Acta Tropica 220 (2021) 105956

Contents lists available at ScienceDirect

Acta Tropica

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

Axenic amastigotes of *Leishmania* species as a suitable model for *in vitro* studies

Geovane Dias-Lopes [a,1], Anabel Zabala-Peñafiel [a,1], Barbara Cristina de Albuquerque-Melo [a], Franklin Souza-Silva [c,d], Laura Menaguali do Canto [a], Léa Cysne-Finkelstein [b], Carlos Roberto Alves [a,*]

[a] Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Laboratório de Biologia Molecular e Doenças Endêmicas, Rio de Janeiro, RJ, Brazil
[b] Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Laboratório de Imunoparasitologia, Rio de Janeiro, RJ, Brazil
[c] Fundação Oswaldo Cruz, Centro de Desenvolvimento Tecnológico em Saúde, Rio de Janeiro, RJ, Brazil
[d] Universidade Iguaçu, Faculdade de Ciências Biológicas e da Saúde, Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Keywords:
Leishmania
Axenic amastigote
Amastigote-like
In vitro culture
Drug screening

ABSTRACT

Leishmania spp. are etiological agents of infection diseases, which in some cases can be fatal. The main forms of their biological cycle, promastigotes and amastigotes, can be maintained *in vitro*. While promastigotes are easier to maintain, amastigotes are more complex and can be obtained through different ways, including infection assays of tissues or *in vitro* cells, and differentiation from promastigotes to axenic amastigotes. Several protocols have been proposed for *in vitro* differentiation for at least 12 *Leishmania* spp. of both subgenera, *Leishmania* and *Viannia*. In this review we propose a critical summary of axenic amastigotes induction, as well as the impact of these strategies on metabolic pathways and regulatory networks analyzed by omics approaches. The parameters used by different research groups show considerable variations in temperature, pH and induction stages, as highlighted here for *Leishmania (Viannia) braziliensis*. Therefore, a consensus on strategies for inducing amastigogenesis is necessary to improve accuracy and even define stage-specific biomarkers. In fact, the axenic amastigote model has contributed to elucidate several aspects of the parasite cycle, however, since it does not reproduce the intracellular environment, its use requires several precautions. In addition, we present a discussion about using axenic amastigotes for drug screening, suggesting the need of a more sensitive methodology to verify cell viability in these tests. Collectively, this review explores the advantages and limitations found in studies with axenic amastigotes, done for more than 30 years, and discuss the gaps that impair their use as a suitable model for *in vitro* studies.

1. Introduction

Leishmaniasis consists in a group of diseases caused by different species of flagellate protozoa of *Leishmania* genus (WHO, 2019). They are considered neglected tropical diseases representing an important health problem with economic and social burden (Okwor and Uzonna, 2016; Herricks et al., 2017). The World Health Organization (WHO) estimates around 1.2 million new cutaneous leishmanisis cases and 0.4 million new visceral leishmanisis cases each year (Alvar et al., 2012). In the most affected countries, limitations in disease surveillance and reporting services underestimate the real situation and current burden (Alvar et al., 2012; Okwor and Uzonna, 2016; Herricks et al., 2017).

The diversity of *Leishmania* spp., sand fly vectors and reservoirs are factors associated with the complex epidemiology of these diseases (WHO 2010). The three most frequent clinical manifestations are: *(i)* cutaneous leishmaniasis (CL) associated with skin lesions, mainly ulcers; *(ii)* visceral leishmaniasis (VL) that affects a variety of organs including the spleen and liver; and *(iii)* mucocutaneous leishmaniasis (ML) associated with destruction of mucosal surfaces such as nasal mucosa (Alvar et al., 2012; WHO 2010). The broad clinical features of these diseases depend on the host immune system and *Leishmania* spp. (Kaye and Scott, 2011). In general, more than 20 *Leishmania* spp. are known to affect humans and transmission occurs through the bite of infected female phlebotomine sand flies (Bates and Rogers, 2004; WHO 2010).

* Corresponding author.
E-mail address: calves@ioc.fiocruz.br (C.R. Alves).
[1] Geovane Dias-Lopes and Anabel Zabala-Peñafiel contributed equally to this work

https://doi.org/10.1016/j.actatropica.2021.105956
Received 21 January 2021; Received in revised form 28 April 2021; Accepted 30 April 2021
Available online 9 May 2021

Interestingly, parasite development location in the insect gut differs between species of the subgenera *Leishmania* and *Viannia* (Lainson and Shaw, 1987; Dostálová and Volf, 2012).

The main etiological agents of VL in the Old World belong to *Leishmania* subgenus such as *L. (L.) donovani* and *L. (L.) infantum,* while in the Americas is caused by *L. (L.) infantum* (syn. *L. (L.) chagasi*) (WHO, 2010; Kaye and Scott, 2011). CL cases, in the Old World, are related to *Leishmania* subgenus such as *L. (L.) major, L. (L.) tropica* and *L. (L.) aethiopica.* While, in the New World, CL is caused not only by *L. (L.) mexicana, L. (L.) pifanoi, L. (L.) amazonensis* and *L. (L.) infantum* but also by parasites of the subgenus *Viannia* such as *L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) panamensis* and *L. (V.) peruviana*. In addition, ML are caused by *L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) panamensis* and *L. (L.) amazonensis* (WHO, 2010; Kaye and Scott, 2011).

