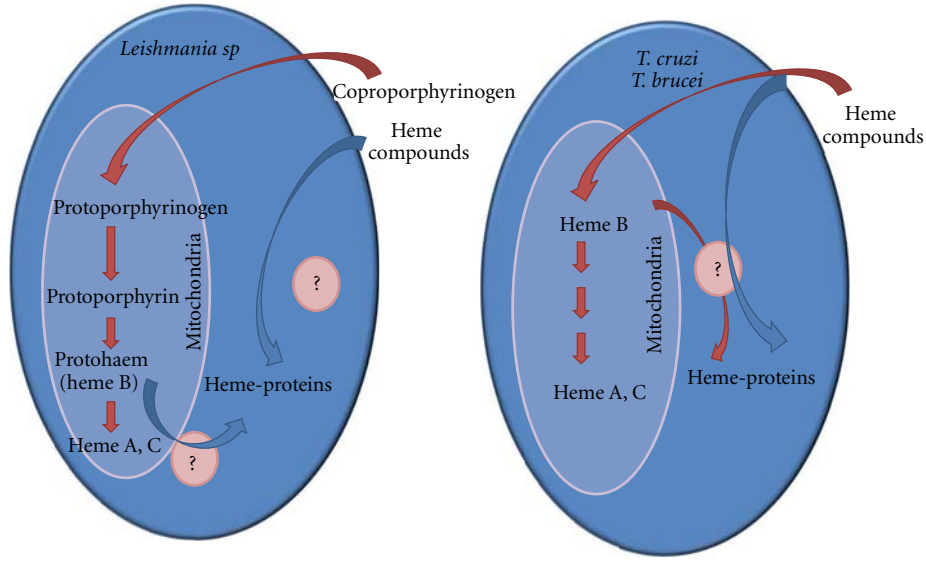

Figure 2: Heme biosynthesis in trypanosomatids (revisited by Kořený et al. [3]). *Leishmania* spp. can perform the last three steps in heme synthesis, catalyzed by coproporphyrinogen oxidase, protoporphyrinogen oxidase, and ferrochelatase, localized into the mitochondria. *T. cruzi* and *T. brucei* cannot perform any step for heme B biosynthesis and must import heme compounds (hemoglobin, hemin, etc.) from their hosts. They can modify heme B to obtain heme A and cytochrome *c*, although this has been described only for *T. cruzi*, it is probable that *L. major* and *T. brucei* have the same capability. Mechanisms by which these trypanosomatids couple heme to apoproteins or regulate heme compounds levels to avoid toxic effects are unknown.

is replaced by protoporphyrin IX [30–32]. This observation provides indirect evidence that at least the last enzyme of the pathway (ferrochelatase) remains functional. There are other experimental data that support that *Leishmania* spp. presents a partial pathway for heme biosynthesis, [31, 33, 34].

The evidence mentioned above indicates that medical relevant trypanosomes are completely (*T. cruzi* and *T. brucei*) or partially (*Leishmania* spp.) deficient in heme synthesis, as it is resumed in Figure 2, and they must therefore scavenge this molecule from their hosts. Once heme is imported, it has to be distributed inside the cell and inserted into the target heme-proteins. As heme is a highly toxic molecule, it is well accepted that heme carriers or chaperons involved in its distribution exist. But, in eukaryotic cells, these type of proteins were not reported yet [35] and the processes of heme transport and distribution in trypanosomatids remain unknown. Besides, these parasites present heme-proteins involved in essential metabolic pathways like biosynthesis of sterols and polyunsaturated fatty acids (PUFAs) carried out in the endoplasmic reticulum (ER) and respiratory complexes in the mitochondrion, as it is shown in Figure 3. The understanding of how they import, distribute, utilize heme, and assemble heme-proteins can help to elucidate the essential metabolic pathway in these trypanosomatids.

3. Heme and Biosynthesis of Polyunsaturated Fatty Acids and Sterols are Connected through Cytochromes

The precise role of heme in the proliferation as well as differentiation of these parasites remains unknown. In the next section, we will focus our discussion on cytochromes and their role in lipid biosynthesis. These heme-proteins have been scarcely studied in trypanosomatids, in spite of being involved in a variety of key pathways. The Table 1 shows a list of genes that were annotated as cytochromes *b5*, *c*, and P450 in the TriTrypDB, most of them assigned by comparison of sequences but without biochemical evidence.

3.1. Cytochrome b5: A Crucial Piece in the Fatty Acid Desaturation Reaction. Fatty acid biosynthesis in trypanosomatids has gained attention in the last few years, since endogenous production of these compounds seems to be essential for the parasite life cycle. A few years ago, the complete pathway for polyunsaturated fatty acid (PUFA) synthesis in trypanosomatids was described [39, 40]. Indeed, it was established that whereas *L. major* is able to obtain docosahexaenoic acid (DHA, 22 : 6) and docosapentaenoic acid (DPA, 22 : 5) from oleate, trypanosomes cannot perform this process. As *Leishmania*, they produce oleate *de novo*, but they have to import precursors from the hosts in order to generate DHA and DPA. The enzymes involved in PUFAs biosynthesis are elongases and the so-called "front end" desaturases.

A common feature of all fatty acid desaturases is the requirement of an electron donor, ferredoxin in plastids and bacteria, and cytochrome *b5* (cyt*b5*) in endoplasmic reticulum [36]. In fact, cyt*b5* is needed not only for the successful desaturation of fatty acids, but also for many other oxidative reactions in the cell. It is a small heme-binding protein that acts as an electron-transfer component in the desaturation reaction [41], with two possible modes of action. In the first one, desaturation can be carried out by a

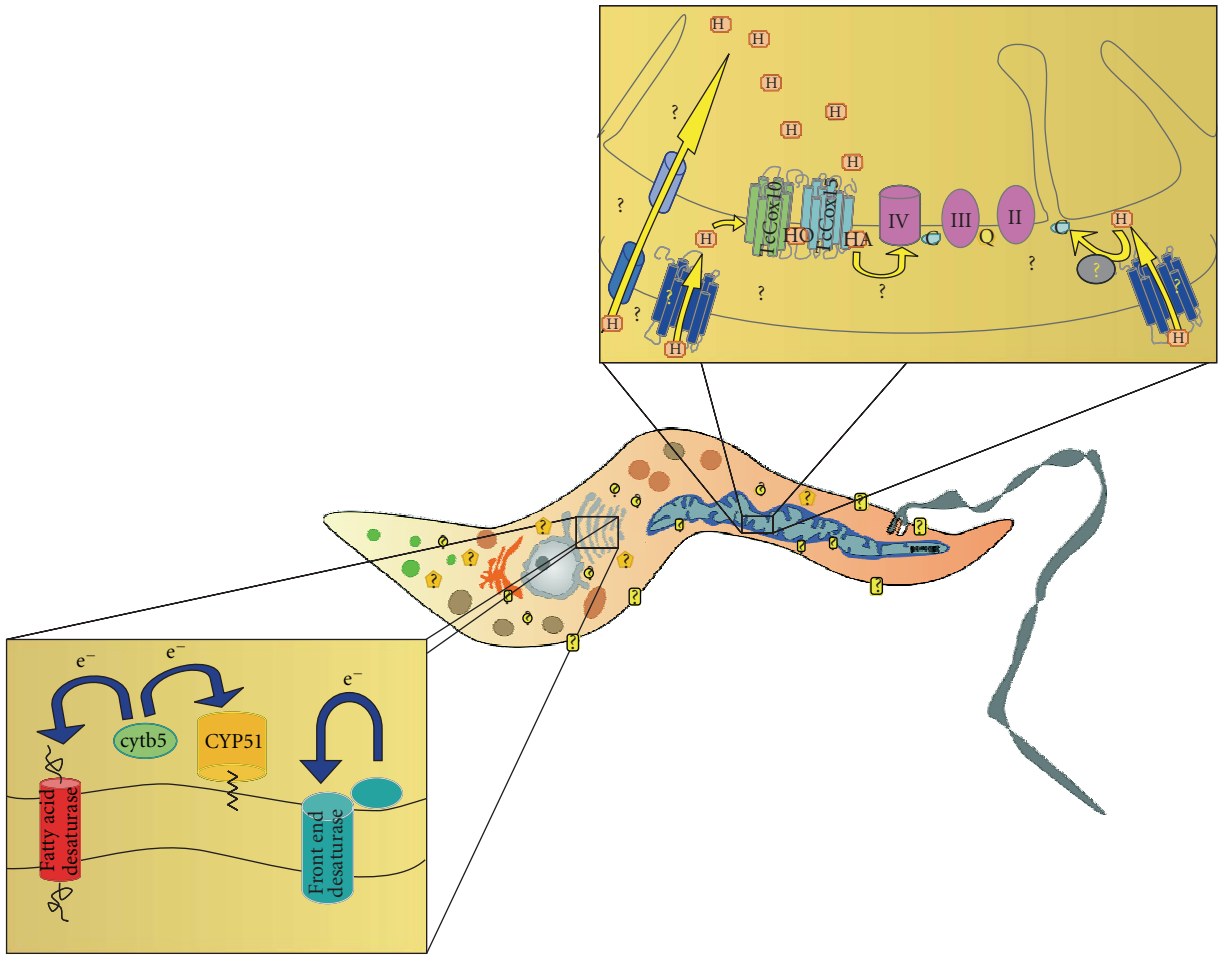

Figure 3: Schematic representation of transport, trafficking, and uses of heme in a trypanosomatid epimastigote. The amplified areas correspond to the mitochondrial and endoplasmic reticulum regions (above and below resp.) where the heme-proteins mentioned in the text are located. In the mitochondrion, heme B is internalized by one or several unknown transport systems. Once in the matrix (or alternatively in the intermembrane space) heme B is transformed into heme O by the membrane-bound heme O synthase (Cox10) enzyme, and immediately later heme O is converted into heme A by the heme A synthase (Cox15) enzyme [17]. Finally heme A is incorporated into the cytochrome *c* oxidase complex by an unknown mechanism. In the endoplasmic reticulum cytochrome *b5* takes electrons from several donors, such as NADH, FADH, and other reduced compounds (not shown), and serves in turn as an electron donor for the various transmembrane fatty acid desaturases and other ER proteins such as CYP51. Alternatively, front-end desaturases contain a cytochrome-type domain which serves as their own electron donor. See text for more details. Abbre*via*tions: H: heme B; HA: heme A; HO: heme O; C: *c*-type cytochrome; Q: quinone; II, III, IV: complexes II, III, and IV of the electron transport chain, respectively.

multienzymatic system, which is composed by a desaturase, NADH cytochrome *b5* reductase and cyt*b5*. The membrane-bound cyt*b5* transfers electrons by lateral diffusion, from NADH cytochrome *b5* reductase to the desaturase [42]. In a second mode, desaturases are modular proteins that include a cyt*b5* domain as a fusion either on the N-terminus in the case of "front end" desaturases [36, 43] or on the C-terminus in the case of fungal Δ9-desaturases [44, 45]. In addition to the desaturases, cyt*b5* domains have been found in a number of unrelated proteins, such as nitrate reductases, sulfite oxidases, and L-lactate dehydrogenases [45].

Desaturases may be classified as type I, II, or III [36]; as they introduce double bonds in the middle of the carbon chain (I), near the methyl-end (II), or at the carboxy (front)-end (III). As mentioned above, all of them require an electron donor, which is cyt*b5* in all trypanosomatids. However, for type I and II desaturases, cyt*b5* acts as an enzyme-independent component of an electron transfer chain (with exception of fungal Δ9-desaturases); whereas type III desaturases have these activities as a domain in the N-terminus. In trypanosomatids, we found the three types of desaturases, but a different route for PUFAs biosynthesis operates in *L. major* and in trypanosomes (Figure 4). *L. major* contains Δ9, Δ12, *ω*3, Δ4, Δ5, and Δ6-desaturases (Δ9Des, Δ12Des, *ω*3Des, Δ4Des, Δ5Des and Δ6Des), whereas *T. cruzi* and *T. brucei* only contain Δ9, Δ12 and Δ4-desaturases (Δ9Des, Δ12Des and Δ4Des). They are enzymes

Table 1: Cytochrome genes present in *L. major*, *T.cruzi* and *T. brucei.* Most of the genes were annotated by similarity in Gene Bank and were published within the TriTryp Genome Project. Only a few of them have been submitted to cloning and characterization.

Type of cytochromes	Organism	Genes	References
Cyt*b5*	*L. major*	**LmjF11.0580, LmjF09.1490, LmjF36.4675, LmjF07.0810, LmjF09.1500** (pseudogene)	Ivens [34]
	T. brucei	**Tb927.3.3470**, **Tb927.7.520, Tb11.02.4485**,**Tb11.01.5225**	Berriman et al. [26]
	T. cruzi	**Tc00.1047053504431.109**, **Tc00.1047053508799.160, Tc00.1047053506773.44**, **Tc00.1047053507951.154, Tc00.1047053503653.60**, **Tc00.1047053510355.269, Tc00.1047053509395.100, Tc00.1047053506753.110**	El-Sayed et al. [27]
Cyt*c*	*L. major*	**LmjF16.1320, LmjF16.1310**	Ivens [34]
	T. brucei	**Tb927.8.1890, Tb927.8.5120**	Berriman et al. [26]
	T. cruzi	**Tc00.1047053511391.160, Tc00.1047053508959.4, Tc00.1047053506949.50**	El-Sayed et al. [27]
CytP450	*L. major*	**LmjF27.0090**, **LmjF30.3550**, **LmjF34.3330LmjF11.1100** (lanosterol 14 α demetilase, putative)	Ivens [34]
	T. brucei	**Tb927.3.680, Tb11.02.4080** (lanosterol 14 α demetilase, putative)	Joubert et al. [37], Berriman et al. [26]
	T. cruzi	**Tc00.1047053509719.40, Tc00.1047053509231.10, Tc00.1047053510101.50, Tc00.1047053506297.260** (lanosterol 14-alpha-demethylase, putative)	El-Sayed et al. [27] Buckner et al. [38]

from the endoplasmic reticulum, with four or six transmembrane segments, as described for mouse Δ9Des [46] and Δ6Des from *Bacillus subtilis* [47].

It is known that PUFAs accumulate at higher levels in the parasite than in the vertebrate hosts. This behavior could be reflecting a requirement for more fluid membranes in order to face with different growth environment and/or to be fully infective. In addition, the presence of linoleate seems to be crucial for both procyclic and bloodstream form of *T. brucei,* as was formerly seen for epimastigotes of *T. cruzi* [48]. In a recent report, linoleate synthesis was blocked by either inhibition or RNA interference of oleate desaturase (OD, a Δ12Des), causing a dramatic drop in parasite growth [49]. This result and the fact that mammals lack OD validate this enzyme as a chemotherapeutic goal. Other potential targets are "front end" desaturases since there is no evidence for the presence of Δ4-Des in mammals. However, the relevance of these enzymes in the life cycle of these parasites awaits further research.

3.2. Cytochrome P450 and Its Role in Sterol Biosynthesis. The sterols of trypanosomatids resemble those of fungi, both in composition and biosynthesis. An important step in the synthesis of ergosterol, an essential component of parasite membranes, is carried out by CYP51. This enzyme belongs to the cytochrome P450 (cytP450) superfamily and is a sterol-14-α-demethylase that performs the oxidative removal of 14-alpha methyl group in lanosterol. It is localized in the endoplasmic reticulum, probably associated by a stretch of hydrophobic residues. CytP450 are mixed function oxidases that catalyze the oxidation of a number of substrates. In the case of CYP51, it has been described that it accepts electrons from NADPH-cytP450 reductase (CPR) and from cyt*b5*. The role of cyt*b5* is to enhance the efficiency of the CYP51 reaction by assisting the interaction and electron transfer between CPR and CYP51 [41].

T. cruzi and *Leishmania* synthesize ergosterol at all life stages, while the bloodstream form of *T. brucei* is able to import cholesterol from the host. Before the conclusion of the Trypanosomatids Genome Project, an enzyme with sterol-14-α-demethylase activity from *T. brucei* was cloned and characterized by complementation assays in the *erg11* yeast mutant cells [37]. A few years later, two alleles with 99% of identity were identified in *T. cruzi,* and they were 83% identical to the *T. brucei* protein [38]. Meanwhile, in *Leishmania* there is a gene annotated as a putative lanosterol 14-α-demethylase (LmjF11.1100) that has not been characterized yet.

A number of antifungal azoles, inhibitors of ergosterol biosynthesis by binding to CYP51, have been experienced in protozoan parasites with some success. Nevertheless, the modification of azoles to enhance the efficiency and circumvent the potential drug resistance has been problematic due to the lack of structural insights into the drug binding site. Recently, the resolution of the crystal structure of *T. cruzi* and *T. brucei* CYP51 cocrystallized with inhibitors (fuconazole or posaconazole) released the opportunity to develop rationally designed anti-trypanocidal drugs [50]. Another work,

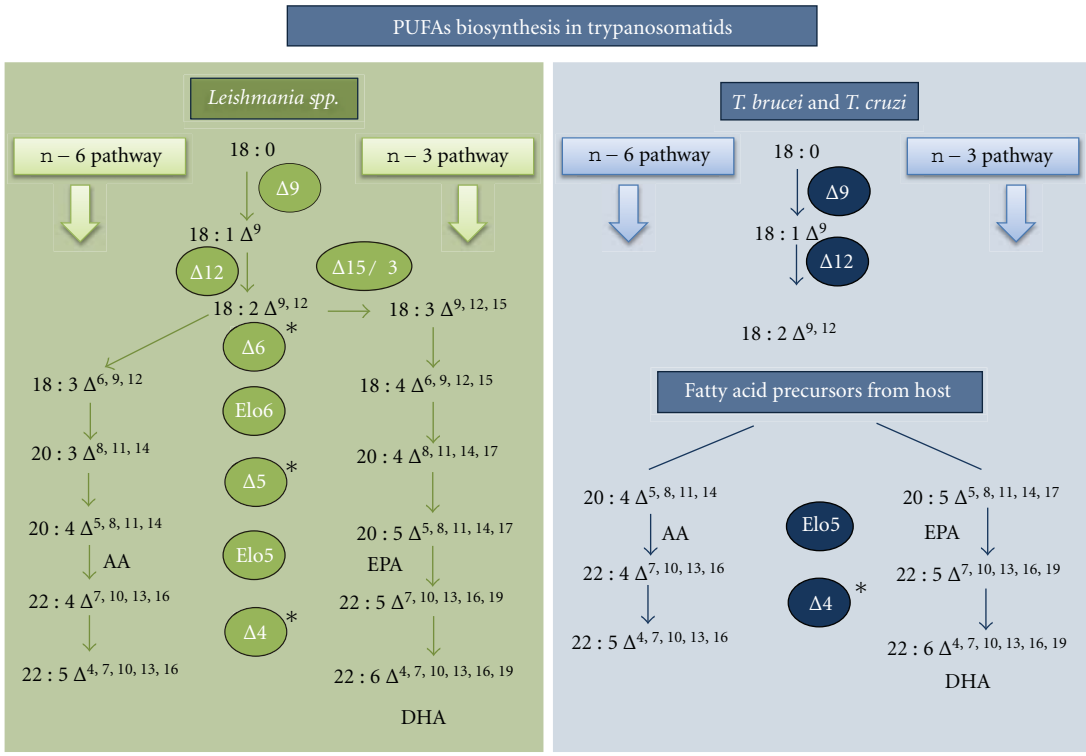

Figure 4: Biosynthesis of polyunsaturated fatty acids in trypanosomatids. This is an important pathway that takes place in trypanosomatids and has been recently described [39, 40]. Cytochrome *b5* operates as the electron donor for desaturation, both as an independent enzyme or as a domain at the N-terminus of the desaturase (wildcards). Δ12, Δ15 (*ω*3) and Δ4-desaturases are absent from mammalian hosts and are potential targets for trypanocidal agents.

that analyze the binding properties of a CYP51 inhibitors library by X-ray techniques, revealed that the *N*-[4-pyridyl]-formamide scaffold group binds in the active site of the enzyme *via* conserved residues and the heme prosthetic group. The use of one of these nonazole inhibitors in a mouse model of *T. cruzi* acute infection triggered the breakdown of membrane parasites and the death of amastigotes [51].