Throughout *Leishmania* spp. life cycle, they develop in different hosts which is directly associated with changes in physicochemical conditions that can be inducers of biochemical and morphological differentiation (Zilberstein and Shapira, 1994; Gomes et al., 2017). These parasites have several developmental stages showing two well-known morphological forms: amastigotes, found in the mammalian hosts; and promastigotes, found in the insect vectors (Bates and Rogers, 2004; Gossage et al., 2003). In the digestive tract of sand flies (26°C), amastigotes, ingested with a blood meal, transform into procyclic promastigotes (Dostálová and Volf, 2012). Then, parasites go through replication and developmental stages including nectomonad, leptomonad and haptomonad (Gossage et al., 2003; Dostálová and Volf, 2012). Promastigotes become non-replicative and infective in metacyclic stage that are able to infect mammalian host cells (Sacks and Perkins, 1984; Gossage et al., 2003). In the vertebrate hosts, parasites are phagocytized by macrophages and other mononuclear phagocytes, and delivered to the phagolysosome (Handman and Bullen 2002). The new physicochemical conditions, where they differentiate into the amastigote forms, are acidic pH (4.5 - 5.5) and higher temperature (32°C - 37°C) (Gossage et al., 2003; Dostálová and Volf, 2012).

Currently, both parasite forms can be maintained *in vitro*, allowing their analysis under controlled conditions. Several culture media are available for *in vitro* growth of promastigotes including Medium 199 (M199), Roswell Park Memorial Institute (RPMI), Grace's insect, Schneider's insect and Novy-MacNeal-Nicolle (NNN) (Santarém et al., 2014; Siripattanapipong et al., 2019). The studies done with amastigotes are performed by obtaining these forms from infected tissues (Pescher et al., 2011), or *in vitro* infected cells, such as murine macrophage cultures (Jara et al., 2017), immortalized cell lines including RAW 264.7, THP-1 and J774 macrophages (Carrió et al., 2000; Rochette et al., 2009; Sánchez-Moreno et al., 2012), fibroblasts (Schwartzman and Pearson, 1985) and dendritic cells (Vargas-Inchaustegui et al., 2008). Amastigote forms can also be obtained by inducing *in vitro* differentiation of promastigotes into axenic amastigotes (Eperon and Mcmahon-Pratt 1989; Bates et al., 1992; Hermoso et al., 1994; Cysne-Finkelstein et al., 1998; Zilberstein and Koren, 2019; Gupta et al., 2001).

This review discusses the advantages and fragilities of using axenic amastigotes, also known as amastigote-like, as a suitable model for *in vitro* studies. Data gathered here provides a critical summary of successful *in vitro* culture strategies to obtain axenic amastigotes, its similarities in gene expression and physiology when compared to the intracellular amastigote forms, and its application in drug tests.

2. Induction strategies of in vitro differentiation

Since the 1980s, several methods for differentiation of promastigotes into axenic amastigotes have been proposed for both subgenera, *Leishmania* and *Viannia*. Currently, at least 12 *Leishmania* spp. have shown sensibility to *in vitro* amastigogenesis (Eperon and Mcmahon-Pratt 1989; Bates et al., 1992; Rainey et al., 1991; Taylor and Williams, 1991; Hermoso et al., 1994; Cysne-Finkelstein et al., 1998; Puentes et al., 2000; Rochette et al., 2009; Cao et al., 2012; Chanmol et al., 2019; Zilberstein and Koren, 2019; Gupta et al., 2001). Among these, *L. (V.) braziliensis* is known for its phenotypic plasticity, producing distinct clinical forms (Cupolillo et al., 2003). Moreover, this parasite shows a low rate of *in vitro* replication, so it represents a challenge for studies (Niño and Camacho 2005). Despite the existence of few studies with this species, there is a great diversity of protocols with at least 12 induction methods proposed (Table 1). These data shows differences in temperature and pH of differentiation culture media, and, in some protocols, cultures are under CO_2 tension (Table 1). It is necessary to highlight that these values varied even among studies using the same strain of *L. (V.) braziliensis* (Sánchez-Moreno et al., 2012; Duarte et al., 2015; Jara et al., 2017; Diaz et al., 2018; Herrera et al., 2020).

In general, these protocols with *L. (L.) braziliensis* apply distinct conditions of temperature and pH. Specifically, in the case of pH, the values varied from 5.0 to 6.4, with a predominance of 5.4 and 5.5. In the case of temperature, the values varied from 28 °C to 37 °C. Only two of these protocols used a gradual temperature change and another maintains constant temperature while increasing pH to increase it later (Eperon and Mcmahon-Pratt, 1989; Balanco et al., 1998; Gomes et al., 2017). Concerning to pH effect in the differentiation culture media, in some protocols of *L. (L.) braziliensis*, pH values have not been described, which could indicate that induction basically depended on temperature changes (Eperon and Mcmahon-Pratt, 1989; Duarte et al., 2015; Diaz et al., 2018). The influence of pH and temperature changes on gene expression during amastigote differentiation has already been observed in *L. (L.) infantum* genomic microarrays (Alcolea et al., 2010). Parasites subjected to only temperature changes have a similar gene expression profile compared to induction that combines pH and temperature changes. However, these conditions present differences in A2 gene expression and presence of the promastigote-specific protein glycoprotein 46, indicating the importance of combining these both factors for a complete amastigote differentiation (Alcolea et al., 2010).