4. Heme in the Mitochondrion

The mitochondria are one of the most relevant heme-protein containing organelles, and it includes the respiratory chain complexes. A characteristic of these parasites is their single and usually well-developed mitochondrion, which presents functional and structural changes depending on the stages of its life cycle [52]. In this part of the review, we describe all the data available about mitochondrial heme and cytochromes in trypanosomatids, highlighting the similitude and differences compared to other organisms.

4.1. Cytochrome c in Trypanosomatids—A Novel Pathway for Cytochrome c Maturation. All eukaryotes, and almost all prokaryotes, that use oxygen as the terminal electron acceptor in the respiratory chain possess cytochromes *c* and *c1* (cytc and cytc*1*). The principal physiological role of mitochondrial cytc and cytc*1* is the electron transfer during oxidative phosphorylation. These *c*-type cytochromes are distinguished from other types (*a* and *b*) because heme is covalently attached to the polypeptide *via* thioether bonds. In the vast majority of the cases, heme is attached to the protein by two thioether bonds between the heme vinyl groups and the thiol of cysteine residues in a CXXCH motif (heme-binding motif) (Figure 1). There are, however, a few exceptions. One is the termed pseudo-*c*-type cytochrome center in the cytochrome *bf* complex of thylakoids where a fourth heme is attached to the cytochrome *b* polypeptide by a novel single-thioether bond [53, 54]. Also, in species from the phylum Euglenozoa, which include the medical relevant trypanosomatids (*T. brucei*, *T. cruzi*, and pathogenic *Leishmania* spp.), heme is uniquely attached to the mitochondrial *c*-type cytochromes by a single-thioether bond within a F/AXXCH heme binding motif [55, 56].

The cytochrome *c* maturation process involves the covalent attachment of heme to the apocytochrome polypeptide. Depending on the mechanism and the enzymes employed for this posttranslational modification, the *c*-type cytochromes-containing organisms are classified into five distinct groups (Table 2).

The system I, also known as the Ccm system (for cytochrome *c* maturation system), is found in *α*- and *γ*-proteobacteria, deinococci, and mitochondria of some plants and protozoa [57]. This is well understood in *Escherichia coli* [58, 59], and it is the most complex system. It consists of eight essential proteins, named CcmA-H, and a number of

Table 2: Different *c*-type cytochromes maturation systems and examples of organisms which possess each one (in the case of eukaryotic organisms is also denoted the organelle where the system operates) [59, 63].

System I	System II	System III	System IV	System V
E. coli *Pseudomonas* *Rhizobium* *Rhodobacter* *Paracoccus*	*B. subtilis* *Mycobacterium tuberculosis* *Helicobacter pylori* *Synechocystis*	*Yeast* *Neurospora* *C. elegans* *Mouse* *Human* Mitochondria	*C. reinhardtii* *B. subtillis* (In some oxygenic phototrophic bacteria and several *Bacillus* spp.)	*T. cruzi* *T. Brucei* *Leishmania* spp. *C. fasciculata* *Euglena gracilis* Mitochondria
Plant/Protozoa Mitochondria	Plant Chloroplasts			

accessory proteins. CcmA-H are all membrane anchored or integral membrane proteins. Heme attachment occurs in the periplasm, after the separate translocation of heme and the polypeptide across the plasma membrane, both synthesized in the cytoplasm [60–62].

The system II is less understood than system I, and it is the responsible for the assembly of the most complex *c*-type cytochromes in which there are multiple hemes *per* polypeptide chain (bacterial cytochromes). This second system is present in Gram positive bacteria, cyanobacteria, and some β-, δ-, and ε-proteobacteria, but also in plant and algal chloroplasts and possibly in archea. The proteins from system II do not share extensive sequence similarities with representatives of system I. The system II model organism is *B. subtilis*, and four proteins, ResA-C and CcdA, are associated to it [64].

The system III for cytochrome *c* maturation is found only in the mitochondrial intermembrane space of some protists, fungi and animals and the enzymes involved are heme lyases. There are separate lyases for the attachment of heme to cyt*c* and cyt*c1* [65].

The system IV has been described for the heme linkage to cytochromes of the *bf* complex from oxygenic phototrophic bacteria and *bc* complex in several *Bacillus* species [53, 66]. Four genes in *Chlamydomonas reinhardtii*, a green alga, have been implicated in the covalent heme attachment to cytochrome *bf* complex (associated to system IV) [62].

The scenario for cytochrome *c* maturation in the protist phylum Euglenozoa seems to be totally different from the aforementioned systems. They contain *c*-type cytochromes with a single covalent bound to the polypeptide chain through the cysteine in the F/AXXCH motif [55, 56, 67].

The trypanosomatids possess cyt*c* and cyt*c1*, but there are no recognizable proteins belonging to the *c*-type cytochrome maturation systems, suggesting the presence of a distinct mitochondrial pathway. The X-ray crystal structure of cyt*c* from the trypanosomatid *Crithidia fasciculata* was solved [68]. It revealed that the protein folding was remarkably similar to that of typical (CXXCH) mitochondrial cyt*c* (in this case cyt*c* from yeast), including the stereochemistry of the covalent heme attachment to the protein. The difference appeared only in the missing thioether bond in the heme attachment site. However, *S. cerevisiae* cyt*c* heme lyase cannot efficiently mature *T. brucei* cyt*c* (containing an AAQCH heme binding motif), or a CXXCH variant, when they were expressed in the cytoplasm of *E. coli* [68]. The later results let the authors propose that a novel, yet unidentified, apparatus for maturation of cyt*c* operates in trypanosomatids [68]. Why the *T. brucei* CXXCH mutant was not able to be maturated by the lyase (system III) is not fully clear.

Analysis of all the available genome sequences and all publicly accessible expressed sequence tag (EST) collections using BLAST reveal that single-cysteine attachment of heme to mitochondrial cyt*c* remains as a unique characteristic to species from the phylum Euglenozoa [63]. In this scenario, this novel system is a good candidate to be validated as a possible target for drug design against pathogenic trypanosomatids, in addition to expanding our understanding about the biogenesis of heme-containing proteins.

4.2. Heme A Biosynthesis. Heme A is the essential cofactor only for the cytochrome *c* oxidases (C*c*O, Complex IV of the eukaryotic mitochondrial respiratory chain). It is synthesized from heme B through two enzymatic steps catalyzed by the heme O synthase (HOS) or Cox10 and heme A synthase (HAS) or Cox15 enzymes (Figure 1) [69]. In eukaryotic cells, the heme A biosynthesis is carried out in the mitochondria, and HOS and HAS are integral mitochondrial inner membrane proteins [70, 71]. After heme A is synthesized, it is inserted into the subunit I of C*c*O. Defects in the maturation of heme groups that are part of the oxidative phosphorylation system are also recognized as important causes of diseases [6, 72, 73]. In the same way, changes in mitochondrial heme A levels have been related to Alzheimer's disease [74, 75].

The presence of an active mitochondrial respiratory chain has been demonstrated in trypanosomatids but its metabolic dependence varies between them [52]. The bloodstream form of *T. brucei* shows a rudimentary mitochondrion with the activity of an alternative oxidase and well-developed glycosomes. In this case, the energetic metabolism depends primarily on glycolysis. But the procyclic form of *T. brucei* presents a well-developed mitochondrion and less glycosomal activity [52, 76]. In *T. cruzi,* there is no evidence of a mitochondrial alternative oxidase activity, and it is proposed that this trypanosomatid depends on the respiratory chain activity throughout the complete life cycle. The presence of electron transport from complex II

to complex IV has been demonstrated, but the contribution of complex I (NADH:ubiquinone oxidoreductase) to energy metabolism remains controversial [77, 78].

Biochemical studies developed in *T. cruzi* epimastigotes showed that the main terminal oxidase is the *aa*3 type [79], the canonical C*c*O for eukaryotic cells. Additionally, proteomic studies demonstrated the presence of subunits of complex IV (C*c*O), other components of the respiratory chain and subunits of the FoF1 ATPase (complex V) [80, 81].

Based on the evidence about the peculiar biogenesis of cyt*c* in trypanosomatids (discussed above), it was expected a possible particular mechanism of heme A biosynthesis in these organisms. However, in a recently published work by Buchensky et al. [17], the first functional characterization of *T. cruzi* ORFs that encoded for enzymes involved in heme A biosynthesis (named TcCox10 and TcCox15) was presented. The sequences of these putative proteins are conserved in others trypanosomatids. The authors showed that the *T. cruzi* Cox10 and Cox15 proteins were recognized by the yeast mitochondrial importing machinery, even though the mitochondrial targeting sequences reported for trypanosomatids are shorter than the ones in other cells, including yeast [82]. These *T. cruzi* proteins were fully active in the yeast mitochondria. Furthermore, the genes encoding TcCox10 and TcCox15 (*TcCOX10* and *TcCOX15*) were differentially transcribed during the parasite life cycle. The authors postulate that the observed changes in the mRNA levels of *TcCOX10* and *TcCOX15* could be a form of regulation reflecting differences in respiratory requirements at different life stages.

It is important to note that, once heme is in the mitochondrion, it could be inserted in different mitochondrial heme-proteins. How heme is transported to the mitochondrion and how it is imported by the mitochondrial membranes to be used in this organelle are still open questions, since carriers, chaperons, and transporters have not been identified yet. Based on the evidences discussed here, while cytochrome *c* biogenesis could proceed from a novel and characteristic mechanism, apparently restricted to Euglenophyta, the heme A biosynthesis might be synthesized by conserved enzymatic pathways.

5. Concluding Remarks

Trypanosomatids are under varying nutritional pressures during their life cycles; consequently, they must adapt their metabolism to different environments. These parasites display auxotrophies for various cofactors, including heme. Heme biosynthesis is absent from trypanosomes, and although *L. major* possesses the last three enzymes of the pathway, it still needs to import precursors from the host. From what we know up to now, a few points in which differences between parasites and hosts might be exploited for rational design of therapeutic compounds exist, for instance, the novel system for maturation of cytochromes *c*, which does not match to any other known system, and the role of cytochromes *b5* as electron donors in at least two essential pathways (PUFAs and ergosterol biosynthesis).

However, in spite of the conclusion of the genome project for TriTryp, there are a number of unknown aspects that need further research. It is not clear, for example, how heme group is internalized into the cell and how it is coupled to heme-proteins. Also it is not known how different cytochromes reach their final intracellular location: mitochondria (cyt*c*) and endoplasmic reticulum (cyt*b5*, CYP51) and how cyt*b5* is organized in the membrane in order to fulfill its role as electron donor for diverse anchored enzymes. New insights into these and other aspects of heme and cytochromes functions would shed light into vital biological processes from these protozoan organisms that could be potential therapeutic targets.

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Review Article

Enolase: A Key Player in the Metabolism and a Probable Virulence Factor of Trypanosomatid Parasites—Perspectives for Its Use as a Therapeutic Target

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Glycolysis and glyconeogenesis play crucial roles in the ATP supply and synthesis of glycoconjugates, important for the viability and virulence, respectively, of the human-pathogenic stages of *Trypanosoma brucei*, *Trypanosoma cruzi,* and *Leishmania* spp. These pathways are, therefore, candidate targets for antiparasite drugs. The glycolytic/gluconeogenic enzyme enolase is generally highly conserved, with similar overall fold and identical catalytic residues in all organisms. Nonetheless, potentially important differences exist between the trypanosomatid and host enzymes, with three unique, reactive residues close to the active site of the former that might be exploited for the development of new drugs. In addition, enolase is found both in the secretome and in association with the surface of *Leishmania* spp. where it probably functions as plasminogen receptor, playing a role in the parasite's invasiveness and virulence, a function possibly also present in the other trypanosomatids. This location and possible function of enolase offer additional perspectives for both drug discovery and vaccination.

1. Introduction

All known organisms belonging to the Trypanosomatidae, a family of protists within the order Kinetoplastida, are parasites of vertebrates, invertebrates, or plants. Species of two trypanosomatid genera, *Trypanosoma* and *Leishmania,* are responsible for several serious diseases of humans and domestic animals in the tropical and subtropical regions of the world [1].

Trypanosoma brucei is the causative agent of human sleeping sickness, a disease that threatens millions of mainly impoverished people in 36 countries of the African continent [2]. This parasite is transmitted by several species of the genus *Glossina*, insects commonly known as tsetse flies, which are endemic in the affected sub-Saharan countries. The two human infective subspecies of *T. brucei*, *T. b. rhodesiense*, and *T. b. gambiense,* live extracellularly, multiplying as so-called long-slender trypanosomes in the bloodstream [3]. By a population-density-dependent mechanism, the slender trypanosomes may transform into nonproliferative stumpy bloodstream forms. Trypanosomes are passed on to the insect vector when a tsetse fly takes blood from an infected host. Once in the tsetse fly gut, the stumpy parasites differentiate into so-called procyclic-form trypanosomes, which subsequently undergo several successive differentiations while migrating from the gut via the proboscis to the salivary glands. From there, the parasites, then called metacyclic forms, are transmitted to a new host [1, 4]. Without treatment, human sleeping sickness is considered fatal. Vaccines or adequate drugs for effective treatment of this disease are currently not available [2].

Trypanosoma cruzi is responsible for Chagas' disease in South and Central America, a form of trypanosomiasis that threatens 15 million people on the American continent. This

parasite is mainly transmitted to humans via the feces of infected triatomine bugs; however, transfusions with infected blood, organ transplantations, and use of contaminated food are also important ways of transmission [5]. *T. cruzi* is an intracellular parasite of humans and many other vertebrates; the parasite is dedicated to invade cardiac and digestive tissues, where infective trypomastigotes differentiate into replicative amastigote forms. The amastigotes differentiate again into trypomastigotes in the cytosol before they burst from the infected host cell, thus liberating parasites that perform a new round of cell infection. Blood trypomastigotes, taken up again by triatomine bugs when they feed on an infected host, differentiate then into replicative epimastigotes in the insect's intestine. A next differentiation occurs in the intestine, producing vertebrate infective, metacyclic trypomastigotes. The parasites are transmitted again to the vertebrate host when the infected bug takes a blood meal, depositing the contaminated feces on the skin of the new host [1]. People infected with *T. cruzi* can only be effectively treated when such treatment is applied shortly after the infection has occurred.

Several species of the genus *Leishmania* are the etiological agents of leishmaniasis, a disease characterized by clinical manifestations with a wide range of symptoms. The disease is distributed in 88 countries of tropical and subtropical areas around the world. It is estimated that currently 350 million people are at risk. The parasite is transmitted by the bite of a female phlebotomine sand fly, which feeds from vertebrate blood [1].

Leishmaniasis can occur as three different forms: cutaneous leishmaniasis is the most common and mild form of the disease characterized by the presence of ulcers that appear at the places of parasite inoculation; mucocutaneous leishmaniasis is manifested by destruction of the mucosa membranes of mouth, nose, and throat after parasite spreading; visceral leishmaniasis is the most dangerous form of the disease in which organs such as liver and spleen are compromised. *Leishmania* species multiply mainly inside host macrophages as amastigote forms; this form of the parasite is present in the phagolysosome where the parasite divides until they burst from the host cells. Subsequently, the released amastigotes invade new cells, in that way spreading the infection. The parasite is taken up by the sand fly when a female phlebotomine feeds on an infected host. The amastigotes then differentiate into proliferative metacyclic promastigote forms in the sand fly gut before migrating to the salivary glands in order to be transmitted to a new host [1]. Cutaneous and mucocutaneous leishmaniases are disabling diseases that affect the quality of life of infected people; however, visceral leishmaniasis is a fatal disease when no treatment is applied.

Although several drugs are available to be used for sleeping sickness, Chagas' disease and leishmaniasis, they do not offer adequate treatment. None has been rationally designed to specifically treat these diseases. As a consequence, toxic side effects, sometimes very serious, are associated with the use of such drugs, and increasing resistance has been reported against some of them. Additionally, the application of some of the treatments requires highly qualified staff and medical equipment, which are usually not available in the regions where most of the people affected by these diseases live [1]. Consequently, there exists an urgent need to develop new drugs that will inhibit the viability of the parasites while causing minimal side effects to the infected people and which can be easily administered and will be affordable for these people usually living in resource-poor areas of the world. In that context, efforts have been made in recent decades to identify, in the parasites, crucial metabolic pathways or cellular structures with features sufficiently different from those in the host in order to exploit them as chemotherapeutic targets.

Several features distinguish members of the Trypanosomatidae family from other eukaryotic cells. A unique feature of all kinetoplastids is that the majority of glycolytic and gluconeogenic enzymes are compartmentalized in unique peroxisome-like organelles called glycosomes [6, 7]. Several lines of evidence have shown that this compartmentalization is absolutely essential for survival of the organisms. Metabolic studies on *T. brucei*, *T. cruzi*, and *Leishmania* spp. have shown that in the human-pathogenic stages of each of these parasites glycolysis and/or gluconeogenesis are crucial—or at least major processes in the provision of free energy and carbon—and consequently essential for parasite viability. Moreover, the peculiar compartmentalization of these pathways in kinetoplastids has endowed the enzymes with specific structural and functional properties that offer potential for development of trypanocidal drugs [8].