Another important point is that only two studies with *L. (V.) braziliensis* used 5% CO_2 tension (Vargas-Inchaustegui et al., 2008; Diaz et al., 2018). The CO_2 environment has been explored mainly in visceral rather than cutaneous species (Zilberstein, 2020). In addition, there are wide variations in the culture media used and in induction conditions. For instance, the growth phases and number of promastigotes as well as time periods to obtain axenic amastigotes (Table 1). These variations in culture conditions for *L. (V.) braziliensis*, as well as for other species, impair parallel comparisons between results (Zilberstein and Koren, 2019).

The *in vitro* differentiation strategy must simulate the sequence of natural events experienced during the parasite life cycle, when it circulates between the phlebotomine to the mammalian host. The environmental changes act as signals, interfering in the pattern of gene expression that allows differentiation of the parasites (Gomes et al., 2017). In this context, it has been assessed whether *in vitro* changes in pH and temperature are capable of triggering responses similar to what happens during host-parasite interactions.

3. Metabolic and regulatory networks in axenic amastigotes

The parasite's responses to different environments are governed by modulation of gene expression (Campbell et al., 2003). The transcription initiation of trypanosomatids presents specificities since they do not have promoter-mediated regulation; thus, clusters of genes undergo polycistronic transcription (Campbell et al., 2003; Clayton, 2016; Iantorno et al., 2017). The transcript levels are related to differential RNA stability generated by post transcriptional and processing events (Clayton, 2016; Cohen-Freue et al., 2007). Interestingly, increased expression of genes involved in drug resistance are correlated with gene copy number variation and aneuploidy (Ubeda et al., 2008; Iantorno et al., 2017). Although other organisms also present differences in mRNA and proteins levels, these *Leishmania* spp. transcription process features makes proteomic and metabolomic analyzes essential to

Table 1
Methods for *in vitro* differentiation of *Leishmania (Viannia) braziliensis* promastigotes into axenic amastigotes.

Maintenance of promastigotes			Amastigogenesis				Characterization criteria	Reference
Conditons	pH	Temperature	Differentiation conditions	pH	Temperature	CO_2 tension		
MHOM/PE/76/SL2 strain were maintained in Schneider's *Drosophila* medium containing 20% FCS.	ND	24°C	Parasites (2×10^5 - 2×10^6/mL) in Schneider's *Drosophila* medium raising the temperature slowly by 2 °C increments.	ND	26°C and 28°C	ND	OMA and I	Eperon and Mcmahon-Pratt 1989
MHOM/BR/75/M2903 strain were maintained in DL-15 medium (1:1 mixture of Dulbecco's modified Eagle's medium and Leibovitz L-15 medium) containing 10% FCS, 25 ug/ml gentamicin sulfate, 20 ug/ml haemin, and 15 mm Hepes.	7.2	22°C	Parasites (2×10^6/mL) from stationary-phase in modified UM-54 medium were incubated at 28°C for 1 week and then raising the temperature slowly. Amastigotes were serially maintained at 34°C.	6.3	28°C, 30°C, 31°C and 32°C	ND	OMA, I and WBA	Balanco et al., 1998
MHOM/BR/3456 strain were maintained in Schneider's *Drosophila* medium containing 20% FCS.	7.2	22°C	Parasites (5×10^6/mL) in Schneider's *Drosophila* medium containing 20% FCS for 3 days.	5.4	34°C	ND	OMA, I and WBA	Teixeira et al., 2002
LC1418 strain were maintained in Schneider's *Drosophila* medium containing 20% FBS, 2 mM L-glutamine and 50 µg/ml gentamicin.	7.0	23 °C	Parasites in Grace's insect cell culture medium containing 20% FBS.	5.0	33°C	5%	OMA	Vargas-Inchaustegui et al., 2008
MHOM/BR/1975/M2904 strain were maintained in trypanosomes liquid medium containing 10% FCS.	ND	28°C	Parasites in M199 medium containing 10% FCS, 1 g/L b-alanine, 100 mg/L L-asparagine, 200 mg/L sucrose, 50 mg/L sodium pyruvate, 320 mg/L malic acid, 40 mg/L fumaric acid, 70 mg/L succinic acid, 200 mg/L a-ketoglutaric acid, 300 mg/L citric acid, 1.1 g/L sodium bicarbonate, 5 g/L 2-(Nmorpholino) ethanesulfonic acid, 0.4 mg/L haemin and 10 mg/L gentamicin for 3 days.	5.4	37 °C	ND	OMA	Sánchez-Moreno et al., 2012
Clinical isolate from the Peruvian Amazon region were maintained in M199 medium containing 20% FBS, 20 mM HEPES, 6 µg/mL hemin, 10 µg/mL folic acid, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 100 uM adenosine.	ND	26°C	Parasites (5×10^7/mL) from day 5 cultivation in RPMI-1640 medium containing 1% D-glucose, 20 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 25 mM MES for 1 day.	5,5	34 °C	ND	OMA	Lambertz et al., 2015
MHOM/BR/1975/M2904 strain were maintained in Schneider's *Drosophila* medium containing 10% FBS, 20 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL of streptomycin.	7.4	24°C	Parasites (1×10^9 promastigotes) from stationary-phase in 5 mL of FBS for 2 days.	ND	37 °C	ND	OMA and I	Duarte et al., 2015
MHOM/BR/1975/M2904 strain was maintained in M199 medium containing 20% FBS, 100 units/mL of penicillin and 100 µg/mL of streptomycin.	7.2	25°C	Parasites (1.5×10^6/mL) from day 4 cultivation in M199 for 4 days.	5.5	34 °C	ND	OMA	Jara et al., 2017
MCAN/BR/1998/R619 strain was maintained in Schneider's *Drosophila* medium containing 10% FCS, 200 IU penicillin, 200 mg/mL, streptomycin and 2% urine.	7.2	26 °C	Parasites (1×10^6/mL) from logarithmic-phase in Schneider's *Drosophila* medium containing 10% FCS, 200 IU penicillin, 200/ml streptomycin and 2% urine incubated at 26 °C for minimum 4 days. When parasites reached the stationary growth phase, a suspension of 1×10^6/mL was transferred to Schneider's insect medium containing 20% FCS, 200 IU penicillin, 200 mg/mL streptomycin and 2% of urine and incubated at 32 °C for 4 days.	5.5	26 °C and 32°C	ND	OMA and UA	Gomes et al., 2017
MHOM/BR/75/M2904 strain were maintained in Schneider's	ND	26°C		ND	35 °C	5%	OMA	Diaz et al., 2018

(*continued on next page*)

Table 1 (*continued*)

Maintenance of promastigotes			Amastigogenesis				Characterization criteria	Reference
Conditons	pH	Temperature	Differentiation conditions	pH	Temperature	CO_2 tension		
Drosophila medium containing 20% FCS and 0.1 μg/ml 6-biopterin.			Parasites from logarithmic-phase in Schneider's *Drosophila* medium for 2 days.					
MHOM/BR/75/M2903 strain were isolated from footpad lesions in BalbC mice previously infected. For culture maintenance was used medium LIT (tryptose 15 g/L, yeast extract 5 g/L, liver extract 2 g/L, hemin–NaOH 0.02 g/L, glucose 4 g/L, NaCl 9 g/L, KCl 0.4 g/L, Na2HPO4 7.5 g/L) containing 10% FBS.	7.4	29°C	Parasites in M199 medium containing 10% FCS, 1 g/L b-alanine, 100 mg/L L-asparagine, 200 mg/L sucrose, 50 mg/L sodium pyruvate, 320 mg/L malic acid, 40 mg/L fumaric acid, 70 mg/L succinic acid, 200 mg/L a-ketoglutaric acid, 300 mg/L citric acid, 1.1 g/L sodium bicarbonate, 5 g/L 2-(Nmorpholino) ethanesulfonic acid, 0.4 mg/L haemin and 10 mg/L gentamicin for 3 days.	5.5	35 °C	ND	OMA	Romero et al., 2018
MHOM/BR/75/M2904 strain were maintained in Schneider's *Drosophila* medium containing 10% FBS.	ND	27°C	Parasites in the medium RPMI-1640 medium containing 10% FBS.	6.4	37 °C	ND	OMA	Herrera et al., 2020

Not described (ND); Fetal Calf Serum (FCS); Fetal Bovine Serum (FBS); Optical microscopy analysis (OMA); Ultrastructural analysis (UA); Infectivity (I); Western blotting assay (WBA); Molecular analysis (MA).

elucidate details of the parasite's life cycle (Cohen-Freue et al., 2007). Therefore, recent studies using omics technologies have explored the information of these parasites genomes by describing their gene transcription profiles (transcriptomics), protein expression and post-translation modifications (proteomics), as well as revealing endogenous metabolites that are part of important cellular processes (metabolomics).

Omics technologies have been explored to compare transcriptome from promastigotes, intracellular, lesion-derived and axenic amastigotes, allowing the detection of similarities and differences between them. It is necessary to highlight that, in several studies, there is no direct comparison between axenic and intracellular or lesion-derived amastigotes. From 19 studies, only six compared two amastigote models (Table 2). In addition, promastigotes were obtained in different growth phases, which may have implications in gene products (Table 2). Metacyclogenesis promotes morphological and biochemical changes that have been studied *in vitro* (Sacks and Perkins, 1984; Sacks et al., 1995; Sádlová et al., 2010; Gomes et al., 2017). In axenic cultures, the number of metacyclic parasites varies from logarithmic to stationary growth phase (Sacks and Perkins, 1984; da Silva et al., 2015). Thus, gene expression profile can change depending on the growth phase used. In fact, omic studies showed differences in gene regulation and protein abundance as well as in protein phosphorylation depending on the growth phase of promastigotes when compared to amastigotes (Almeida et al., 2004; Nugent et al., 2004; Tsigankov et al., 2014).

3.1. Parasite transcriptomic evaluation

The shotgun DNA microarray expression of lesion-derived amastigotes and logarithmic promastigotes of *L. (L.) major* showed a difference of 3.1% in transcript abundance of these forms (Akopyants et al., 2004). The genomic microarray of promastigotes and axenic amastigotes of *L. (L.) donovani* presented about 3% of variation in gene expression, with overexpression of genes encoding for HSP83 and amastin (Srividya et al., 2007). Similarly, microarray-based expression of *L. (L.) donovani* showed ~5.5% genes were regulated and ~3.5% underwent transient changes during the transition from promastigotes to axenic amastigotes (Saxena et al., 2007). Among the up-regulated genes are small hydrophilic ER-associated protein (SHERP), HSP83 and tryparedoxin (TXN), proteins related to stress response and metabolism (Figure 1). Meanwhile, the down-regulated set of genes encoded for paraflagellar rod protein 1D, actin and β-tubulin, related to cell growth and motility (Saxena et al., 2007).