Trypanosomatid parasites are also equipped with several potent mechanisms that allow them to evade the immune response by the host, as well as to migrate through several tissue barriers to reach their final destination. *T. brucei* is present in the bloodstream of the mammalian host during the initial phase of the infection. A sophisticated mechanism of antigenic variation at the level of their surface coat allows the parasites to escape continuously from the host's immune defense system [4]. At a second stage of the disease, the parasites reach the central nervous system by crossing the blood-brain barrier via a multistep mechanism of which the details remain to be established [9]. In contrast, *T. cruzi* lives only briefly in the bloodstream of the vertebrate host before invading the cardiac muscle and cells of the digestive system. It is known that metacyclic trypomastigotes initially adhere to the host cell through several proteins exposed on the parasites' cell surface that function as ligands and adhering molecules. Then, the parasites control their invasion by exploiting phagocytic and nonphagocytic mechanisms that lead to their internalization into the parasitophorous vacuole. Once there, the parasites differentiate into amastigotes, which excrete potent pore-forming peptides and transsialidases/neuraminidases that mediate the vacuolar membrane's disintegration to let the parasites free in the cytosol in order to multiply [10]. Metacyclic promastigotes of *Leishmania* spp. are exposed during a very short time to the lytic factors present in the bloodstream. Surviving parasites are able to adhere through different mechanisms to the host cell, which belongs to the monocyte/macrophage family. The presence on the parasites' surface of lipophosphoglycans, metalloproteases,

and specific ligands for macrophage receptors allows the parasites to invade the host cells, as well as migrate through the extracellular matrix [11]. Once in the phagolysosome, promastigotes differentiate into amastigotes that are better equipped with molecules that provide resistance against the stress conditions encountered in the phagolysosome, such as the presence of hydrolases and the acidic pH. The surface molecules of the amastigotes play an important role in inhibiting the mechanism that triggers the macrophage's activation as well as the proper antigen presentation through the MHCI and II complexes, which eventually would activate a host protective T_H1-cell response [12].

Studies of the metabolic pathways essential for parasite survival and the mechanisms by which the trypanosomatids establish an infection in the human host are important both for future chemotherapy and vaccination. Such studies may identify molecules—unique or at least highly different from host molecules—that are interesting as candidate drug targets and the subsequent discovery of selective inhibitors of these molecules to combat the parasitic diseases, as well as molecules that could be exploited as potential antigens to induce immunoprotection against the trypanosomiases and leishmaniases.

In this paper, we will argue that enolase, a protein involved both as enzyme in glycolysis and gluconeogenesis, metabolic processes essential for *T. brucei*, *T. cruzi*, and *Leishmania* spp., and probably also as a virulence factor in the pathogenesis caused by *Leishmania* spp., *T. cruzi* and possibly also *T. brucei*, represents both an interesting drug target and vaccine candidate.

2. General Aspects of Enolase

Enolase is the enzyme responsible for the reversible conversion of D-2-phosphoglycerate (2PGA) and phosphoenolpyruvate (PEP) in glycolysis and gluconeogenesis, two metabolic pathways that are often vital for cellular function [13]. Enolase is a highly conserved protein found in Archaea, Bacteria, and eukaryotes with catalytic properties that are similar among divergent organisms. The ubiquitous presence of the enzyme and the sequence homology between enolases from extant organisms belonging to different phyla indicate that an enolase gene has already been present in the common ancestor and diversified by speciation of organisms and gene duplication within organisms [14].

Since enolase catalyzes the reversible conversion of 2PGA into PEP, the forward reaction being the ninth step of glycolysis and the reverse reaction being the first one of gluconeogenesis, an organism, tissue, or cell may need to optimize the properties of this enzyme in such a way that the flux in either one or the other direction is facilitated, dependent on whether it should adapt its metabolism to performing the catabolic or anabolic process. Indeed, in many organisms (vertebrates, *Saccharomyces cerevisiae*, *Toxoplasma gondii*) the presence of different enolase isoforms has been reported, often with kinetic properties favouring a flux in the glycolytic or gluconeogenic direction. In vertebrates, it has been shown that expression of the different enolase isoforms, formed by homodimers and heterodimers of α, β, and γ subunits, is regulated in a developmentally and/or tissue-specific manner [13, 15]. In yeast, the expression of the two enolase isoforms is dependent on the carbon source used to propagate the cells, glucose, or a nonglycolytic substrate; however, the kinetic properties of homodimeric enzymes of both isoforms are very similar [16]. In the case of *T. gondii,* the specific expression of enolase genes is linked to life-stage conversion. While enolase 1 is expressed during encystations where anaerobic glycolysis is promoted, enolase 2 is expressed in the actively dividing and invasive tachyzoite [17].

Some highly conserved proteins perform multiple functions that are different from their "classical", well-known activities, and, therefore, are called "moonlighting" functions. These latter functions are often revealed when these proteins are found at cellular localizations different from where the primary function is exerted. A considerable number of glycolytic enzymes, including enolase, exhibit nonglycolytic functions in several organisms, both bacteria and eukaryotes. Enolase is located mainly in the cytosol of all eukaryotic cells where, besides its function in glycolysis and gluconeogenesis, it is also involved in regulation of cell morphology and material trafficking by interacting with the cytoskeleton system [18]. Enolase has also been detected in the nucleus of mammalian cells where it participates in transcriptional regulation of genes involved in morphological transformation and cell proliferation [19]. Bacterial enolase has been reported as a main component of the degradosome where it functions, in a still poorly understood manner, in the regulation of mRNA stability [20]. The mammalian α-enolase is also found as a main structural component of the eye lens, where it is found as an inactive monomer [13].

Additionally, a considerable number of reports described that enolase can be expressed at the cell surface of several nonpathogenic and pathogenic organisms. The enzyme has been found at the cell wall of *S. cerevisiae* and also at the surface of the pathogenic yeast *Candida albicans* where it constitutes an immunodominant antigen during invasive candidiasis [21]. In some bacterial pathogens, such as *Bacillus anthracis*, *Streptococcus pneumoniae,* and *Streptococcus mutans* [22–24], surface enolase has been highlighted as an important virulence factor. In Apicomplexan parasites like *Plasmodium* spp. [25], cell surface enolase has been suggested to participate in the tissue invasion process. Additionally, in *Entamoeba invadens,* enolase expression is induced by environmental signals, and it shows association with cytoplasmic vesicle-like structures that transport the protein to the cyst wall where it plays an essential but so far unknown function [26]. Human and animal parasites of the genus *Schistosoma* have been reported to express enolase also on the surface of the adult worms [27, 28], where it could exert an important role in the inhibition of clot formation during host infection [28].

Enolase may also be excreted to the extracellular environment where it mediates degradation of host tissues and immune evasion, such as has been observed for the human pathogenic *Streptococcus pyogenes* and the insect parasite *Aphidius ervi* [29, 30].

3. Enolase of Trypanosomatids

African trypanosomes living in their mammalian host are entirely dependent on glucose, abundantly available in the blood. Metabolic studies performed on bloodstream-form *T. brucei* have shown that glycolysis represents the only process through which ATP is synthesized by the parasite. Inhibition of glycolysis, therefore, leads to rapid death of these parasites. The same is probably true for the human pathogenic stages of *T. cruzi*. Axenically cultured amastigotes have an essentially glycolytic metabolism, fermenting glucose to succinate and acetate [32], strongly suggesting that also in these cells enolase might be a candidate drug target. Glycolysis is probably also essential in the trypomastigote form of these parasites, as it lives in a glucose-rich bloodstream where it shows a highly upregulated glucose uptake activity [33]. Interestingly, the pathogenic stage of *Leishmania*, the amastigotes living intracellularly in phagolysosomes of macrophages, utilize amino sugars as a source of carbon and energy, indicating that glycolysis is important [34], while even during hexose uptake gluconeogenesis has been shown to be an essential pathway for the synthesis of glycoconjugates and β-mannan, both required for the virulence of the parasite [35, 36]; therefore, enolase could also be considered as vital, and thus a drug target, in this organism. The fact that even during uptake of (amino) sugars gluconeogenesis is required has been interpreted as an indication that the sugar levels in the phagolysosome are generally too low to sustain fluxes through essential pathways such as N-glycosylation, the pentose-phosphate pathway, inositol synthesis, and the catabolism of major carbohydrate reserve material [34].

In these three parasitic trypanosomatid species, as in all kinetoplastids, the majority of the glycolytic enzymes—dependent on the life-cycle stage, the 6 or 7 enzymes from hexokinase to glyceraldehyde-3-phosphate dehydrogenase or phosphoglycerate kinase, converting glucose into 1,3-bisphosphoglycerate or 3-phosphoglycerate, respectively—are compartmentalized in peroxisome-like organelles called glycosomes [6, 37, 38]. Only the last enzymes of the pathway—phosphoglycerate mutase, enolase, and pyruvate kinase—were not found to be associated with these organelles. More detailed studies specifically devoted to enolase have confirmed that its activity is exclusively present in the cytosol of *T. brucei* bloodstream forms [39], *T. cruzi* epimastigotes (W. Quiñones, and J. L. Concepción, unpublished results), and *L. mexicana* promastigotes [40]. In *T. cruzi*, the enzyme was detected by peptide mass fingerprinting in the different developmental stages with a higher expression in trypomastigotes and amastigotes compared to epimastigotes [41]. In contrast, mass-spectrometric analysis of differentiating *L. mexicana* showed that the glycosomal glycolytic enzymes were 1.4-fold upregulated during axenic differentiation of promastigotes to amastigotes whereas the cytosolic glycolytic enzymes including enolase were 2-fold downregulated [42].

In bloodstream-form *T. brucei*, enolase has been genetically validated as a drug target by RNA interference (RNAi) [43]. Upon induction of RNAi, enolase activity gradually decreased during the first 24 h to 16%, leading to growth arrest followed by trypanosome death after two days.

The genes coding for enolases of *T. brucei*, *T. cruzi*, *L. mexicana,* and *L. major* have been identified in the genome sequence database (TriTrypDB: http://tritrypdb.org/tritrypdb/) for a variety of African trypanosomes (*T. brucei*, *T. gambiense*, *T. congolense*, *T. vivax*), the American trypanosome *T. cruzi,* and several *Leishmania* species (*L. major*, *L. mexicana*, *L. infantum*, *L. braziliensis*). In each case, a single gene copy per haploid genome has been found, except in *T. gambiense* where two tandemly linked genes coding for identical proteins were found, clearly the result of a recent gene duplication in this species. They all encode polypeptides of 429 amino acids (including the initiator methionine) with relative molecular masses of 46 kDa. Only the predicted *T. congolense* enolase polypeptide has 430 residues due to the presence of an additional Lys residue at the C-terminus. The *Trypanosoma* amino-acid sequences show approximately 80% identity with the *Leishmania* sequences. Comparison of the three isoenzymes of the parasite's human host and trypanosomatid enolases reveals between 60 and 63% identity. Residues essential for substrate binding, catalytic activity, as well as those constituting the binding sites of substrates, and two Mg^{2+} ions are invariably present in all sequences (see the multiple sequence alignment in Figure 1).

The protein has been more extensively studied in *T. brucei* and *L. mexicana* [39, 40, 44]. Both enzymes have been purified from the parasites and expressed as N-terminally His-tagged proteins in *Escherichia coli*. The kinetic properties of both the natural and bacterially expressed enzymes were determined for the forward (glycolytic) and reverse (gluconeogenic) reactions. In each case, the enzyme displayed standard Michaelis-Menten kinetics for both substrates with very similar K_m and V_{max} values (Table 1). Mg^{2+} is essential for the activity of all enolases (with K_a values varying between 0.26 and 0.45 mM; optimal concentration for activity between 1 and 2 mM), from trypanosomatids and other organisms, but inhibits the enzymes at high concentrations. The K_i^{app} values of the trypanosomatid and yeast enzymes for Mg^{2+} are similar (43–50 mM), but the mammalian enzyme is more susceptible to inhibition; the inhibition of this latter enzyme, but not that of trypanosomatids and yeast, is pH dependent and seems to involve two processes with K_i^{app} values of 7.5 (40% inhibition) and >100 mM. Furthermore, other divalent metals such as Co^{2+}, Mn^{2+}, and Cu^{2+} inhibit all enolases tested. Enolases are also generally inhibited by the monovalent ions Li^+ and Na^+; rabbit muscle enolase is activated by K^+, but the trypanosomatid and yeast enzymes are not. Furthermore, the *L. mexicana* enzyme is inhibited by fluoride with K_i values of 1.9 mM (versus 2PGA) and 6.3 mM (versus PEP) for the natural enzyme and 13.0 mM (versus 2PGA) and 10.0 mM (versus PEP) for the bacterially expressed enzyme, in agreement with reports about other enolases. Similarly, enolase purified from *T. cruzi* epimastigotes is inhibited by fluoride with a K_i value of 2.6 mM (versus 2PGA; L. González-González, W. Quiñones, and J. L. Concepción, unpublished results). The *L. mexicana* enolase is also inhibited by inorganic pyrophosphate (PPi): K_i = 0.232 mM (versus 2PGA) and 0.238 mM (versus PEP)

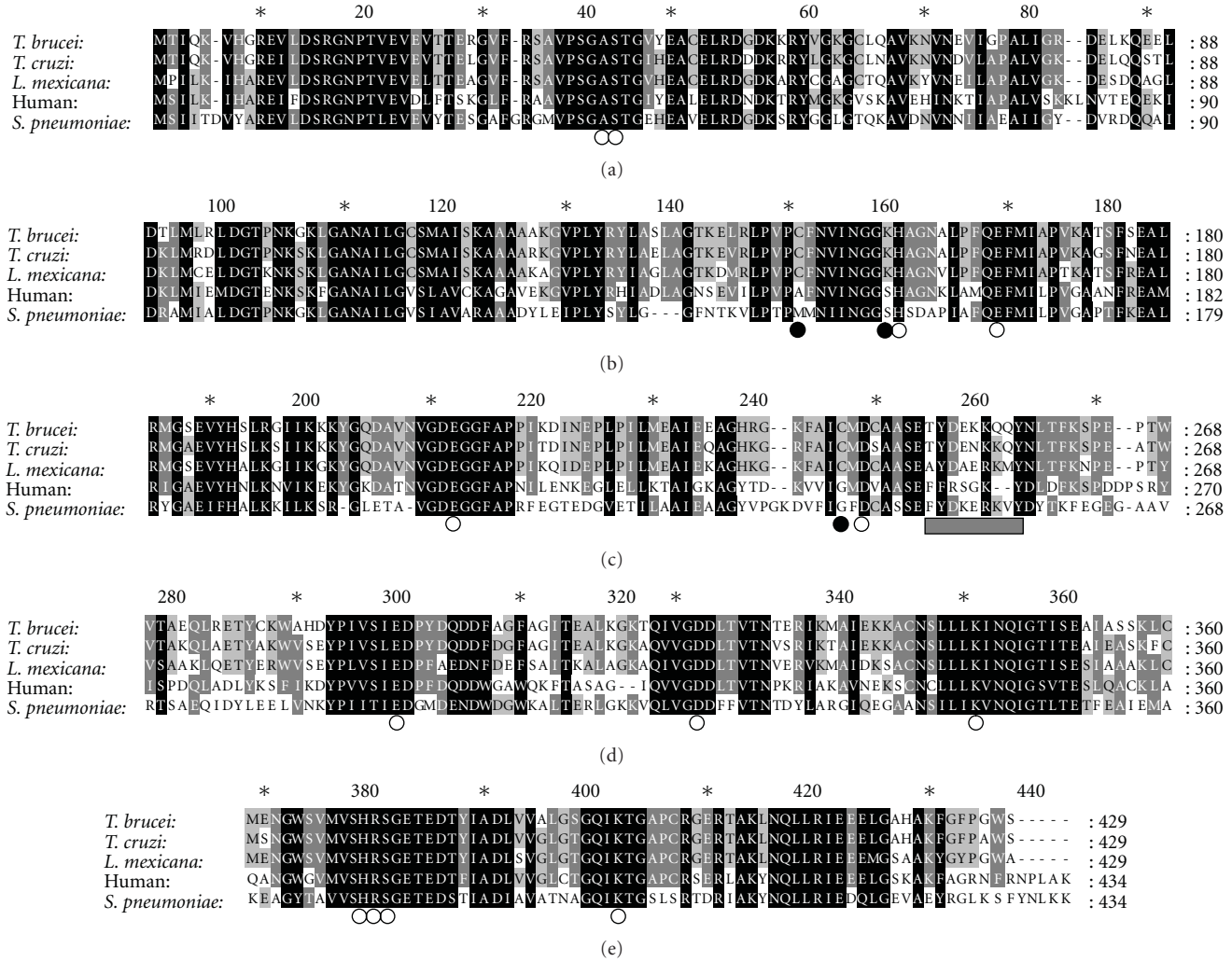

Figure 1: Characteristics of the enolase sequences of trypanosomatids. Alignment of the amino-acid sequences of enolase from *L. mexicana* (accession number ABA64522), *T. brucei* (accession number XP_822542), *T. cruzi* (accession number XP_819700), human α-enolase (accession number NP_001419), and *S. pneumoniae* (accession number Q97QS2). Open circles indicate residues involved in ligand binding (PEP, metals), closed circles unique reactive active-site residues found in enolases of trypanosomatids. The bar indicates the plasminogen-binding motif in *S. pneumoniae*. This plasminogen-binding peptide contains both positively and negatively charged residues flanked by hydrophobic amino acids, important for plasminogen binding [31].

for the natural enzyme and K_i = 0.127 mM (versus 2PGA) and 0.260 mM (versus PEP) for the recombinant enzyme. For the natural *T. cruzi* enzyme, inhibition by PPi with a K_i = 0.21 mM (versus 2PGA) was found (L. González-González, W. Quiñones, and J. L. Concepción, unpublished results). The susceptibility of *T. brucei* enolase to PPi has not been tested. It should be noted that, previously, several other *Leishmania* and *T. cruzi* glycolytic enzymes have been shown to be inhibited by PPi [45, 46] whereas no such inhibition was found for *T. brucei* enzymes.

4. Enolase Three-Dimensional Structure: Unique Features of the Trypanosomatid Enzyme

Crystal structures of enolase—the enzyme in its apo-form and with bound ligands—are available for a variety of organisms: *S. cerevisiae*, lobster, and *E. coli*. The structural analysis of these different enolases has shown that the enzyme usually folds as a homodimer, although, for enolase of some bacteria, an octameric quaternary structure has been reported. The overall structure and catalytic site are highly conserved, explaining the very similar kinetic properties between the enzymes from highly different organisms, as described in the previous section. Each subunit of the dimer is composed of two domains, a small N-terminal domain containing three α helices and four β sheets and a C-terminal domain which folds as an atypical eight-fold α/β barrel at the end of which the active site is found. Although the catalytic site lies at the dimer interface, each site involves residues of only a single subunit, explaining that the monomeric form is catalytically active, despite earlier claims of the contrary [47].

The early studies, particularly with the yeast enzyme, had shown that the enzyme is naturally activated by two divalent

Table 1: Kinetic characteristics of the natural and recombinant enolases of *T. brucei*, *T. cruzi*, and *L. mexicana*.