A comparative profile of gene expression of cDNAs and open reading frames from *L (L.) major* lesion-derived amastigotes and procylic promastigotes showed a higher percentage, ~35%, of differentially expressed genes while between amastigotes and metacyclics was ~20% (Almeida et al., 2004). Even using the same species, two studies comparing *L (L.) major* transcriptome of metacyclic promastigotes and lesion-derived amastigotes found difference in the percentage of regulated genes, Almeida et al. (2004) described 20% while Akopyants et al. (2004) reported 0.6 – 3.1%. This difference might be due to the strain used (LV39 vs. Friedlin strain), statistical cut-offs and cluster analysis to determine significance.

Additionally, *L. (L.) mexicana* transcriptome analysis by *L. (L.) major* oligonucleotide whole-genome microarray design showed differences of 2.9% between promastigotes and lesion-derived amastigotes and 0.8% between promastigotes and axenic amastigotes (Holzer et al., 2006). Comparison revealed up-regulation in lesion-derived and axenic amastigotes compared to promastigotes of genes related to proteolysis processes such as calpain-like cysteine protease, and resistance responses such as sodium stibogluconate resistance protein resistance (Holzer et al., 2006) (Fig. 1). Of 175 genes statistically different between the axenic and lesion-derived amastigotes, 126 were highly expressed in axenic amastigotes (Holzer et al., 2006). An interesting gene found in the intracellular forms was fatty acid elongase-like which is related to metabolism since, during differentiation, parasites change their carbon source from glucose and proline to beta-oxidation of fatty acids (Holzer et al., 2006) (Fig. 1).

While an assay using Illumina sequencing to select and compare RNA from promastigotes and intracellular amastigotes of *L. (L.) mexicana* found 2176 differentially expressed genes. Around 41% were different transcripts between promastigotes and intracelular amastigotes while 13% between axenic and intracellular amastigotes (Fiebig et al., 2015). Among the 2-fold up-regulated genes in the latter forms they found amino acid transporters, TXN and aquaglyceroporin1 (AQP1) (Fig. 1); the latter gene is related to protection of amastigotes, influx of antimony and greater susceptibility to these drugs (Figarella et al., 2007). It is important to highlight that greater variation between axenic and intracellular amastigotes was observed in studies where differences between promastigotes and amastigotes were also greater (Holzer et al., 2006; Fiebig et al., 2015). In fact, transcriptomic studies showed

Table 2
Omics studies comparing promastigotes with intracellular, lesion-derived and/or axenic amastigotes of *Leishmania* spp.

Omics approach	Species and strain	Parasite stages	Method	Findings	Highlights	Reference
Genomic	*L. (L.) major* (MHOM/JL/80/ Friedlin)	**P:** Procyclic and metacyclic **LA:** Mice lesion-derived amastigotes	Genomic microarrays for *L. (L.) major* with 11484 probes	Difference between lesion amastigotes and logarithmic promastigotes: 3.1% of the genes in ≥ 1 experiment and 0.6% in 3 experiments Difference between metacyclic and logarithmic promastigotes: 3.2% of the genes in ≥ 1 experiment and 0.5% in 3 experiments	NH	Akopyants et al., 2004
	L. (L.) donovani (K59)	**P:** Not described **DS:** 24h during differentiation **AA**	Genomic microarrays for *L. (L.) donovani* with 4224 probes	Difference between 24h diferential stage and promastigotes: ~1% of the genes were upregulated with expression ≥ 2.0-fold Difference between amastigote and promastigote: ~3% of the genes were upregulated with expression ≥ 2.0-fold	HSP83 and amastin were up-regulated in AA compared to P	Srividya et al., 2007
	L. (L.) donovani (MHOM/SD/00/ 1SR)	**P:** Late-logarithmic phase DS: 5, 10, 24h during differentiation **AA**	Genomic microarrays for *L. (L..) major* with 10468 probes	Difference between amastigote and promastigote: ~3% of the genes were up-regulated in amastigotes, and 2.5% down-regulated Transient changes of ~3.5% of the genes during differentiation	SHERP, HSP83 and TXN were up-regulated in AA compared to P	Saxena et al., 2007
	L. (L.) major (RHO/SU/59/P/ LV39)	**P:** 3, 7, 10 days of culture **LA:** Mice lesion-derived amastigotes	cDNA microarrays of *L. (L.) major* with 1830 probes	Difference between amastigote and procylic promastigote: ~35% of the genes were regulated Difference between amastigote and metacyclic promastigote: ~20% of the genes were regulated	NH	Almeida et al., 2004
	L. (L.) mexicana (WHO strain MNYC/BZ/62/ m379)	**P:** Logarithmic phase **LA:** Mice lesion-derived amastigotes **AA**	Genomic microarrays for *L. (L..) major* with 8156 probes	Difference between promastigote and lesion amastigote: 2.9% of the genes were regulated with expression ≥ 2.0-fold Difference between promastigote and axenic amastigote: 0.8% of the genes were regulated with expression ≥ 2.0-fold Difference between axenic amastigote and lesion amastigote: 0.9% of the genes were regulated with expression ≥ 2.0-fold	Calpain-like cysteine protease and sodium stibogluconate resistance protein were up-regulated in LA and AA compared to P Fatty acid elongase-like was up-regulated in LA compared to AA	Holzer et al., 2006
	L. (L.) mexicana (MNYC/BZ/62/ M379)	**P:** Late exponential growth phase **IA:** *Leishmania*-infected bone marrow-derived macrophages **AA**	Illumina sequencing of poly-A selected RNA	Difference between promastigotes and intracellular amastigotes: 41% of the genes were regulated Difference between axenic and intracellular amastigotes: 13% of the genes were regulated	Among the 2-fold up-regulated genes in the IA are TXN and aquaglyceroporin1 (AQP1)	Fiebig et al., 2015
	L. (L.) infantum (MOHM/MA/ 67/ITMAP-263)	**P:** Not described **IA:** *Leishmania*-infected THP-1 cell **AA**	Genomic microarrays for *L. (L..) major* and *L. (L.) infantum* with 8978 probes	Difference between intracellular amastigotes compared to promastigotes: 3.8% of the genes were up-regulated in amastigotes, and 3.3% down-regulated Difference between axenic amastigotes compared to promastigotes: 6.3% of the genes were up-regulated in amastigotes, and 6.2% down-regulated	NH	Rochette et al., 2009
Proteomic	*L. (L.) donovani* (Lo8)	**P:** 72h of culture **AA**	2-DE and MS	Proteins (n=49) were identified from spots differentially expressed	TIM, Prx and HSP60 were up-regulated in AA compared to P	Bente et al., 2003
	L. (L.) infantum (MHOM/MA/ 67/ITMAP-263)	**P:** Mid-logarithmic phase **AA**	2-DE and MS	Proteins (n=2) were identified from spots differentially expressed	TIM and IDH were up-regulated in AA compared to P	Fakhry et al., 2002
	L. (L.) infantum (MHOM/MA/ 67/ITMAP-263)	**P:** Late-logarithmic phase **AA**	2-DE and MS followed by microarray analyses of genomic sequences corresponding to the proteins identified	Proteins (n=71) were identified from spots differentially expressed Amastigote-specific protein and mRNA expression correlated 53%	TIM, Prx and HSP60 were up-regulated in AA compared to P	McNicoll et al., 2006

(*continued on next page*)

Table 2 (*continued*)

Omics approach	Species and strain	Parasite stages	Method	Findings	Highlights	Reference
	L. (L.) mexicana (MNYC/BZ/62/M379)	**P:** Procyclic and metacyclic **AA**	2-DE and MS	Proteins (n=47) were identified from spots differentially expressed	TIM, IDH, Prx, HSP60, TXN, TXNPx and CPB were up-regulated in AA compared to P	Nugent et al., 2004
	L. (V.) panamensis (MHCOM/CO/86/1166)	**P:** Logarithmic phase **AA**	2-DE and MS	Proteins (n=12) were identified from spots differentially expressed	PrX, HSP60 and HSP83 are up-regulated in AA compared to P	Walker et al., 2006
	L. (L.) donovani (MHOM/SD/62/1S-CL2D)	**LA:** Hamster spleens lesion-derived amastigotes **AA**	2-DE and MS	Proteins (n=10) were identified from spots differentially expressed	NH	Pescher et al., 2011
	L. (L.) donovani (MHOM/SD/62/1S-CL2D)	**P:** Logarithmic phase **AA**	2-DE and MS	Phosphoproteins (n=73) purified by immobilized metal affinity chromatography were identified	Cyclo40 and MAP kinase were expressed in both stages but phosphorylated mostly at the amastigote stage	Morales et al., 2008
	L. (L.) donovani (MHOM/SD/62/1S-CL2D)	**P:** Not described **AA**	2-DE and MS	Difference of 38% of the phosphoproteome	HSP90, HSP70 and Cyclo40 were phosphorylated during differentiation	Morales et al., 2010
	L. (L..) donovani (1SR)	**P:** Mid-logarithmic phase **DS:** 5, 10, 24 h during differentiation **AA**	MS using iTRAQ-labeled peptides	Difference in 163 phosphopeptides (from 106 proteins) during differentiation	NH	Tsigankov et al., 2014
	L. (L.) infantum (MHOM/BR/1972/BH46 and MCAN/BR/2000/BH400)	**P:** Not described **LA:** Hamster spleens lesion-derived amastigotes	Two peptide labeling techniques (DIGE and TMT) and MS	Differentially abundant proteins (n=165) were identified	NH	Junior et al., 2021
Metabolomic	*L. (L.) mexicana* (MNYC/BZ/62/M379)	**P:** Mid-logarithmic and stationary growth phases **LA:** Mice lesion-derived amastigotes **AA**	^{13}C-Nuclear magnetic resonance spectroscopy	Both amastigotes showed similar metabolic profile compared with promastigote	Decrease in the uptake and utilization of glucose and amino acids and increased fatty acid β-oxidation in amastigotes	Saunders et al., 2014
	L. (V.) braziliensis (MHOM/BR/75/M2904 and MHOM/PE/03/PER206)	**P:** Logarithmic and stationary **IA:** *Leishmania*-infected mice peritoneal macrophages **AA**	MS	Both amastigotes showed the same metabolic profile compared with promastigote	Lower capacity of biosynthesis, a reduced bio-energetic level compared to promastigotes	Jara et al., 2017

Promastigote (P); Differentiation stage (DS); Axenic Amastigote (AA); Intracellular amastigote (IA); Lesion-derived amastigote (LA); Two-dimensional gel electrophoresis (2-DE); Mass spectrometry (MS); differential gel electrophoresis (DIGE); tandem mass tag (TMT); Not highlighted (NH); Small hydrophilic ER-associated protein (SHERP); Tryparedoxin (TXN); Triosephosphate isomerase (TIM); Peroxiredoxin (Prx); Isocitrate dehydrogenase (IDH); Tryparedoxin peroxidase (TXNPx); Cysteine protease B (CPB); Cyclophylin 40 (Cyclo40); Mitogen activated protein kinases (MAPK).

differences related to gene products between intracellular and axenic amastigotes. These differences may be related to *(i) Leishmania* spp. used; *(ii)* differences in promastigotes developmental stages (procyclic or metacyclic) and in protocols to obtain axenic amastigotes; *(iii)* omic technologies and statistical criteria used to evaluate differential gene products (Saxena et al., 2007).