Source of enzyme	K_m (μM)		V_{max} (μmol·min^{-1}·mg^{-1})	
	2PGA	PEP	2PGA	PEP
T. brucei (natural)	54	244	ND	ND
T. brucei (recombinant)	49	289	63	6.3
T. cruzi (natural)	55	147	97	42
T. cruzi (recombinant)	50	410	0.87	12.35
L. mexicana (natural)	80	216	16.0	3.38
L. mexicana (recombinant)	51	200	80.0	3.64
Rabbit muscle	16	238	31	6.4
Yeast	57	264	65	7.8

Data of *T. brucei*, rabbit muscle, and yeast enolases were taken from Hannaert et al. [39], those of *L. mexicana* enolases from Quiñones et al. [40], and the *T. cruzi* data have not yet been reported before (L. González-González, W. Quiñones, and J. L. Concepción, unpublished results). Abbreviations: 2PGA: D-2-phosphoglycerate; PEP: phosphoenolpyruvate; ND: not determined.

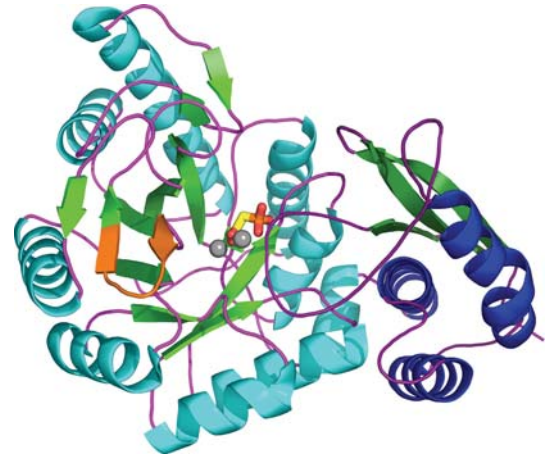

Figure 2: Cartoon view of *T. brucei* enolase in complex with the enolase inhibitor phosphonoacetohydroxamate (PAH; PDB code 2ptz [50]). Secondary structure is coloured blue (α-helices), green (β-strands) or purple (loops) with the N-terminal domain to the right of the figure drawn in darker hues. Ligand is shown as sticks and bound metals as isolated spheres. The putative plasminogen-binding site is shown in orange.

metal ions that bind to each monomer in two distinct metal-binding sites (I and II) near the active site. These studies also revealed the importance of sequential binding of a first metal ion, the substrate and a second metal ion, and associated local conformational changes, notably movements of three loops near the active site, for catalysis to occur. The loops define "open," "closed," and "incompletely closed" active-site structures [48]. Structures of the apo-enzyme and the enzyme in complex with its substrate or product indicate that, in the "open" conformation, the active site is totally exposed to solvent. When the substrate or product and the divalent ions are bound, an important conformational change occurs, bringing the loops one (comprising residues 37–44), two (150–166), and three (248–272) to the catalytic active site producing the "closed" enzyme conformation [48]. The catalytic mechanism of the reaction implies an acid-base chemistry involving a Lys-Glu dyad.

It had also already initially been found that enolase activity is inhibited by high metal concentrations, which suggested the presence of a third inhibitory metal binding site. Notwithstanding the agreement about the kinetic data, no structural data were available to support this hypothesis.

More recently, seven structural determinations have been performed for the *T. brucei* enzyme in ligation states with substrates, inhibitors, sulphate, phosphate, and metal ions–and in various conformations [49, 50]. A subunit structure of *T. brucei* enolase is shown in Figure 2. All these structures together have provided a profound insight into the structure-function relationship and the catalytic mechanism of the enzyme, and they solved some questions raised by the earlier structure determinations.

First, the different structures of the *T. brucei* enzyme and complementary molecular dynamics simulations revealed a strikingly larger conformational variability for the catalytic loops than initially thought. Secondly, in the studies of the parasite's enzyme, also an unexpected diversity of metal binding was found, and two additional metal-binding sites were detected near the active site. The occupancy of one of these sites, site no. III, explained the inhibitory effect of Mg^{2+} at high concentrations; the contribution of site IV to this inhibition remains to be determined.

Importantly, all available structures together, from trypanosomes and other organisms, and a sequence comparison of trypanosomatid and human enolases have revealed that, despite the high similarity (approximately 60% positional amino-acid identity), similar overall fold and active-site architecture and identical catalytic residues, potentially important differences exist between parasite and host enzymes that might be exploited for drug discovery. First, each of the trypanosomatid enolases contains three unique residues, absent from the enolases of mammals and almost all other organisms studied (Figure 1), which might be targets for irreversible reaction with active-site inhibitors [39]. A pair of cysteine residues, numbered 147 and 241 (see Figure 3), is present in a water-filled cavity near the active site. Despite their proximity, no disulphide bond between these residues was observed in the structure, nor is expected to be formed *in vivo* because of the low redox potential in the cytosol. One of these residues, and probably both, could be chemically modified by iodoacetamide with concomitant loss of enzyme activity indicating their accessibility to potentially reactive ligands [49]. Substrate analogues extended with reactive groups reaching either one of these Cys residues underneath the catalytic site may thus result in trypanosomatid-enzyme specific, irreversible inhibitors. In addition, a unique Lys, at position 155, was located on one of the active-site loops (Figure 3) and could also potentially interact with ligands in the active site. Although the side chain of this Lys residue points away from the catalytic site in original *T. brucei* enolase crystal structures, later structures, backed up by molecular dynamics simulations, show the Lys

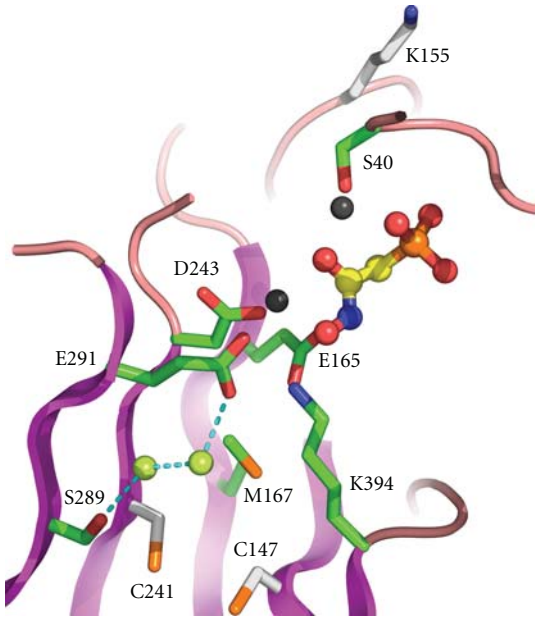

Figure 3: Close-up view of the catalytic site and neighbourhood of *T. brucei* enolase in complex with the inhibitor phosphonoacetohydroxomate (PAH) (PDB code 2ptz) [50]. Ligand is shown in ball and stick representation and bound metal as grey spheres. Bound water molecules near the Cys residues are shown as lime green spheres. Selected secondary structure is shown as magenta (β-strands) or pink (loops). Selected residues are shown as sticks and labeled with those discussed in the text as potentially relevant to trypanosomatid-specific drug design coloured white.

to approach the catalytic site more closely; it may, therefore, be available for irreversible binding of active-site inhibitors. Despite the closed catalytic site being small, the studies showed that occupation of the site by ligands does not need to lead to full closure, leaving open a tunnel near which Lys 155 is closely located. This offers thus a possibility for the design of enlarged ligands that interact with this potentially modifiable residue and inhibit the enzyme irreversibly.

5. Plasma-Membrane Bound and Secreted Enolase in *Leishmania* Parasites

A study of enolase localization in *L. mexicana* by differential centrifugation detected this protein, as expected from its function in glycolysis/gluconeogenesis, mainly in the cytosol. However, some enolase was also present in the microsomal fraction that contains predominantly the plasma membrane [40]. Similar results were obtained upon selective permeabilization of cells with digitonin; Western blot analysis showed that enolase was present in both the supernatant and pellet of each cell suspension, independent of the digitonin concentration added, indicating that the detergent was unable to fully release the enzyme from the permeabilized cells. These results suggested that enolase might be associated with membrane constituents of the cell. The presence of enolase at the plasma membrane was further assessed by immunofluorescence experiments using nonpermeabilized parasites [40]. These experiments not only confirmed the plasma-membrane localization of enolase but also showed that it is associated to the external surface of the parasite. In agreement with these results is the recent visualization of enolase in the proteome of the plasma membrane from *Leishmania chagasi*, prepared by two different approaches: live cell biotinylation followed by streptavidin affinity purification and octyl glucoside extraction [51]. Prior to these studies, subcellular fractionation of *L. infantum* with digitonin followed by proteomic analysis of the different fractions had predicted that enolase could have an additional localization, apart from the cytosol, also in this species [52].

Measurement of the enolase activity in the fractions obtained by differential centrifugation of an *L. mexicana* lysate showed that it was all present in the cytosolic fraction; no activity whatsoever was found in the microsomal fraction [40], indicating that the enolase on the parasite surface is inactive. This was corroborated by the digitonin solubilization experiment: all enolase activity was released with the cytosolic marker and no activity was detected in the pellets [40]. Another indication for the lack of activity of this surface enolase was recently provided by the report that its localization in *L. donovani* and *L. major* is regulated by oligopeptidase B (OPB), a serine-protease that is highly expressed in the amastigote form [53]. Although loss of this peptidase in $OPB^{-/-}$ parasites resulted in the accumulation of enolase at the cell surface of the parasites, no increased enolase activity was detected in total cell lysates. The authors hypothesized that oligopeptidase B causes clearance of surface enolase, once in the macrophage, to avoid macrophage activation [53]. This hypothesis is consistent with enolase having multiple functions.

Enolase is also present at the cell surface of several mammalian cells [54–56]. In addition, enolase can be found at the cell surface of numerous prokaryotic and eukaryotic pathogenic organisms including, among others, group A streptococci [57], *S. pneumoniae* [58], *L. mexicana* [40, 53, 59], *C. albicans* [60], *Schistosoma bovis* [61], and *Plasmodium falciparum* [25]. In the majority of these cases, enolase could act as a plasminogen-binding protein. Plasminogen is the zymogenic form of the serine-protease plasmin that is part of the fibrinolytic system. This molecule possesses five kringle domains that provide lysine-binding sites responsible for its binding to protein substrates and cell surfaces [62]. Surface-located enolases of mammalian cells and some pathogens bind plasminogen through a C-terminal lysine that is recognized by the lysine-binding sites of the plasminogen molecule [63]. However, in the amino-acid sequence of enolase from *S. pneumoniae,* an internal motif (FYDKERKVYD) was identified as being responsible for the plasminogen recognition [23]. Similar internal motifs were identified in the enolases of *L. mexicana* (AYDAERKMY) and *Aeromonas hydrophila* (FYDAEKKEY) [59, 64]. None of these two latter enolases possesses a C-terminal lysine residue. Enolases from other trypanosomatids (Figures 1 and 2) as well as from other pathogenic organisms have similar internal motifs at the same position that could contribute to plasminogen binding [65]. A similar motif has been shown

to be responsible for plasminogen binding by another *L.* protein, the parasite's homologue of receptors for activated C-kinase [66].

In addition to its localization on the membrane surface, enolase is secreted by several pathogenic microorganisms, such as the bacteria *S. pyogenes* [30], *S. mutans* [24], and *Lactobacillus crispatus* [67]. In *Leishmania* parasites, enolase has been found as a part of the secretome/exosome of *L. donovani* [68, 69], *L. major* [69], and *L. braziliensis* [70]. Enolase has also been found in the secretome of *T. brucei* [71, 72] and as a protein secreted by other parasites: *Eimeria tenella* [73], *Fasciola hepatica* [74], *Echinostoma caproni* [75], *Giardia lamblia* [76], and *Schistosoma japonicum* [77]. Different exosome proteomes from mammalian cell types indicate that enolase is commonly present in this structure [78].

Enolase does not contain a detectable secretion signal or membrane anchor region that can explain its membrane localization. Studies with *S. pneumonia* showed that soluble enolase can bind to the pneumococcal surface, suggesting that one possibility for the surface localization of enolase in this bacterium is the reassociation of secreted enolase with the plasma membrane [58]. One may thus speculate that, in the case of *Leishmania* parasites, enolase, after its secretion via exosomes, might bind to the cell surface. Further studies are necessary to elucidate the mechanism of translocation of enolase to the cell surface in these parasites.

6. The Role of Plasminogen/Plasmin and Enolase in the Virulence of *Leishmania* Parasites

The binding of plasminogen to the cell surface has been demonstrated for several mammalian cells [54, 63] including metastatic tumor cells. Equally, over the last 15 years, a steadily increasing number of reports have been published about plasminogen binding by both prokaryotic and eukaryotic pathogens, and even viruses [79]. This interaction has been implicated as part of their mechanisms of invasion and establishment in the host. However, in contrast to the large number of reports about plasminogen binding by mammalian cells and unicellular organisms, only few analyses have been reported thus far that provide evidence for the importance and physiological role of this interaction. Evidence for the physiological role and importance in infection and pathogenesis of the plasminogen-pathogen interaction has been provided by studies of animal models infected with group A streptococci [80], *Yersinia pestis* [81], *Borrelia* spp. [82–84], and *S. pneumoniae* [23]. The binding of plasminogen through their plasminogen-binding proteins and its activation made the pathogens acquire a potent protease for their own purpose by kidnapping the fibrinolytic system of the host. Plasmin is involved in several physiological processes, such as the degradation of fibrin and other extracellular matrix proteins. Acquisition of this host protease allows the pathogens to invade and disseminate in the host. In the case of the streptococci, *Staphylococcus aureus,* and *Y. pestis,* the conversion of plasminogen to plasmin is achieved by their own plasminogen activators [85]. The bound plasminogen can also be activated by the host plasminogen activators, the tissue plasminogen activator (t-PA), and urokinase (u-PA). Once the plasminogen is activated, the surface-bound plasmin cannot be inhibited by the host plasmin inhibitors (for reviews on bacteria-plasminogen interaction see [30, 85–89]).

Besides *L. mexic*ana, surface plasminogen binding has also been demonstrated in several other parasites: *T. cruzi* [90], *Trichomonas vaginalis* [91], *S. bovis* [28, 61], and *S. japonicum* [27]. In most of them, enolase has been proposed as the plasminogen receptor. For *L. mexicana,* it was demonstrated that plasminogen and plasmin bind to both the promastigote and amastigote forms of this parasite [92, 93]. The K_d for this interaction in promastigotes is 2.4 μM for plasminogen and 1.2 μM for plasmin. These values are consistent with an *in vivo* interaction since the concentration of plasminogen in plasma and the extracellular milieu is around 2 μM [94]. Moreover, enolase seems to contribute for 60% to this binding, suggesting that it is the principal plasminogen receptor on the cell surface [59]. This parasite does not appear to have its own plasminogen activator, thus requiring an exogenous source of activators similar to several other plasminogen-binding pathogens such as *Borrelia burgdorferi* [95]. t-PA, urokinase, and streptokinase are able to activate the bound plasminogen of *L. mexicana*. Indeed, the activation of plasminogen by t-PA is facilitated in the presence of the parasite (up to nine-fold enhancement of this activation was observed) [92]. Interestingly, there are different plasminogen-binding capacities among the morphotypes present in axenic cultures of *L. mexicana* promastigotes. Round forms bind more plasminogen than the slender forms. The proportion of the former is higher in heat-shocked and aged cultures [93].

Although the role of the interaction with plasminogen is considered to be important in bacterial pathogenesis, it is not clear what the function of this interaction is in the case of *Leishmania* parasites, particularly because this organism thrives within macrophages. However, this parasite can find plasminogen, as promastigotes, at the moment of host inoculation or as amastigotes when it is liberated from a macrophage to infect other cells. A clue about a possible function of the interaction of *Leishmania* with plasminogen was provided by studies of cutaneous lesions caused by *L. mexicana* in plasminogen-deficient mice [96]. In the lesion of $plg^{-/-}$, mice, the parasites were limited to isolated foci in contrast to the $plg^{+/+}$ mice lesion where a scattered pattern was observed. The parasites were less infectious in the plasminogen-deficient mice. In the lesion of $plg^{-/-}$, increased deposits of fibrin could be observed. These deposits are present in some organs in plasminogen-deficient mice [97, 98]. Thus, in natural conditions, fibrin could be limiting the encounter between *Leishmania* and macrophages. Fibrin is part of the host defense mechanism providing a barrier limiting invasion and dissemination and is also linked to inflammatory response [81, 99]. In addition, fibrin is one of the most important targets for plasmin *in vivo* [100]. The inflammatory response initiated by *Leishmania* parasites may increase capillary permeability, a process

associated with inflammation [101], resulting in the deposition of fibrin matrix within the lesion. This fibrin matrix would provide, together with other extracellular matrix proteins, the medium for parasite-macrophage interaction. Activated plasminogen on the parasite surface, or on secreted plasminogen-binding proteins, might thus help to degrade this fibrin matrix favoring the encounter with macrophages. In the latter case, secreted plasminogen-binding proteins would act as "torpedo mines" mediating clearance of fibrin. Indeed, secreted proteins of *L. mexicana* are able to enhance the plasminogen activation by t-PA [66]. Macrophages have also plasminogen receptors [102] and produce plasminogen activator [103]. The plasmin formed on these cells, together with metalloproteases, mediate macrophage migration during the inflammatory process [104, 105]. This relation plasminogen-*Leishmania*-macrophage might even be more complex. It is known that plasmin can have a cytoprotective effect on macrophages by inhibiting apoptosis in these cells [106] and could so support more infection by the parasite. It is clear, however, that more information is needed to assess the function of the interaction of *Leishmania* parasites with plasminogen. Equally, more studies of the role and dynamics of fibrin in the leishmaniasis lesion are needed as well as of the effect of this molecule and plasminogen on the inflammation process within the lesion.

Although fibrinolysis seems to be the most important function of plasmin [100], one may not discard the possibility that other known functions of this enzyme may play a role in the case of *Leishmania* infection and pathogenesis. Plasmin can function in procollagenase activation and release of peptides for nutrition [85]. Since both *Leishmania* parasites and macrophages have plasminogen receptors, it is tempting to speculate that plasminogen might act as a bridge between these two cells. Moreover, it has been demonstrated previously that the pathogen *Mycoplasma fermentans* is able to adhere and invade HeLa cells in a plasminogen-dependent manner [107]. Although the real function of the interaction plasminogen-*Leishmania* is not known, this interaction suggests that plasminogen-binding proteins, such as enolase, could be virulence factors.

7. Discussion and Conclusions

As described above, enolase may have one or more of three important functions in the different trypanosomatid parasites, in glycolysis, in gluconeogenesis, and/or as plasminogen receptor. In bloodstream-form *T. brucei,* enolase is involved in glycolysis, a process crucial for the ATP supply of the parasites. In intracellular *Leishmania* amastigotes, amino sugars are utilized as carbon and energy source, while also gluconeogenesis is an essential pathway for the synthesis of glycoconjugates and β-mannan, both required for virulence of the parasite; therefore, enolase should also be important for the parasitic life of this organism. Less information exists about the nature of the carbohydrate and energy metabolism of the human-pathogenic stages of *T. cruzi*, but the available data indicate that axenically cultured trypomastigotes and amastigotes are highly dependent on glucose catabolism, strongly suggesting that, also in these cells, enolase fulfills an essential function in metabolism. Therefore, in each of these trypanosomatid species, enolase may be considered as a validated or at least a highly likely candidate drug target.