Furthermore, a study with *L. (L.) infantum* showed that, from a total 1022 differentially expressed genes, 12% of upregulated transcripts were common to both axenic and intracellular amastigotes (Rochette et al., 2009). These common upregulated genes were related to metabolic processes, such as trypanothione synthetase (TryS); membrane/surface proteins, such as small hydrophilic ER-associated protein (SHERP) and hydrophilic acylated surface protein b (HASPB); amastins and the A2 gene (Fig. 1). Interestingly, SHERP and HASPB are expressed in the plasma membrane of infective stages, metacyclic promastigotes and amastigotes, which was validated mainly for Old World species and *L. (V.) braziliensis* (Sádlová et al., 2010).

The A2 gene family, as well as amastins, have been studied as biomarkers (Zhang et al., 1996; Nasereddin et al., 2010). The characterization of axenic amastigotes of *L. (L.) tropica* showed expression of amastins in axenic and intracellular amastigotes but not in promastigotes (Nasereddin et al., 2010). Therefore, differential expression of these genes could be used as stage specific markers to assess differentiation or for immunodiagnostics assays (Dayakar et al., 2012). These assays using axenic amastigotes to detect proteins, i.e. A2 and amastin, can be employed for serodiagnosis of leishmaniasis (Kaul et al., 2000; Salotra et al., 2002; Duarte et al., 2015). In fact, the omic studies of *L. (L.) infantum* demonstrate that these two gene families are common for axenic and intracellular amastigotes (Fig. 1). Besides being biomarkers of the amastigote stage, the A2 gene family are related to VL manifestation caused by *L. (L.) infantum* (Zhang and Matlashewski, 2001).

3.2. Parasite proteomics and metabolomics evaluation

The first studies regarding stage-specific proteins abundance used 2-dimensional gel electrophoresis spot identification, which has limitations since it does not constitute a large-scale analysis to separate and quantify *Leishmania* spp. proteins during differentiation (Bente et al., 2003; Fakhry et al., 2002; McNicoll et al., 2006; Nugent et al., 2004; Walker et al., 2006). A study with *L. (L.) infantum* revealed two proteins related to energy metabolism, triosephosphate isomerase (TIM) and isocitrate dehydrogenase (IDH), significantly more abundant in axenic amastigotes than promastigotes (Fakhry et al., 2002). TIM is essential in the glycolytic pathway within the *Leishmania* spp. glycosome and IDH is part of the tricarboxylic acid pathway, both pathways generate ATP, which efflux during infection is crucial for parasite survival and escape of the host macrophage immune defense (Basu et al., 2020). Similarly, in *L. (L.) donovani* axenic amastigotes, TIM was up-regulated when compared to promastigotes along with peroxiredoxin (Prx) and HSP60, which are proteins related to stress response (Bente et al., 2003), and this was also the case for *L. (L.) infantum* axenic amastigotes (McNicoll