In addition to this essential metabolic role played by enolase in the cytosol of the parasites, the enzyme was also shown to be present, in a catalytically inactive form, at the outer surface of *L. mexicana* and *T. cruzi* (W. Quiñones, and J. L. Concepción, and L. Avilan, unpublished results), where it probably acts as a plasminogen receptor. Although pathogens may have also other means to bind plasminogen, in *L. mexicana*, enolase was proposed to be the predominant receptor for this zymogen, responsible for 60% of its total binding. This plasminogen binding, and the subsequent activation of the plasminogen into the protease plasmin, could be important for the parasite's virulence.

Several moonlighting functions are known for the glycolytic/gluconeogenic enzyme enolase (discussed in Section 1). In some cases, organisms have multiple enolase isoforms, and some moonlighting functions have been attributed to specific isoforms. In each of the trypanosomatids, except in *T. gambiense*, only a single enolase gene copy is present, thus the encoded protein must be responsible for both the glycolytic/gluconeogenic activity and the plasminogen-binding function. The absence of activity of the surface-associated plasminogen-binding form may be due to processing of the enzyme during or after its translocation across the plasma membrane. This remains to be determined. How the enzyme arrives at the surface, and is bound to the membrane, is not clear yet. The protein does not possess a predicted transmembrane region or glycosylphosphatidylinositol (GPI) anchor site, nor a detectable N-terminal transit peptide, suggesting that it is not transported via the classical secretion pathway. Moreover, enolase was detected in the secretome of *L. donovani* [68], and also in that of *T. brucei,* both bloodstream-form and procyclic cells [71, 72]. Strong experimental evidence has been provided for both *Leishmania* and *Trypanosoma* that these parasites use multiple nonclassical secretion pathways, including active exocytosis via microvesicles or exosomes. This suggests that the enolase is first secreted and subsequently retrieved by *Leishmania* to associate in a still unknown manner with its outer surface.

In addition to a gene coding for a typical enolase, some trypanosomatids possess genes coding for hypothetical proteins with regions homologous to those of authentic enolases. It is unlikely that these proteins, which are usually considerably longer than typical enolases, possess enolase activity, because some catalytically important residues seem to be missing. Their location in the cell and their function—a receptor function or something different—remain to be determined.

Enolases from a variety of pathogenic organisms have the ability to bind plasminogen via a C-terminal Lys residue. However, in *S. pneumoniae* enolase an internal peptide located in a loop at the surface of the protein was identified as the primary site for interaction with plasminogen [31]. For *L. mexicana* that, like almost all other trypanosomatid enolases (except *T. congolense*), lacks a C-terminal Lys, a peptide was found with high sequence similarity to, and

at the corresponding position as a plasminogen-binding peptide of, *S. pneumoniae* enolase. This sequence is highly conserved in the enolase of all other *Leishmania* species. The plasminogen binding of *Leishmania* enolase and the involvement of this peptide in this interaction, remain to be proved by biochemical and/or structural studies.

The peptide is also well conserved in the enolase of *T. cruzi* as well as, intriguingly, in that of all species of African trypanosomes. The necessity of *T. cruzi* to recruit host plasminogen can be understood in view of the fact that this parasite, like *Leishmania* spp., invades tissues and enters cells and may require extracellular proteases for these activities. However, the conservation of the candidate zymogen-binding peptide and the presence of enolase in the secretome of the extracellularly living *T. brucei* raises the question as to whether it plays also a role as plasminogen-receptor in African trypanosomes, and if so, what could be the function of recruiting a host protease. It should be noted that, whereas in *L. mexicana* the enolase is bound to the cell surface, it has not been detected in the proteome of the *T. brucei* plasma membrane [108]. The ability of African trypanosomes to degrade fibrinogen and fibrin has already been reported several decades ago [109]. It may be that the infection is accompanied with an increased synthesis of fibrinogen by the host and formation of microthrombi that have to be dealt with by the parasites. Alternatively, the parasites may recruit plasminogen from the host to enable them to cross the blood-brain barrier. This crossing is a still poorly understood process, but may occur in a paracellular way. In this case, plasmin, as a host-derived protease, can help to degrade adherens junctions and tight junctions in the trypanosome-induced blood-brain barrier dysfunction model as proposed by Grab et al. [110].

The facts that enolase plays an essential function in the metabolism of probably all trypanosomatids and is probably an important virulence factor for, at least, the intracellular parasites offer two possibilities for using this protein to combat or prevent an infection. First, the presence of the plasminogen-receptor form of enolase could be used in two different ways to interfere with the virulence of the parasites. Compounds may be developed as drugs that prevent or disrupt the receptor-plasminogen interaction. Alternatively, the trypanosomatid enolase, or unique parts of its sequence, may be used either as an antigen for an infected person to boost his immune system in combating the parasites, or it may be administered as a vaccine to protect people at risk of being infected. Secondly, as discussed above, the availability of the crystal structure of *T. brucei* enolase has opened the possibility to design parasite-enzyme selective inhibitors. Substrate analogues may be synthesized that exploit unique physicochemical and/or steric features of the catalytic site of the parasite's enzyme and, therefore, provide high affinity and selectivity. Moreover, incorporating substitutions that interact with the reactive Lys and Cys residues (Figure 3) specific of the trypanosomatid enolases will not only increase the selectivity but also render the inhibition irreversible. Irreversible and uncompetitive inhibitors are particularly attractive because, 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 [111]. Because of the high similarity between the enolases of the different trypanosomatids, it is likely that inhibitors developed in this way for the *T. brucei* enzyme may also affect the activity of the enzymes of *T. cruzi* and *Leishmania* spp. Alternatively, the availability of systems for the easy production of purified bacterially expressed enolases of all three trypanosomatids allows high-throughput screening of large libraries of drug-like compounds. Any hits obtained by such screenings will subsequently be optimized through successive cycles of structure-activity relationship (SAR) analysis. 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. Finally, lead compounds thus obtained may serve for their further development to trypanocidal or leishmanicidal drugs.

Abbreviations

GPI: glycosylphosphatidylinositol
OPB: oligopeptidase B
PAH: phosphonoacetohydroxamate
PEP: phosphoenolpyruvate
PGA: phosphoglycerate
PLG: plasminogen
PPi: inorganic pyrophosphate
RNAi: RNA interference.

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Review Article

Phospholipases A in Trypanosomatids

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Phospholipases are a complex and important group of enzymes widespread in nature, that play crucial roles in diverse biochemical processes and are classified as A_1, A_2, C, and D. Phospholipases A_1 and A_2 activities have been linked to pathogenesis in various microorganisms, and particularly in pathogenic protozoa they have been implicated in cell invasion. Kinetoplastids are a group of flagellated protozoa, including extra- and intracellular parasites that cause severe disease in humans and animals. In the present paper, we will mainly focus on the three most important kinetoplastid human pathogens, *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania spp.*, giving a perspective of the research done up to now regarding biochemical, biological, and molecular characteristics of Phospholipases A_1 and A_2 and their contribution to pathogenesis.

1. Introduction

Kinetoplastids are a group of flagellated protozoans distinguished by the presence, in their single large mitochondrion, of a DNA-containing region known as kinetoplast. These unicellular organisms have a similar genomic organization and cellular structures and undergo morphological changes during their life cycles, being transmitted by different insect vectors. The members of this group include extra- and intracellular parasites that cause severe diseases in humans and animals, as well as various free-living forms. Herein, we will mainly focus on the three most important human pathogens *Trypanosoma brucei*, *Trypanosoma cruzi,* and *Leishmania spp.*

T. brucei and its subspecies are extracellular parasites transmitted by tsetse flies and responsible of human African trypanosomiasis (HAT), also known as African sleeping sickness, and Nagana in cattle. The life cycle of African trypanosomes is complex and represented by extracellular stages found in blood, lymph, and spinal fluid in the mammal [1]. The disease threatens over 70 million people and uncounted numbers of cattle in 36 countries of sub-Saharan Africa, having a devastating impact in human health and economy in affected areas [2]. HAT symptoms occur in two stages; in the first, haemolymphatic phase, parasite invasion of the circulatory and lymphatic systems is associated with severe swelling of lymph nodes. If untreated, the disease overcomes the host's defences and can cause more extensive damage. The second stage, neurological phase, begins when the parasite invades the central nervous system by passing through the blood-brain barrier; without treatment, it is fatal and the damage caused can be irreversible [1].

T. cruzi in contrast, is an intracellular parasite that invades all types of nucleated cells in the mammalian host. This protozoa, transmitted through the faeces of bloodsucking insects of the Triatominae family, enters the mammalian host via damage to the skin and causes Chagas disease in humans, a chronic inflammatory condition characterized by cardiomyopathy, megacolon, and mega-esophagus [3]. The disease is prevalent throughout America and according to WHO estimations, 25 million people are at risk and 10 million are infected worldwide [4]. Chemotherapy against Chagas disease is limited and unsatisfactory. The two available drugs, nifurtimox and beznidazole, are capable of curing at least 50% of recent infections and both produced side effects [5].

The genus *Leishmania* comprises 20 species of intracellular protozoan that are transmitted by phlebotomine sandflies and infect specifically cells of the mononuclear phagocyte system in mammals. These parasites cause various diseases

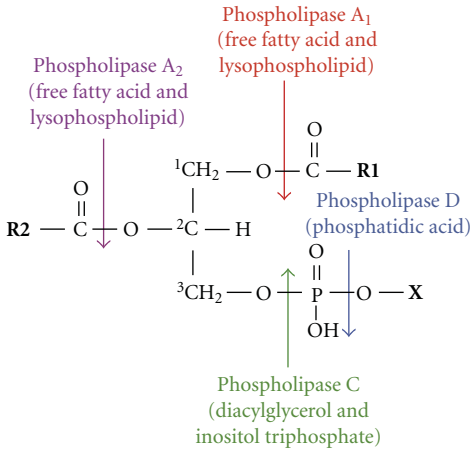

Figure 1: Hydrolysis of phospholipids by phospholipases. Arrows indicate the sites of attack for hydrolytic cleavage of phospholipases type A_1, A_2, C, and D. The main products generated by their action are also shown. **R1/R2**: free fatty acids in *sn*-1 or *sn*-2 positions; **X**: choline, ethanolamine, serine, inositol, and so forth.

ranging from self-healing cutaneous leishmaniasis, mucocutaneous leishmaniasis, with partial or total destruction of the mucous membranes, to severe and lethal (if untreated) visceral leishmaniasis, also known as kala-azar. Leishmaniasis is wide spread in Southern Europe, Africa, Asia, and America, threatening 350 million people in 88 countries around the world and represents an important global health problem that results in a significant economic burden [6].

One of the major components of biomembranes present in all living organism are phospholipids (PL), which form the lipid bilayer and serve as hydrophobic anchors of membrane proteins. These compounds can be enzymatically modified by the action of phospholipases (PLAs), with generation of bioactive lipid molecules that can act as second messengers and also modulate the immune response [7–9]. Moreover, PLAs have been considered virulence factors for many pathogenic bacteria like *Escherichia coli*, *Helicobacter pylori, Neisseria spp. Yersinia spp.*, and so forth [10–14].

PLAs are a complex and important group of enzymes, widespread in nature, that play crucial roles in diverse biochemical processes and are classified as A_1, A_2, C, and D, depending on the site of hydrolysis [15]. These enzymes cleave cell membrane and intracellular PL releasing a variety of products such as lysophospholipids (LPL), free fatty acids (FFA), diacylglycerols (DG), choline phosphate, phosphoinositides and phosphatidic acid, among others (Figure 1).

Phospholipase A_1 (PLA_1) (EC 3.1.1.32) specifically hydrolyses acyl groups from PL at the *sn*-1 position, producing FFA and LPL (Figure 1) [15]. PLA_1 activities have been detected in various cell types and tissues from a wide range of organisms by measuring hydrolysis of phosphatidylcholine (PC) to lysophosphatidylcholine (LPC) [15]. But despite their apparent ubiquity and diversity, up to now a limited number of PLA_1s have been cloned and characterized [16–18]. Increasing evidence indicates that some PLA_1s are capable of generating bioactive lipids, a role traditionally assigned to PLAs A_2, C, and D, though the biological relevance of this particular PLA deserves to be deeply studied [16, 19, 20].

At present time, nine PLA_1 molecules are known in mammals, being six extracellular and three intracellular enzymes, sharing no sequence homology between them and probably having distinct functions [7]. The extracellular PLA_1s belong to the pancreatic lipase gene family, that is conserved in an extensive range of organisms from insects to mammals and have been biochemically characterized and classified, according to their substrate specificities, structures, expression patterns, and possible functions [7]. Some PLA_1s have a broad substrate specificity and might also have triacylglycerol lipase activity (EC 3.1.1.3) [21]. Others, such as phosphatidylserine-specific PLA_1s from rat platelets and membrane-associated phosphatidic acid $PLA_1\alpha$ and $PLA_1\beta$, show strict substrate specificity [20]. The latter PLA_1s have specific roles in producing bioactive LPL, lysophosphatidylserine, and lysophosphatidic acid [7].

Phospholipases A_2 (EC 3.1.1.4), in contrast to PLA_1, are the most widely studied. Great advances in understanding the structure and function of the superfamily of Phospholipase A_2 (PLA_2) has occurred in the last decades [9, 22–25]. This superfamily includes fifteen groups, comprising four main types including secreted PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), Ca^{2+}-independent PLA_2 ($iPLA_2$), and platelet activating factor acetyl hydrolase/oxidized lipid lipoprotein associated PLA_2 ($LpPLA_2$) [9]. The classification is based upon the following characteristics: source, secreted, or cytosolic, availability of structural information, molecular weight, cofactors, and inhibitor specificity [9]. $sPLA_2$s have a low molecular weight (~14 kDa) and contain a large number of disulphide bridges, consistent with their extracellular environment, and require millimolar concentrations of Ca^{2+} for optimum catalytic activity [9]. $cPLA_2$s have a high molecular weight (~85 kDa) and preferentially hydrolyze PL containing arachidonic acid, therefore playing a key role in the biosynthesis of eicosanoids, precursors of prostaglandins and thromboxane [26]. Full activation of these enzymes requires Ca^{2+} binding to an N-terminal C2 domain and phosphorylation on serine residues [23]. $iPLA_2$s have a high molecular weight (85–88 KDa), contain seven to eight ankyrin repeats, one of the most common sequence motif, and the consensus lipase motif GXSXG, being detected mainly in human tissues [27].

Considering the important and various roles that PLAs possess, in the present review, we will summarize the research done to date in Trypanosomes regarding biochemical, biological, and molecular characteristics of PLAs and their contribution to pathogenesis.

2. Biochemical and Biological Characteristics of the Trypanosomatids Phospholipases A

2.1. Phospholipase A_1. In 1978, Tizard et al. described the presence of a haemolytic activity in *T. congolense,* due to the FFA generated by the action of PLA on endogenous PC, meanwhile in the nonpathogenic *T. lewisi*, no FFA generation

FIGURE 2: Multiple sequence alignment of trypanosome PLA_1. Alignment of the protein sequences of cloned *T. brucei*, *T. cruzi*, and *L. braziliensis* PLA_1 and *T. congolense* and *T. vivax* putatives PLA_1 was performed with NTI 10 Software (Invitrogen) using the Clustal W algorithm. The lipase consensus pattern is underlined. Letters indicate: identical (red), conserved (blue), similar (green), weakly similar (grey), and nonsimilar (black) amino acids. *Leishmania braziliensis* PLA_1 (TriTrypDB LbrM31_V2.2750), *Trypanosoma brucei* PLA_1 (TriTrypDB Tb.927.1.4830), *Trypanosoma congolense* putative PLA_1 (TriTrypDB TcIL3000.1.2010), *Trypanosoma vivax putative* PLA_1 (TriTrypDB TvY486_0102170), and *Trypanosoma cruzi* PLA_1 (TriTrypDB Tc00.1047053510679.100).

was observed and therefore, nonhaemolytic activity was detected [28]. Further, it was determined in four different species of African trypanosomes that Ca^{2+}-independent PLA_1 was the predominant PC-degrading activity. The levels of PLA_1 varied widely, with very high activity in the pathogenic *T. brucei* and relatively low activity in the nonpathogenic *T. lewisi* species [29]. Other authors found that *T. brucei* bloodstream forms possess high levels of PLA_1 activity, whereas in the procyclic culture forms PLA_1 specific activity was only 15% of that of bloodstream forms, suggesting an important physiological role for the enzyme in the mammalian stage [30]. Bloodstream trypomastigotes are covered with a dense layer of Variant Surface Glycoprotein (VSG), which protects the parasite from lysis by host complement via the alternative pathway [31]. It has been suggested that the high activity of PLA_1 in these forms, may play a role in the acquisition of fatty acids for synthesis of the VSG and also provide a source of myristate that can be employed for remodelling the lipid anchor of the VSG [32].

PLA_1 was purified from *T. brucei* bloodstream forms, where the major portion was found as a soluble activity in the cytosol and the minor as a particle-bound activity associated with lysosomal markers. Both enzymes had optimal activity at acid pH and were activated by Triton X-100 [30]. Although cultured procyclic trypomastigotes also possess PLA_1 activity, the levels were significantly reduced compared to bloodstream forms, due to a decrease in soluble PLA, similar levels of lysosomal activity were present in both stages [30]. Other authors reported that PLA_1 activity eluted together with a lysophospholipase activity (LPLA)

(EC:.3.1.1.5), suggesting that a single enzyme displays both activities [33].

T. brucei PLA_1 ($TbPLA_1$) has been recently cloned and expressed as recombinant protein [34]. This intracellular enzyme is localized in the cytosol and has optimal activity at neutral pH and a predicted size of 34.8 kDa. $TbPLA_1$ deacylates choline-containing PLs, with greater efficiency than those containing ethanolamine, inositol, serine, or just phosphorous at the *sn*-3 position [34]. The enzyme also displayed LPLA activity towards LPC, as previously reported [33].

As regards *T. cruzi*, we previously determined that when epimastigotes were grown at 28°C and then transferred to 37°C, a fatty acid exchange occurs between PL and neutral lipids [35]. This mechanism of membrane lipid adaptation suggested for the first time the action of PLA activity as part of a deacylation-reacylation cycle in *T. cruzi* [35]. Other authors reported membrane PLA_1 and PLA_2 activities in epimastigotes, but acting only on anionic PL such as inositolphospholipids and inositolphosphoceramides [36]. We further determined the presence of a PC-PLA_1-degrading activity in all parasite stages, being up to 20-fold higher in the infective amastigotes and trypomastigotes than in the noninfective epimastigotes, as occurs in *T. brucei,* where the mammalian stages possess the highest levels of PLA_1 activity [30, 37]. Interestingly, in both infective stages membrane-bound PLA_1 activity was remarkably higher than those detected in organelle bound or soluble fractions [38]. This localization does not appear to have a similar counterpart in *T. brucei*, where the major proportion of activity (more than 90% of the total) corresponds to a soluble cytosolic fraction [30]. In *T. cruzi* epimastigotes, in contrast, the enzyme was only detected in lysosomes [37]. It is remarkable that only infective stages secreted PLA_1 to the extracellular media [38], similarly to other enzymes that participate in *T. cruzi* endocytic pathway, such as cruzipain and trans-sialidase [39–42]. We purified *T. cruzi* PLA_1 ($TcPLA_1$) from epimastigote and amastigotes, obtaining in both cases a unique band of ~38 kDa. These enzymes proved to be independent of the bivalent cations Ca^{2+}, Mn^{2+}, and Mg^{2+}, had an optimum acidic pH, and were activated by Triton X-100. The biochemical characteristics of $TcPLA_1$ activities were similar to those reported for $TbPLA_1$ and other PLA_1s from mammalians [43, 44]. As previously demonstrated in *T. brucei* we also determined the presence of LPLA activity in autolysing parasites [17, 33, 45].

As concerns secreted $TcPLA_1$, we determined during metacyclogenesis, process in which epimastigotes differentiate into the infective metacyclic trypomastigotes, an increase in secreted enzyme activity simultaneously with the appearance of metacyclic forms, as expected [38]. Accordingly, it has been reported that membrane PLA_1, A_2, and C, may act in remodelling reactions needed for plasma membrane transformation during *T. cruzi* differentiation; these enzyme activities may be acting in remodelling reactions leading to the anchor of the mature glycoproteins of *T. cruzi* [46].

In the case of *Leishmania spp*., preliminary results of our laboratory have also demonstrated in *L. braziliensis* promastigotes a PLA_1 activity hydrolyzing PC. Moreover, we detected by Immunoblot two bands of ~37 and 41 kDa, using polyclonal antibodies against both $TcPLA_1$ and $TbPLA_1$ [47]. These antibodies were obtained in our laboratory, since no commercial antibodies against any PLA_1 were available until this year [48].

2.2. Phospholipase A_2. The eukaryotic PLA_2 were the first of the PLAs to be recognized. The pancreatic PLA_2 has been known to degrade PC since 1878, and at the turn of the century cobra venom was shown by Keyes in 1902 to have haemolytic activity directed towards the membranes of erythrocytes [49]. Secreted and membrane-bound PLA_2 activity has been described in Bacteria, Fungi and Protozoa [50–54], but in the case of Trypanosomes just a few reports are available.

PLA_2 was isolated from *T. congolense*; the enzyme appeared to exist in a dimeric form with subunit molecular weights of 16.5 and 18 kDa, had optimum pH of 6.8, and showed specificity for 1,2,dimyristoyl-*sn*-PC and 1,2,dioleoyl-*sn*-PC [55]. Inhibition studies implicated a thiol group at the catalytic site of the enzyme, which was stable to heat treatment and possessed haemolytic and anticoagulating properties [55].

In *T. brucei* bloodstream forms, other authors reported a PLA_2 activity that could be stimulated by Ca^{2+} or by the amphiphilic peptide melittin, and that was responsible of the release of arachidonic acid, a prostaglandins precursor, being a pivotal enzyme in the control of Ca^{2+} influx [56, 57]. In addition, it was demonstrated in *T. brucei* procyclic forms, that the arachidonic acid generated endogenously could induce both Ca^{2+} entry and Ca^{2+} release from the intracellular compartments acidocalcisomes, suggesting that PLA_2 activity participates in *T. brucei* signalling events [58]. On the other hand, in *L. donovani* promastigotes and *T. cruzi* amastigotes, these authors found that arachidonic acid only induced Ca^{2+} entry, possibly due to low generation of arachidonic acid or to the low amount of releasable Ca^{2+} in the acidocalcisomes of these cells [58].

Previous reports in *T. cruzi* suggested that PLA_2 could mediate the association between the parasite and macrophages, but the authors did not clearly establish the source of the enzyme [59]. In epimastigotes, it has been described a haemolytic activity that destabilize *in vitro* red blood cells membranes and that could be attributed to PLA_2 activity [60]. However under our experimental conditions using zwitterionic PL such as PC or PE as substrates, no secreted PLA_2 was detected in the supernatants of living epimastigotes [38]. In this concern, other authors have described in this protozoa a membrane-bound PLA_2 activity acting only on anionic PL such as inositolphospholipids and inositolphosphoceramides [36].

PLA_2 degrading activities have been also reported in *L. major*, and they could be involved in the biosynthesis of lipophosphoglycan, the main macromolecule on the surface of the procyclic promastigote [61]. Other authors showed that parasite pretreatment with a low dose of pachymatismin, a glycoprotein extracted from a marine sponge, increased PLA_2 activity, however macrophage invasion was partially inhibited [62].

Table 1: Phospholipase A_2 putative genes found in TriTrypDB.

Gene	Organism	Product	Syntenic	Comments
LbrM34_V2.2930	*L. braziliensis*	phospholipase A_2-like protein, putative	yes	no
LinJ35_V3.3070	*L. infantum*	phospholipase A_2-like protein, putative	yes	no
LmjF35.3020	*L. major*	phospholipase A_2-like protein, putative	yes	no
LmxM34.3020	*L. mexicana mexicana*	phospholipase A_2-like protein, putative	yes	no
Tbg972.9.7760	*T. brucei gambiense*	phospholipase A_2-like protein, putative	yes	no
TclL3000.0.00740	*T. congolense*	product unspecified	no	no
Tc00.1047053510743.60	*T. cruzi CL Brener Esmeraldo-like*	phospholipase A_2-like protein, putative	yes	no
Tc00.1047053510659.250	*T. cruzi CL Brener Non-Esmeraldo-like*	phospholipase A_2-like protein, putative	yes	no
TvY486_0906130	*T. vivax*	phospholipase A_2-like protein, putative	yes	no

On the other hand, in *L. amazonensis* the modification of PL composition of infected macrophages has been described, with increasing levels of LPC, an effect that may reflect indirectly, the action of an endogenous/parasite PLA_2 on the macrophage [63]. Furthermore, other studies showed PLA_2 activity in supernatants and lysates of *L. (L.) amazonensis* promastigotes and suggested that this enzyme may be a progression factor for cutaneous leishmaniasis [64].

In summary, there are increasing evidences of the presence and possible roles of PLA_2 in the pathogenic Trypanosomes, so far however, these enzymes have not been purified or characterized in deep.

3. Phospholipases A of Trypanosomatids and Pathogenesis

PLA activity has been linked to pathogenesis in various microorganisms such as *Escherichia coli*, *Yersinia spp, Helicobacter pylori, Neisseria spp, Legionella spp.,* and *Campylobacter spp.*, which cause different disease syndromes; however, the exact mechanism of the PLA action has not been definitively determined [10–14, 65, 66]. PLA toxicity has been associated to cytolytic activity resulting from the accumulation of membrane-destabilizing products or by the extensive destruction of membrane phospholipids [10]. In pathogenic protozoa PLAs have been implicated in cell invasion [54, 67, 68]; in *Toxoplasma gondii* it has been described that PLA_2 inhibition protected human monocytic cells from parasite invasion [53]; in *Entamoeba histolytica,* PLA_2 is one of the several factors related to virulence [54] and in *Cryptosporidium parvum* the use of PLA_2 inhibitors as well as specific anti PLA_2 antibodies significantly reduced invasion of human enterocytes [68].

The role of PLA_1 in the pathogenesis of African trypanosomiasis has been intensively studied [29, 45, 69, 70]. Hambrey et al. described in the tissue fluids of *T. brucei* infected rabbits large amounts of PLA_1 activity that increased with parasite burden, whereas in blood plasma this activity was also detected, but at a considerably lower level [70]. The enzyme seemed to be of trypanosomal origin, being either secreted by living parasites or released from dying organisms [70]. In intravascular locations PLA_1 could contribute to the pathology of trypanosomiasis by causing cell membrane damage and could account for some or all of the connective tissue cell destruction, which is a prominent feature of infections with *T. brucei* [71].

The high level of PLA_1 found in *T. congolense* and *T. brucei*, in comparison to other pathogens like *Escherichia coli* (1000 times fold higher) [33], and its relatively low level in the nonpathogenic rat trypanosome *T. lewisi*, suggested the importance of the enzyme in the pathology of African trypanosomiasis [28, 29, 45, 69, 72]. Given that *T. lewisi* and *T. congolense* are restricted almost entirely to the blood stream of the host, whereas *T. brucei* develops mainly in the connective tissues [71], it was suggested that PLA_1 could help the latter to penetrate blood vessels endothelium and other barriers hindering and contributing to tissue damage [29].

In the pathogenic *T. brucei* and *T. congolense* it has been determined that PLA_1 activity increased greatly during the autolytic process and large quantities of FFA were accumulated, whereas the non pathogenic *T. lewisi* failed to increase the enzyme activity even on prolonged autolysis [69]. PLA_1 yields FFA and LPC, which is then further degraded by the LPLA to yield more FFA and glycerophosphorylcholine. FFA are cytotoxic and haemolytic as a result of their detergent-like properties [73], and they could account for the immunosuppression and the structural disturbances in lymphoid organs observed in African trypanosomiases [69]. These observations deserve to be updated and deeply studied.

The first evidences related to phospholipid degrading enzymes in *T. cruzi*, was associated to the inflammatory responses that appear surrounding degenerating amastigote nests in various tissues of Chagas' disease patients [74]. This finding strongly suggested that autolytic processes generate factors, possibly PL-breakdown products, which

cause inflammation [11]. In this regard, it was demonstrated that FFA and LPL released from killed trypomastigotes have toxic effects on culture cells [12]. These facts are in agreement with the pathogenic mechanism proposed for African trypanosomiasis [29, 69]. Accordingly, we determined in all *T. cruzi* stages the rapid and extensive breakdown of endogenous PL in autolysing parasites [37]. A major increase in FFA was observed, significantly higher than the generation of LPC, indicating not only the presence of PLA_1 activity but also LPLA activity [37]. We also found that living *T. cruzi* infective stages were able to hydrolyze LPC, confirming the presence of a LPLA activity (Belaunzarán et al. unpublished observations). It is well known that LPC is potentially toxic for the cells [75, 76] though this activity would thereby contribute significantly to the parasite self-protection against lysocompounds. Similarly, in living *T. brucei* it has been demonstrated that PLA_1 is active against LPL [77]. Other authors reported that bloodstream forms can acquire substantial amounts of exogenous LPL through a pathway consisting of three enzymes associated with the plasma membrane: PLA_1, acyl-COA ligase, and LPC acylCOA-acyl transferase [32]. These cytotoxic compounds can change the ionic permeability of the plasma membrane, though they are rapidly metabolized to ensure tolerable levels in the cell. Thus a membrane-bound PLA_1 would protect *T. brucei* against the high levels of plasma LPC [32].

In *T. cruzi,* we already showed the involvement of PLA_1 in the early events of parasite-host cell interaction preceding parasite invasion. We demonstrated that either intact infective parasites or purified PLA_1 significantly modified the host cell lipid profile with generation of second lipid messengers (DG, FFA, and LPC) and concomitant protein kinase C activation [38], an enzyme that has been implicated in the upregulation of *T. cruzi* invasion [78].

With respect to *Leishmania spp*., it has been observed that LPC, which is scare in the macrophage, increased significantly after infection with *L. amazonensis* [63]. As LPC and arachidonic acid are the products of PC cleavage by PLA_2, the increase in the levels of LPC may suggest the action of the enzyme on the macrophage PC, producing prostaglandin E2 [63]. In this regard, it has been shown that this lipid mediator is increased after 1-2 hours of infection with *L. donovani* and can exacerbate the infection [79]. Nevertheless, whether the LPC generation was due to parasite PLA_2 or to the activation of macrophage PLA_2 remains unclear [63]. It is possible that the LPC could also be generated by a PLA_1 activity, similarly to that we detected in *L. braziliensis* [47].

4. Bioinformatic Analysis of the Trypanosomatid Genomes for Phospholipases A

The publication of the genomes of the kinetoplastid parasites *T. brucei* [80], *T. cruzi* [81], *Leishmania spp*. [82], and other related organisms, allowed the scientific community to perform comparative analyses giving insight into the evolutionary similarities/differences among trypanosomatids.

T. brucei PLA_1 (Tb.927.1.4830) has been cloned and characterized and the analyses of its protein sequence indicated that this enzyme is not homologous to neutral lipases [34]. The only similarity to them was in the amino acidic sequence that contains a lipase consensus pattern harbouring a conserved GXSXG motif, a marker of the serine hydrolase superfamily [34]. In these enzymes, the catalytic triad is typically constituted by a base residue (Histidine), an acid (Aspartic), and a nucleophile (Serine), belonging to the latter to the GXSXG motif. No eukaryotic homologues of $TbPLA_1$ were found in *T. cruzi* and *Leishmania* spp., but orthologues of this enzyme were identified in *T. congolense* (TcIL3000.1.2010) and *T. vivax* (TvY486_0102170) [34]. Interestingly, $TbPLA_1$ resembled a putative PLA_1 homologue from *Sodalis glossinidius,* a proteobacterium endosymbiont of tsetse flies. These findings suggested that a *T. brucei* ancestor acquired the PLA_1 gene through horizontal gene transfer after/during its adaptation to a parasitic lifestyle in the insect vector [34].

Regarding *T. cruzi*, we previously reported the presence in the *T. cruzi* data base (http://www.tcruzidb.org/) of at least sixteen different genes encoding putative lipases and the identified sequences presented a high degree of similarity among them (70–80%), may be haplotype variants [38]. When we further performed a search in the Kinetoplastid Genomic Resource TriTryDB (http://tritrypdb.org/tritrypdb/) using only the lipase consensus pattern of $TbPLA_1$ and considering the biochemical characteristics of *T. cruzi* PLA_1, the number of putative genes was reduced to eight [38]. One of them (Tc00.1047053510679.100) was cloned and expressed in *E. coli*, being the recombinant enzyme recognized by both anti-$TcPLA_1$ and anti-$TbPLA_1$ antibodies [48]. The eight sequences are currently under study in our laboratory, to elucidate the identity of each of these genes that codify for *T. cruzi* PLA_1 and to obtain the active recombinant enzyme.

We extended these analyses to *Leishmania spp.*, searching in the TriTryDB database for homologues of *T. cruzi* PLA_1 putative genes and have identified in *L. braziliensis*, *L. infantum,* and *L. major,* three, nine and eight putative genes with the conserved lipase motif, respectively. One of the putative genes from *L. braziliensis*, LbrM31_V2.2750, was cloned and expressed in *E. coli*, being the recombinant protein recognized by both anti-$TcPLA_1$ and anti-$TbPLA_1$ antibodies [47]. At present, we are running assays with the aim of obtaining and characterizing the active recombinant enzyme.

The alignment of the protein sequences corresponding to the cloned *T. brucei*, *T. cruzi,* and *L. braziliensis* PLA_1 (Tb.927.1.4830, Tc00.1047053510679.100, and LbrM31_V2.2750, resp.), with the putative PLA_1s proteins of *T. congolense* and *T. vivax*, (TcIL3000.1.2010 and TvY486_0102170), shows that the sequences of *T. brucei*, *T. congolense,* and *T. vivax* are closely related, whereas *T. cruzi* and *L. braziliensis* only share with all of them the lipase motif (Figure 2). The fact that *T. cruzi* and *L. braziliensis* PLA_1 protein sequences do not share significant homologies with African trypanosomes, particularly with $TbPLA_1$, is in

agreement with that previously observed by Richmond and Smith [34].

Although PLA_2 activity was detected in *T. brucei*, *T. congolense*, *T. cruzi*, *L. major,* and *L. amazonensis* years ago, still little is known about the identity of the genes that codify for them [36, 55, 56, 61, 63, 83]. We have performed a search in the TriTrypDB database and identified at least 9 putative PLA_2-like proteins in the different Trypanosomes species (Table 1), but at the moment no PLA_2 of trypanosomal origin has been identified in the genomes or cloned.

5. Inhibitors of Trypanosomatid Phospholipases A

As described above, parasite PLAs participate in diverse and relevant cellular processes such as membrane remodelling, modification of membrane permeability, generation of lipid second messengers and parasite invasion. All these facts emphasize the interest of these enzymes as potential chemotherapeutic targets that could contribute to the control of parasite proliferation and survival.

A number of compounds with potential inhibitory activity on parasite PLA_1 have been investigated. $TbPLA_1$ activity was inhibited by several heavy metals through an undefined mechanism, being the most potent at lower concentrations cadmium and copper. Iron produced partial to total inhibition depending on the concentrations employed, whereas moderate inhibition was detected in the presence of relatively high concentrations of nickel and zinc [34]. As the active-site residue for $TbPLA_1$ is Serine 131, the active-site serine modifiers iPr2*P*-F (di-isopropyl fluorophosphate), PMSF (phenylmethylsulfonyl fluoride), and E-600 (diethyl-*p*-nitrophenyl phosphate) were also assayed. Relatively little inhibition of the enzyme activity was observed but at very high concentration of inhibitors, suggesting that the catalytic triad active site of $TbPLA_1$ is buried inside the enzyme and sheltered by a lid domain, a property shared with other lipases [34].

Other compounds with potential inhibitory activity on $TcPLA_1$ were investigated in our laboratory, including the antimalarial drugs quinine and chloroquine, the antiarrhythmic drugs amiodarone and chlorpromazine, and the local anaesthetics dibucaine, procaine, and xylocaine. Among all of them, only chlorpromazine had an inhibitory effect, but at concentrations that induce cell toxicity [37].

As previously mentioned, in *T. brucei* Ca^{2+} influx can be regulated by PLA_2 [56, 58]. Various inhibitors of this enzyme such as thioetheramide-PC, manoalide, arachidonyl trifluoromethyl ketone, and aristolochic acid were tested in this protozoa, being the most effective in blocking Ca^{2+} influx 3-(4-octadecyl)-benzoylacrylic acid (OBAA), a potent inhibitor of secreted PLA_2 [56]. On the other hand, *T. brucei gambiense* and *T. brucei brucei* PLA_2s were inhibited in a noncompetitive fashion when using organotin compounds like fatty acid derivatives of dibutyltin dichloride [83]. In the case of *T. cruzi*, it has been suggested that quinacrine, which inhibited erythrocyte lyses, blocked PLA_2 activity [60].

Concerning *Leishmania spp.*, up to now, there are no reports about the use of specific PLA inhibitors. However, it has been reported that the lysophospholipid analog (LPA) miltefosine, affects lipid metabolism in *Leishmania donovani* promastigotes, with reduction in PC and enhancement in PE and LPC, a process in which PLAs could participate among other enzymes [84]. The usefulness of lipid biosynthesis inhibitors has gained great interest in the last years to fight parasitic Trypanosomes [5, 85–88]. These compounds, initially developed to be antitumor agents, have proved to be highly effective in the treatment of visceral leishmaniasis [87, 89, 90]. Although their effectiveness is known, the mode of action against this parasite is not completely understood [91]. In *T. cruzi*, the synergy of the LPAs edelfosine, ilmofosine, and miltefosine with the ergosterol biosynthesis inhibitor, ketoconazole, induced alterations in the plasma membrane, reservosomes, and mitochondrion, indicating that these organelles are potential targets of these drugs, probably through interference with lipid metabolism [92].

Considering that PLAs are present in both, trypanosomes and mammalian host, it will be of relevance to achieve the knowledge of their three-dimensional structures to determine the differences/similarities among them. This would allow the rational design of specific inhibitors that could be employed as potential chemotherapeutic agents in the diseases caused by kinetoplastid pathogens.

6. Concluding Remarks

In summary, as presented in this paper PLAs of pathogen trypanosomes mediate a variety of processes in both protozoan and host cell lipid metabolism, being also considered virulence factors. However, the knowledge of these enzymes is far from complete, though in the future, continued biochemical, biological, and structural research are needed to obtain a full understanding of the molecular mechanism in which these enzymes participate. Unravelling the differences between parasite and host PLAs may contribute, besides, to the design of specific enzyme inhibitors that could be used in the treatment of the neglected diseases that trypanosomes cause.

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Review Article

Singular Features of Trypanosomatids' Phosphotransferases Involved in Cell Energy Management

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Trypanosomatids are responsible for economically important veterinary affections and severe human diseases. In Africa, *Trypanosoma brucei* causes sleeping sickness or African trypanosomiasis, while in America, *Trypanosoma cruzi* is the etiological agent of Chagas disease. These parasites have complex life cycles which involve a wide variety of environments with very different compositions, physicochemical properties, and availability of metabolites. As the environment changes there is a need to maintain the nucleoside homeostasis, requiring a quick and regulated response. Most of the enzymes required for energy management are phosphotransferases. These enzymes present a nitrogenous group or a phosphate as acceptors, and the most clear examples are arginine kinase, nucleoside diphosphate kinase, and adenylate kinase. *Trypanosoma* and *Leishmania* have the largest number of phosphotransferase isoforms ever found in a single cell; some of them are absent in mammals, suggesting that these enzymes are required in many cellular compartments associated to different biological processes. The presence of such number of phosphotransferases support the hypothesis of the existence of an intracellular enzymatic phosphotransfer network that communicates the spatially separated intracellular ATP consumption and production processes. All these unique features make phosphotransferases a promising start point for rational drug design for the treatment of human trypanosomiasis.

1. Introduction

Protozoan pathogens constitute an important group of parasites with medical and veterinary importance. Among them, *Leishmania spp. and Trypanosoma spp.* are examples of mammalian parasites. About half a million people are infected by parasites of the *T. brucei* group in Africa, 11–18 million with *T. cruzi* in the Americas, and 12 million with Leishmania in Africa, Asia, Europe, and Americas [1, 2]. The life cycles of Leishmania and *T. cruzi* involve an obligatory intracellular stage in mammals, in contrast to the exclusively extracellular parasites of the *T. brucei* group. Both Leishmania and *T. cruzi* invade host cells, while Leishmania lives inside parasitophorous vacuoles and *T. cruzi* escapes from the vacuole and lives in the cytoplasm of the host cell. In both cases, the parasites have to adhere to the host cell surface in order to invade the cell and survive under harsh conditions of the host cytoplasm. Trypanosoma and Leishmania also present an insect stage during its life cycle, all *T. brucei* group organisms are transmitted by tsetse flies of the genus Glossina, *T. cruzi* is transmitted by haematophagous insects belonging to the family Reduviidae, and *Leishmania spp.* are transmitted from man to man by different species of sandflies. Therefore, a common feature amongst parasitic protozoan organisms is their ability to adapt their metabolism to a wide range of environmental conditions and selection pressures, which include the availability and quality of carbon sources in the different mammalian and insect hosts [3]. Therefore, enzymes associated in energy metabolism are important candidates to rational designing of trypanocidal therapeutic drugs.

2. A General View of Trypanosomatids' Energy Metabolism

Even though trypanosomes share energy metabolism features with higher eukaryotes, they present unique characteristics which differentiate them from their metazoan host. Furthermore, the exact nature of their energy metabolism varies sensibly not only between trypanosomatid species [3] but also between different life cycle stages of any given specie [4]. It has been considered that these differences evolved from the variable nutrient supply in the particular environments of each trypanosomatid [5]. However, the metabolic disparities among different trypanosomatid species, which share the same host, indicate that metabolite availability alone cannot be the reason for the energy metabolism strategy exploited in each case [6].

To date, the most extensive experimental studies of trypanosome energy metabolism have been conducted in *T. brucei*, more precisely on the mammalian host associated to the bloodstream form and procyclic trypomastigote present in the tsetse fly midgut. Both of these life cycle stages are easily cultured in defined media in vitro.

The completion of the respective genome projects has enabled to in silico deduce a general metabolic pathway map for these trypanosomes; however this strategy alone misses to determine the presence and importance of single metabolic steps in each life cycle stage [5].

Although trypanosomes possess all enzymatic components needed for the glycolytyc pathway, the first seven enzymes are contained inside specialized microbodies from the peroxisome class called glycosomes [7]. These are rounded single membrane-bound organelles with a diameter of approximately 300 nm [8]. Many proteins that are localized to the interior of glycosomes contain specific targeting signals called PTS1 and PTS2 [9]. Furthermore the specialized matrix protein import system shares mechanistic similarities with the endoplasmic reticulum/proteasome degradation process which suggests that glycosomes, as well as peroxisomes and glyoxysomes, all share a common evolutionary origin [10, 11]. The key role of glycosomes in trypanosome energy metabolism becomes evident with the fact that the bloodstream form of *T. brucei* depends exclusively on glycolysis for ATP generation. The end metabolite of this pathway of hexose sugars corresponds to excreted pyruvate [12]. Intraglycosomal redox balance is maintained using a glycerol-3-phosphate dehydrogenase shuttle. The reoxidation of the glycolysis-derived NADH coenzyme is accomplished inside this organelle through an NAD-linked glycerol-3-phosphate dehydrogenase which reduces dihydroxyacetone phosphate to glycerol-3-phosphate [7]. In the presence of molecular oxygen, after exportation from the glycosome this molecule is directed to the mitochondria where it becomes reoxidized to dihydroxyacetone phosphate by the cyanide-insensitive trypanosome alternative oxidase [13] which then returns to the glycosome. On the other half of the glycolytyc pathway the final product that leaves the glycosome is 3-phosphoglycerate. In the cytosol the remaining three glycolitic steps take place producing pyruvate as end-product, thus the net ATP yield corresponds to two molecules per glucose. However in anaerobic conditions the yield is halved due to the inability to reoxidize glycerol-3-phosphate, and glycerol becomes an end-product equimolar to pyruvate [5]. The essential role of glycolysis in trypanosome energy metabolism and its particular and divergent strategy of glycosomal confinement, which distinguishes them from other eukaryotes, constitute a clear and plausible target for chemotherapeutic molecules [14]. RNAi-induced down regulation of components of the glycosomal matrix protein import system produces a relocalization of glycolytyc enzymes to the cytosol which is accompanied by a lethal phenotype [15, 16]. One of the possible explanations for the essential compartmentalization of glycolytyc enzymes relies on the lack of feedback regulation determined for the trypanosome enzymes hexokinase and phosphofructokinase [17–19]. Both of these initial steps of the glycolytyc pathway consume ATP that is recovered in later steps as well as the net ATP gain derived from the pathway. In the absence of specific regulation ATP produced by glycolysis would boost the flux through these enzymes above the capacity of the enzymes downstream with lethal accumulation of intermediate metabolites and cellular depletion of ATP. In this sense confinement within a membranous organelle from the final ATP synthesis steps constitutes an alternative regulatory strategy to unregulated enzymes [16].

Returning to the metabolism of bloodstream form *T. brucei*, the end-product of glucose metabolism is pyruvate. It cannot be further metabolized because during this stage pyruvate dehydrogenase, the tricarboxylic acid (TCA) cycle, and the respiratory chain are absent from the mitochondrial compartment. All the members of the order Kinetoplastida are characterized by a single large mitochondrion which contains a unique structure named kinetoplast [12]. This structure is constituted by a gigantic network of concatenated circular DNAs which represent the mitochondrial genome. Among these molecules, those termed maxicircles encode mitochondrial rRNAs and respiratory chain subunits [20]. Evidence for the expendable nature of mitochondrial metabolic pathways during bloodstream stage derive from the findings that *T. equiperdum* and *T. evansi* actually correspond to *T. brucei* mutants which, respectively, contain relics or completely lack kinetoplast DNA [21]. Although ATP synthase is present, it hydrolyzes ATP in order to maintain proton gradient across the inner mitochondrial membrane essential for the translocation of nuclear-encoded proteins into the mitochondrial matrix [22, 23].

On the other hand, *T. brucei* procyclic trypomastigotes contain a complete set of mitochondrial respiratory chain complexes and all the enzymes responsible for the tricarboxylic acid cycle. Despite the suggestive potential aerobic metabolism, glucose catabolism end-products indicate a predominant fermentation activity. Additionally inhibition of respiration and F0/F1-ATP synthase has no effect on intracellular ATP concentration [24]. Apart from carbon dioxide, succinate and acetate are the main excreted metabolites [25]. A fraction of the succinate derives from intraglycosomal redox balance maintenance. Glycosomal NADH is reoxidized by a glycosomal malate dehydrogenase which reduces oxaloacetate to malate, and after the subsequent production

of fumarate, another glycosomal reducing reaction yields succinate which is then secreted [26]. The remaining succinate is produced inside the mitochondria through a set of the enzymes relative to the tricarboxylic acid cycle, during the degradation of proline and glutamate [12]. Furthermore mitochondrial pyruvate is not oxidized to carbon dioxide and water [27, 28]. This molecule is decarboxylated, and the resulting acetyl-CoA is converted to acetate yielding an additional molecule of ATP [29, 30]. Acetate represents the essential precursor for lipid biosynthesis in procyclic form of *T. brucei* [31]. The diverse functions of components of the tricarboxylic acid cycle allow concluding that in these organisms there is no cycle [12].

Trypanosomes in culture universally prefer glucose as carbon source for energy metabolism; however in the digestive environments endured during the insect stages, it is accepted that carbohydrates are only available in limited quantities. Therefore it has been demonstrated that amino acids, such as proline and threonine, can be metabolized for ATP production. This has also been studied in *T. cruzi* epimastigotes which although prefer to use glucose over amino acids as an energy substrate [32]. Under aerobic conditions they produce, in addition to CO_2, considerable amounts of succinate, L-alanine, and acetate [33]. Epimastigotes produce ammonia only after the glucose in the medium has been exhausted [32]. An axenic culture model suggests that *T. cruzi* amastigotes mostly use glycolytyc metabolism for ATP production [34]. Amastigotes also ferment glucose to succinate and acetate, but do not seem to excrete ammonia and have little need for the oxidation of amino acids. All these metabolic pathways are summarized in Figure 1.

Maintenance of energy homeostasis requires coordinate regulatory responses according to the surrounding media composition inside the hosts. Most of the enzymes required for energy management, participating in these adaptation processes, are phosphotransferases with a nitrogenous group or a phosphate as acceptors (ECs 2.7.3 and 2.7.4, resp.), such as arginine kinase (AK), nucleoside diphosphate kinase (NDPK), and adenylate kinase (ADK).

3. Phosphotransferases in Trypanosomatids

Phosphotransferase families related to cell energy management are highly represented in trypanosomatid organisms; for example, since most of the organisms express one to three adenylate kinase isoforms in each cell, *T. brucei* has seven isoforms targeted to different subcellular structures, such as flagellum, glycosome, mitochondrion, and cytoplasm [35, 36]. *L. major* and *T. cruzi* also have six putative adenylate kinase isoforms according to our data and the currently available genome projects [37, 38]. The presence of such number of phosphotransferases and the predicted subcellular localization of each isoform support the hypothesis of the existence of an enzymatic phosphotransfer network that communicates the spatially separated intracellular ATP consumption and production processes [35, 39, 40]. In other organisms, energetic homeostasis is maintained by remodeling this phosphotransfer network. For example, in mammals the lack of muscle creatine kinase is complemented by glycolytyc enzymes and adenylate kinase; in a similar way, the suppression of the adenylate kinase gene produces an upregulation of glycolytyc enzymes and creatine kinase [41–43].

Since phosphotransferases participate in a variety of metabolic routes leading to many crucial compounds essential for trypanosomatid organisms, these families of enzymes become interesting targets for drug design.

3.1. Arginine Kinases. Phosphoarginine and phosphocreatine, generally called phosphagens, play a critical role as energy reserve because the high-energy phosphate can be transferred to adenosine diphosphate (ADP) when the renewal of adenosine triphosphate (ATP) is needed. It has been proposed that phosphoarginine supports bursts of cellular activity until metabolic events such as glycogenolysis, glycolysis, and oxidative phosphorylation are switched on [44]. Phosphoarginine synthesis also allows the cells to operate with low ATP levels since it may constitute a usable pool of the high-energy phosphate. Phosphagens act as reservoir, not only of ATP, but also of inorganic phosphate that is mostly returned to the medium by the metabolic consumption of ATP [45]. Arginine kinase (ATP: arginine phosphotransferase; EC: 2.7.3.3) catalyzes the reversible transphosphorylation between N-phospho-L-arginine and ADP [44]:

$$\mathrm{Mg \cdot ATP + L\text{-}arginine} \longleftrightarrow \mathrm{P\text{-}L\text{-}arginine + Mg \cdot ADP + H^{+}} \quad (1)$$

From an evolutionary viewpoint, arginine kinase was included in a family of conserved proteins with phosphotransferase activity, with creatine kinase as the best known member. Arginine kinase is the most widely distributed phosphagen kinase, which is found in Annelida, Coelenterata, Platyhelminthes, Nemertea, Mollusca, Phoronida, Arthropoda, Echinodermata, Hemichordata, and Chordata [46, 47]. In addition, arginine kinases are considered the most closely related member to the ancestral guanidino kinases [48].

In the last decade, the molecular and biochemical characterizations of arginine kinases in *T. cruzi* and *T. brucei* have been reported [49–53]. Since arginine kinase, an important enzyme involved in the energy supply for the parasite, is absent from mammalian tissues, it becomes a possible target for the future development of chemotherapeutic agents against Chagas' disease and other parasitic diseases caused by related organisms. For this purpose, a rational approach would involve the validation of the enzyme as a therapeutic target and the search for specific enzyme inhibitors. It was also postulated that arginine kinase could be a useful chemotherapeutic target in pesticides development for the control of cockroach proliferation [54].

Multiple evidence indicates that *T. cruzi* arginine kinase is strongly regulated by intra- and extracellular conditions: (1) the arginine kinase protein and the associated-specific activity increase continuously along the epimastigote growth curve, suggesting a correlation between the enzyme activity, and the nutrient availability or parasite density [32];

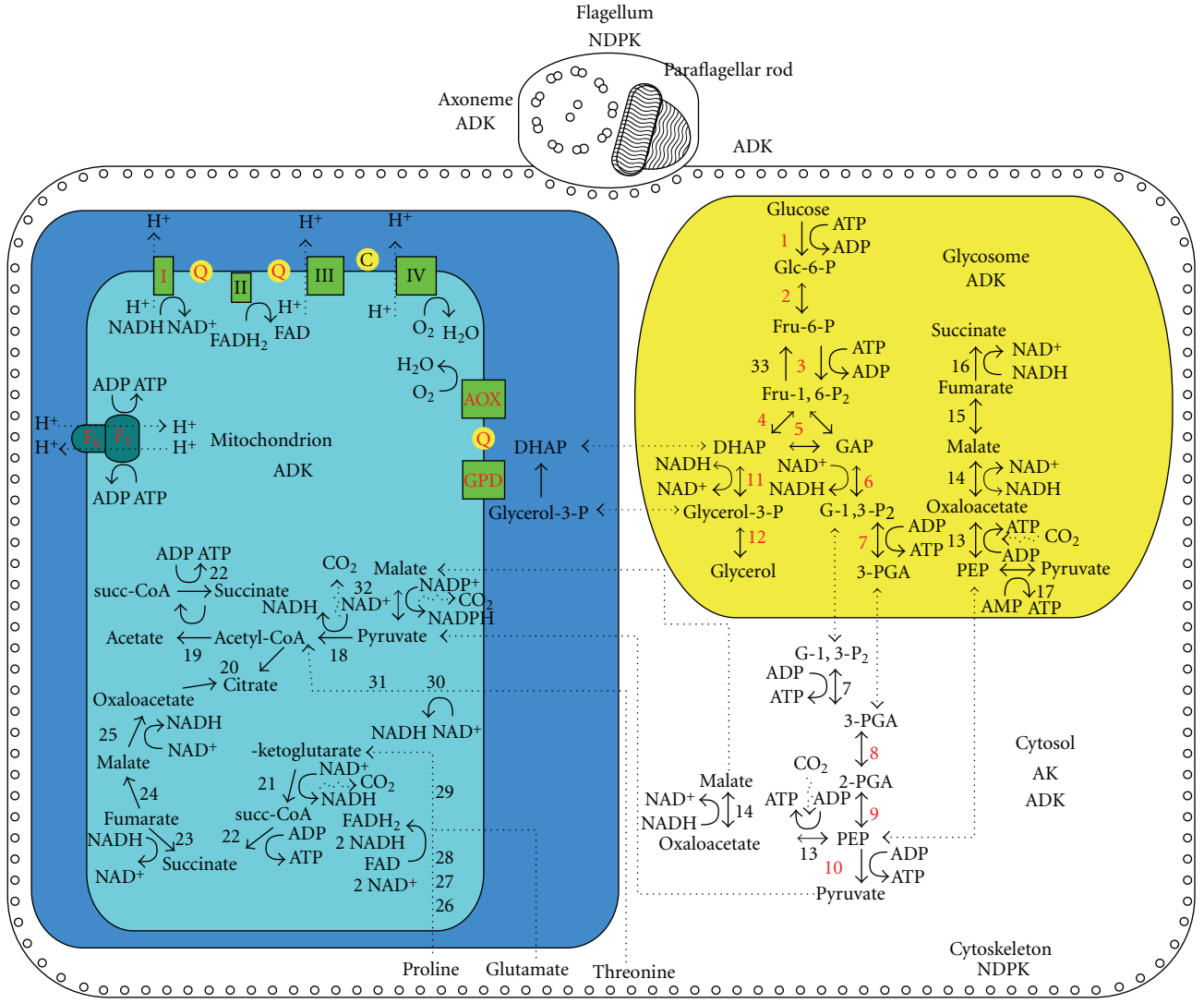

Figure 1: Schematic representation of trypanosomatids' energy metabolism. Enzymes are indicated with numbers: 1, hexokinase; 2, glucose-6-phosphate isomerase; 3, phosphofructokinase; 4, aldolase; 5, triosephosphate isomerase; 6, glyceraldehyde-3-phosphate dehydrogenase; 7, phosphoglycerate kinase; 8, phosphoglycerate mutase; 9, enolase; 10, pyruvate kinase; 11, glycerol-3-phosphate dehydrogenase; 12, glycerol kinase; 13, phosphoenolpyruvate carboxykinase; 14, malate dehydrogenase; 15, fumarase; 16, fumarate reductase; 17, pyruvate phosphate dikinase; 18, pyruvate dehydrogenase complex; 19, acetate:succinate CoA transferase; 20, citrate synthase; 21, α-Ketoglutarate dehydrogenase; 22, succinyl-CoA synthetase; 23, succinate dehydrogenase; 24, fumarase; 25, malate dehydrogenase; 26, proline oxidase; 27, Δ'-pyrroline-5-carboxylate reductase; 28, glutamate semialdehyde dehydrogenase; 29, glutamate dehydrogenase; 30, threonine dehydrogenase; 31, acetyl-CoA : glycine C-acetyltransferase; 32, NADP-linked decarboxylating malic enzyme; 33, fructose-1,6-bisphosphatase. Enzymes present in bloodstream forms only are indicated in red, procyclic forms enzymes are in red and black. AOX: alternative oxidase; GPD: FAD-dependent glycerol-3-phosphate dehydrogenase; I: NADH-ubiquinone oxidoreductase (complex I); II: succinate dehydrogenase (complex II); III: cytochrome c reductase (complex III); IV: cytochrome c oxidase (complex IV); c: cytochrome c; Q: ubiquinone; F0/F1: F0/F1-ATP synthase; Glc-6-P: glucose-6-phosphate; Fru-6-P: fructose-6-phosphate; Fru-1,6-P2: fructose-1,6-bisphosphate; GAP: glyceraldehyde-3-phosphate; G-1,3-P2: 1,3-bisphosphoglycerate; 3-PGA: 3-phosphoglycerate; 2-PGA: 2-phosphoglycerate; PEP: phosphoenolpyruvate; DHAP: dihydroxyacetone phosphate; glycerol-3-P: glycerol-3-phosphate; succ-CoA: succinyl-coenzyme A; acetyl-CoA, acetyl-coenzyme A. For a detailed explanation see the text.

(2) the existence of a relationship between the arginine transport rate, arginine kinase activity and the parasite stage and replication capability was recently described, indicating a critical role of arginine kinase as a regulator of energetic reserves and cell growth [55]; (3) the homologous overexpression of *T. cruzi* arginine kinase improves the ability of the transfectant cells to grow and resist nutritional and pH stress conditions [51]. Arginine kinase would play a role as a stress resistance factor when expressed in organisms that lack this enzyme, such as yeast and bacteria. Recombinant yeast, expressing crab muscle arginine kinase, showed improved resistance under stress challenges that drain cellular energy,

which were transient pH reduction and starvation [56, 57]. *T. cruzi* epimastigotes treated with hydrogen peroxide presented a time-dependent increase in arginine kinase expression, up to 10-fold, when compared with untreated parasites. Among other oxidative stress-generating compounds tested, only nifurtimox produced more than 2-fold increase in arginine kinase expression [52]. Moreover, parasites overexpressing arginine kinase showed significantly increased survival capability during hydrogen peroxide exposure. These findings suggest the participation of arginine kinase in oxidative stress response systems. It is important to remark that the insect stage of the *T. cruzi* life cycle is frequently exposed to nutritional and pH stress conditions, depending on the feeding status of the vector. For example, the pH of excreted material of the *T. cruzi* vector *T. infestans* varies between 5.7 and 8.9, accordingly with the time after feeding [58]. All these data suggest that arginine kinase is involved in the adaptation of the parasite to environmental changes and stress conditions. Recently, the crystal structure of ligand-free TcAK was determined by molecular replacement methods and refined at 1.9 Å resolution [59]. This information could be a new relevant tool for rational trypanocidal drug design.

Until today no outstanding arginine kinase inhibitors have been found. Only a few compounds have been reported which present a partial inhibition of arginine kinase. For example, the trypanocidal action of green tea (*Camellia sinensis*) catechins against two different developmental stages of *T. cruzi* was demonstrated by Paveto et al. [60]. Furthermore, recombinant *T. cruzi* arginine kinase was 50% inhibited by nanomolar concentrations of these polyphenols (catechin gallate or gallocatechin gallate). In silico docking studies indicated that the flavonoid rutin is an arginine kinase noncompetitive inhibitor and interacts mainly by a hydrophobic force forming an intermolecular complex with the enzyme [61]. Arginine kinase was also inhibited by the arginine analogs, agmatine, canavanine, nitroarginine, and homoarginine [62]. In addition, canavanine and homoarginine also produce a significant inhibition of the epimastigote growth in culture.

3.2. Nucleoside Diphosphate Kinases. Nucleoside diphosphate kinases (EC: 2.7.4.6) are enzymes involved in the intracellular nucleotide maintenance that catalyze the reversible transference of high energy phosphates from a nucleoside triphosphate donor to a nucleoside diphosphate acceptor as follows [63]:

$$\text{N1TP} + \text{N2DP} \longleftrightarrow \text{N1DP} + \text{N2TP} \quad (2)$$

Although the high energy phosphate is mainly supplied by ATP, these enzymes have broad spectrum substrate specificity and are able to use other ribo- and deoxyribonucleotides having preference for GTP formation [64].

NDPKs are ubiquitous and widely studied enzymes, and they can be divided in two groups according to the primary structure [65]. Group I is composed of canonical NDPKs, which are broadly studied and found in prokaryotes and eukaryotes. They form homotetramers in prokaryotes and homohexamers in eukaryotes [66]. The monomers have molecular mass between 15 and 18 KDa and are highly conserved during evolution; for example, there is about 40% identity between NDPKs from *Escherichia coli* and humans [64]. In contrast, group II is formed by divergent NDPKs that are present only in eukaryotes. Proteins included in this group are still poorly characterized and contain one or more NDPK's canonical domains and N-terminal or C-terminal extensions. Some domains such as DM10 domains are present in single copy in this type of NDPKs at the N-terminus. These domains are also contained in other unrelated proteins which have three repeats of this domain. There are only a few studies made on these proteins, in one of them they could associate DM10 domains to protein-axoneme connection [67]. However, the function of NDPK's DM10 domain remains unexplored. Thioredoxin domains are also found in this group of NDPKs, and it was postulated its participation in regulation of NDPK activity by a redox mechanism [68].

NDPKs are also involved in numerous and diverse biological processes. Because of the phosphohistidine enzyme formation in the transference reaction, the phosphate can also be transferred to different acceptors such as other proteins in serine or threonine residues and was postulated to participate in protein G activation by GTP supplement, so NDPKs are implicated in transduction pathways [69–74] playing many functions in, for example, development, proliferation, differentiation, and apoptosis [65, 72, 75–79]. Interestingly, it was also observed that some NDPKs have several roles in DNA processing. Human beings have ten NDPK isoforms (NM23-H1 to 10), five of which were found to interact to nucleic acids. NM23-H2 is considered to be a transcription factor because of its capacity to bind to specific DNA sequences, and NM23-H1, H5, H7, and H8 had 3′-5′exonuclease activity *in vitro*. All these characteristics together with others, as recognition, cleavage, and structural modification of DNA molecules, allow the association between NDPKs and DNA repair mechanisms [77, 80–82].

In the context of studying the biological function of an enzyme, the subcellular localization may be a critical point to establish. Being multifunctional enzymes, NDPKs have been found in numerous subcelular compartments such as cytoplasm, nucleus, mitochondria, intermembrane space, plasmatic membrane, and flagellum from different organisms [82–88]. Considering that NDPKs are involved in phosphotransfer networks their subcellular distribution might be related to an efficient energy distribution inside the cells [39].

Trypanosomatid's NDPKs are of particular interest due to its inability to synthesize purines de novo relying on NDPKs for nucleotide recycling [89]. For this reason these enzymes are considered potential therapeutic targets for trypanosomiasis such as Chagas disease. *T. cruzi* has four putative isoforms of NDPK, TcNDPK1–4. TcNDPK1 is the unique canonical isoform, while TcNDPK2, 3, and 4 correspond to group II variants. Isoforms 2 and 3 have one DM10 domain preceding the catalytic region, and variant 4 has unknown N- and C-terminal extensions. The orthologous

genes of these enzymes are also present in the genomes of the related parasites *T. brucei* and *L. major*, except for the absence of TcNDPK4 in the latter [90]. The first report of NDPK activity in trypanosomatids was published in 1995, where Ulloa et al. detected activity in different subcelular fractions including membranes and purified a soluble NDPK from *T. cruzi* epimastigotes with biochemical properties similar to canonical enzymes, probably corresponding to TcNDPK1 [91]. TcNDPK1 has a molecular mass of 16 KDa and like eukaryotic NDPKs forms homohexamers [92]. In addition, it is expressed in trypomastigote and amastigote stages [90]. This is an interesting enzyme because it showed not only phosphotransference activity but also DNAse activity with similar rates to commercial nucleases [93]. This new activity was extensively characterized in NM23-H2, the human orthologous of TcNDPK1, and a Lys inside the catalytic site seems to be responsible for it [94]. As *T. cruzi* genomic DNA is also susceptible to TcNDPK1 nuclease activity, it evidences that TcNDPK1 could act at nuclear level, for example, being component of programmed cell death machinery in trypanosomatid organisms [86, 95]. Reinforcing this idea, *T. brucei*-related NDPK was localized mainly in the nucleus of the parasites [96]; conversely it was also identified as a secreted protein [97]. Other results were obtained for members of Leishmania genus; in *L. major* this isoform was associated to microsomal fractions, and in *L. amazonensis* it is secreted and involved in macrophage infection [98, 99]. In *T. cruzi* it is still not clear the localization of the unique canonical isoform. It could be possible that the enzyme has several positions inside the cell and can move from one to another in response to stimuli. In this context a regulation by compartmentalization or phosphorylation is expected as was reported for *T. brucei* [100].

TcNDPK2, a longer NDPK isoform from *T. cruzi*, is a protein of 37 KDa whose first 88 amino acids correspond to the DM10 domain. It is expressed in the three major stages of *T. cruzi* life cycle, and apparently it has distinct regulation from TcNDPK1 because it is inhibited at high substrate concentration [90]. Using immunofluorescence and biochemical techniques we recently demonstrated that TcNDPK2 isoform is a microtubules-associated enzyme mainly localized in the cytoskeleton and flagellum (Miranda et al., unpublished results). TcNDPK2-like NDPKs are conserved in a wide range of eukaryotes with motile axoneme, from unicellular to superior organisms. For example, *T. brucei* orthologous genes codify for an NDPK found in parasite's flagella [101, 102], and in humans the related protein (NM23-H7) is also expressed in flagella-containing cells such as spermatozoids [103]. These are interesting results because they suggest a common possible function for TcNDPK2-like enzymes such as GTP supplying for tubulin polymerization and thus being involved in microtubule dynamics. DM10 domains present in this type of NDPK have not been investigated till the moment. However, we recently demonstrate, by expression of truncated and fusion variants of TcNDPK2 in *T. cruzi*, that the DM10 domain is sufficient and necessary for cytoskeleton delivery of the enzyme (Miranda et al., unpublished results). In addition, it is possible that DM10 domains were implicated in flagella-targeting machinery, a poorly understood process yet. Importantly, trypanosomatids are considered model organisms for the study of human illness based on cilia and flagella disorders, since NM23-H7 is associated to cone rod dystrophy, a progressive retinal disorder, then the study of TcNDPK2-related enzymes opens new insights in this interesting field.

There is an increasing amount of information about NDPKs that evidence their participation in many diverse biological processes. NDPKs seem to be key metabolic enzymes, thus further studies need to be made in trypanosomatids to understand how they work, to understand their role in metabolism and pathogenesis.

3.3. Adenylate Kinases. One of the enzymes related to cell energy management is adenylate kinases, which are ATP:AMP phosphotransferases. These enzymes are involved in the homeostasis of adenine nucleotides by interconversion of the adenine nucleotide pool, which includes ATP synthesis from ADP and an increase in the ATP energetic potential. They catalize reversible phosphotransfer between ADP, ATP, and AMP molecules, which have been implicated in processing metabolic signals associated with cellular energy utilization [104–106]:

$$Mg^{2+} \cdot ADP + ADP \longleftrightarrow Mg^{2+} \cdot ATP + AMP \qquad (3)$$

Recent evidence indicates that adenylate kinases facilitate intracellular energetic communication. In typical mammalian cells the loss of adenylate kinase function can be complemented by activation of creatine kinase phosphortransfer [40, 107]. Furthermore a similar role can be attributed to creatine and adenylate kinases, being both implicated in the renewal of ATP from ADP and a phosphorylated compound. In some organisms they participate in muscle contraction, metabolic sensing of K^+-ATP channels [106–108], and cell motility. In unicellular organisms such as *Tetrahymena* and *Paramecium*, adenylate kinases are involved in the ATP-regenerating system required for ciliary and flagellar movement.

They can be considered key enzymes in life support as they are present in almost all living organisms; they are distributed from bacteria to vertebrates.

The tridimensional structure of adenylate kinases can be decomposed into three subdomains, based on the functional roles and induced fit movements: the NMP-bind and LID domains, the moving parts, and the CORE domain that is unaffected by substrate binding. The "long" and "short" adenylate kinases classification is based on the differences in the LID domain; LID is an 11-residue segment in the short type, whereas that in the long type consists of 38 residues and the difference leads to drastic changes in the conformation of the LID domain. They are small globular proteins that suffer conformational changes when they interact with their substrate [109]; moreover, in most of the cases they are found as monomers [110].

Adenylate kinases isolated from prokaryotes belong to the long type [111]. Similarly the ADK localized in the chloroplast is also the long type [112]. Considering the chloroplast ADKs and the mitochondrial ADKs to have

Table 1: Characteristics of trypanosomatids' phosphotransferases. Summary of the main features of arginine kinases (AKs), adenylate kinases (ADKs), and nucleoside diphosphate kinases (NDPKs) isoforms from trypanosomatids. Predicted subcellular localizations are indicated with a question mark. The existence or not of human equivalents (human), N- or C-terminal extensions (N-t and C-t), peroxisomal targeting signal (PTS-1), and DM10 motifs are also detailed.

Enzyme	Organism	Isoform	Localization	Human	Features
AK	*T. cruzi/T.brucei*	1	Cytosol	No	Canonical
	T. brucei	2	?	No	N-t
		3	?	No	C-t
ADK	*T. cruzi/T. brucei/L. major*	1	Flagellum	Yes	N-t
		2	Reservosome (?)	Yes	—
		3	Glycosome	Yes	PTS-1
		4	Flagellum	Yes	N-t
		5	Cytosol	Yes	Canonical
		6	Mitochondria	Yes	—
	T. brucei	7 (A)	Flagellum	Yes	N-t
NDPK	*T. cruzi/T. brucei/L. major*	1	Nucleus (?)	Yes	Canonical
		2	Cytoskeleton	Yes	DM10
		3	?	Yes	DM10
	T. cruzi/T. brucei	4	?	Yes	Putative

a prokaryotic origin in view of the endosymbiont hypothesis, the classification can be subdivided into the eukaryotic short type and the prokaryotic long type. As always there are exceptions to this classification. For example, the cytosolic ADK from *S. cerevisiae* is the long type [113]. Another interesting case is the long type ADK from Giardia [114]. *Giardia* belongs to the most primitive group in the Eukaryotae, it does not have mitochondria. Consequently it is highly impossible, and Giardia's ADK has derived from mitochondria. Lastly the third exception is the short type ADK from the bacteria, Micrococcus [115]. Gathering all the information many authors postulate that the long and short types of adenylate kinase have diverged before the appearance of eukaryotes; this hypothesis could perfectly explain why both types of ADKs are found in prokaryotes and eukaryotes [116].

Their function can be related to their subcellular localization. They have been found in the cytoplasm, mitocondrial matrix, chloroplasts [117], and hydrogenosomes [118], structures that can be directly related to energy consumption and generation places. There is absolutely no doubt that adenylate kinases in those structures are responsible for the interconversion of the adenosine nucleotides. On the other hand essential functions have been related to adenylate kinases; for example, null growth is observed in *E. coli* [119] and *S. pombe* [120], lacking in their adenylate kinases. On the other hand they can be linked to consumption sites of ATP. For example, in Paramecium it has been proposed that they would be involved in the fast interconversion of ADP to ATP in the process of cilia reactivation [121].

In a few words adenylate kinases can be considered as key enzymes in cell energetic with the ability of doubling the ATP energy potential. They are a key sensor in cell energetic status sensing; thanks to their catalytic activity small variations in the nucleotide pool of ATP and ADP can be reflected as big changes in the AMP pool, increasing in this way the sensibility and response of the AMP responding mechanisms [107]. Lastly they can be linked to cellular energetic communication; under highly demanding energy process in some subcellular structures such as nucleus or flagella, fast relocalization of organelles involved in energy synthesis has been observed [39, 40]. These movements have been considered as mechanisms for reducing the distances between energy consumption and generating places. Even so these mechanisms would not be enough, diffusion processes are slow, so it has been proposed that the energy transport is catalyzed enzymatically. Key enzymes in the phosphotransferase network would be adenylate kinases [122].

In parasitic protozoa adenylate kinases have been detected and characterized. In *L. donovani* a long LID domain adenylate kinase has been cloned and characterized [123]. The unicellular malaria parasite, *P. falciparum* (Apicomplexa), presents two adenylate kinase isoforms [124].

In other parasitic protozoa a larger number of isoforms have been characterized. *L. major* has six putative adenylate kinase isoforms according to the genome project data. In other trypanosomatids, *T. brucei* and *Phytomonas spp*, adenylate kinases have been detected in diverse organelles, microbodies, glycosomes [125]. Recently two flagellar, cytoplasmic and mitocondrial associated adenylate kinases were characterized in *T. brucei* [35, 36]. A large number of isoforms, six in total, have also been described in *T. cruzi*, with possible flagellar, glycosomal, mitocondrial, and cytoplasmatic subcellular localization [37]. The data presented reveals an enormous variability within organisms, which are reflection of their adaptation to their life cycle. In the case of *T. cruzi* the high number of adenylate kinases can be attributed to the complex life cycle it goes through which involves distinct environments (insect vector gut, mammalian blood, and mammalian host cell cytoplasm) and consequently variable nutritional conditions. Another possible explanation to the highly unusual number of

adenylate kinase can be that they are not only in different subcellular localization but that they are stage specific.

A summary of the main characteristics from each phosphotransferase mentioned in the text is presented in Table 1.

It is evident that hosts and parasites have a large number of differences in terms of their energy metabolism. Trypanosomatids' enzymes completely absent in mammals, different isoforms in subcellular localization, structure, and number are some of these divergences. Phosphotransferases are mainly involved in crucial processes such as the maintenance of the ATP balance in the cell. Slight disruptions on this equilibrium are usually lethal for all living organisms; in consequence we consider that trypanosomatids' phosphotransferases are promising targets for rational drug design.

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