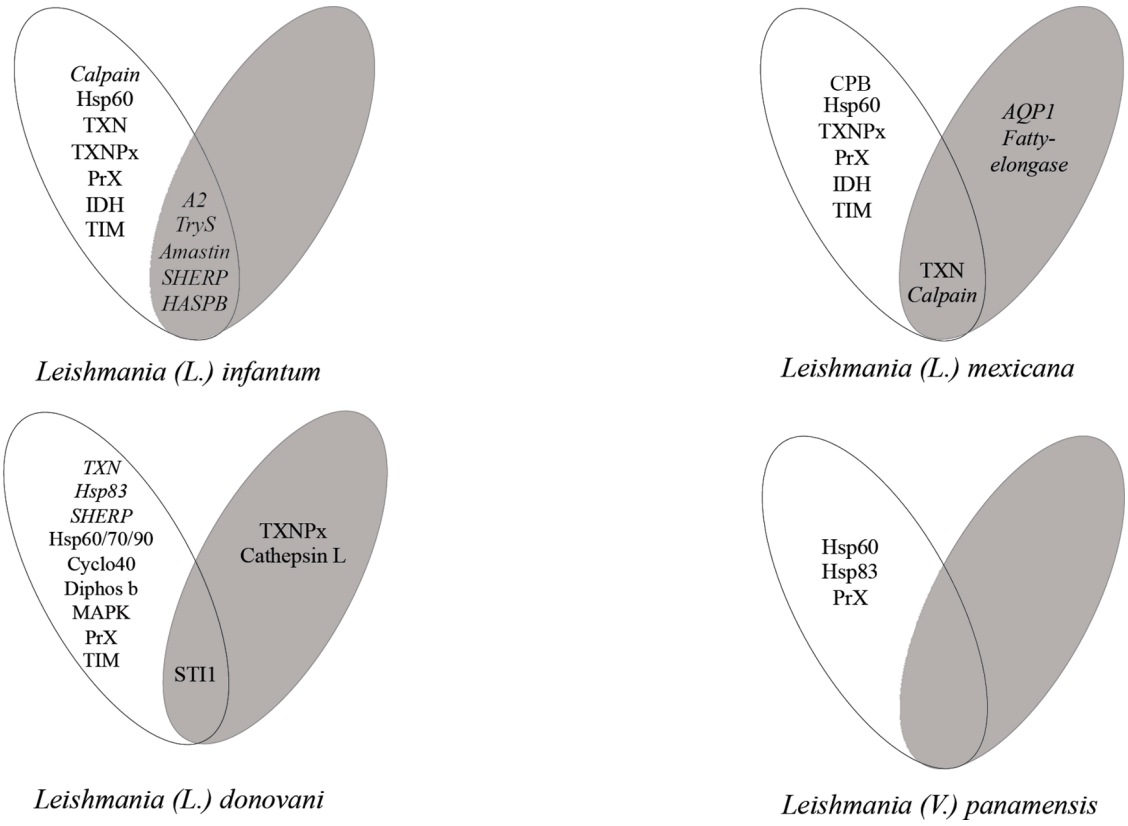

Fig. 1. Venn diagrams of relationships between axenic and intracellular amastigotes of *Leishmania* spp. reported through omics approaches. The stage-specific or up-regulated transcripts, proteins, and phosphorylated proteins are represented for each condition: axenic amastigotes (white ellipse) and intracellular amastigotes (grey ellipse). Genes identified by transcriptomics (italic fonts), and proteins identified by proteomics and/or phosphoproteomics (non-italic fonts) are presented. Genes and proteins: Amastin, Calpain, Cathepsin L, Trypanothione synthethase (TryS); Small hydrophilic ER-associated protein (SHERP), Hydrophilic acylated surface protein b (HASPB), Isocitrate dehydrogenase (IDH), Tryparedoxin peroxidase (TXNPx), Tryparedoxin (TXN), Cysteine protease B (CPB), Triosephosphate isomerase (TIM), Heat shock protein (HSP), Cyclophilin 40 (Cyclo40), Mitogen activated protein kinases (MAPK), Peroxiredoxin (PrX), and Aquaglycoporin 1 (AQP1).

et al., 2006) (Fig. 1). It was observed a correlation of only 53% between amastigote-specific protein and mRNA expression of *L. (L.) infantum* (McNicoll et al., 2006). Transcriptome studies demonstrated that a limited number of genes presented differential expression during life cycle of these parasites, therefore, the regulation at the translation and post-translational levels are important to understand the biological processes involved in amastigogenesis (McNicoll et al., 2006).

Additionally, another proteomic study with *L. (L.) mexicana* found not only TIM, IDH, Prx and HSP60 but also tryparedoxin (TXN), tryparedoxin peroxidase (TXNPx) and cysteine protease B (CPB) up-regulated in the axenic amastigote forms (Nugent et al., 2004). Peroxiredoxin, TXN and TXNPx are part of the trypanothione system, a trypanosomatids unique detoxification and defense system against macrophage' reactive oxygen species and stress (induced by pH and temperature shock) (Krauth-Siegel et al., 2003). Proteomic analysis of amastigote and promastigote forms of *L. (L.) infantum* corroborated previous studies on the main biological processes regulated during differentiation. This study showed changes in proteins related to metabolic process, stress response as well as transport and cytoskeleton organization (Junior et al., 2021). In addition, phosphoproteomic comparison between *L. (L.) donovani* axenic amastigotes and splenic amastigotes from infected hamsters revealed that these forms shared 93% of abundant proteins (Pescher et al., 2011). Interestingly, among the differential proteins are cytosolic TXNPx, a vital enzyme for survival and detoxification of peroxides within macrophages (Krauth-Siegel et al., 2003; Iyer et al., 2008), and cathepsin L-like protease a virulence factor vital for differentiation and virulence in other *Leishmania* spp. (Williams et al., 2006; Gomes et al., 2017). Similarly, the first proteomic comparison done in *L. (V.) panamensis* found seven distinct proteins more abundant in axenic amastigotes which were also related to energy metabolism and heat/stress response, including PrX, HSP60 and HSP83 (Walker et al., 2006) (Fig. 1).

Studies evaluated changes in the phosphoproteome from *L. (L.) donovani* promastigotes versus axenic amastigotes and revealed 73 novel proteins in both stages but with more phosphorylation in the latter forms (Morales et al., 2008). Within the stage-phosphorylated proteins they found cyclophylin 40 (Cyclo40) related to protein folding, and mitogen activated protein kinases (MAPK) which is expressed in both stages but phosphorylated mostly in the axenic amastigote stage (Morales et al., 2008). Similarly, increased phosphorylation of chaperones such as HSP90 and isoforms, HSP70 and Cyclo40 revealed that there are phosphorylation stoichiometry changes during differentiation of *L. (L.) donovani* (Morales et al., 2010) (Figure 1). The phosphoproteome differences at specific times during differentiation revealed higher kinases phosphorylation during the amastigote maturation phase (Tsigankov et al., 2014). This suggests that *Leishmania* spp. response to stress is very-well organized through signals exchange and regulatory mechanisms, and phosphorylation is a stage-specific mechanism rather than constitutive of the differentiation process (Tsigankov et al., 2014). Kinases activity increase while phosphorylation patterns change during differentiation of *L. (L.) major* and *L. (L.) mexicana*, even though the former species differentiates at a much lower rate than the latter (Hermoso et al., 1994).

Lastly, metabolomics studies have been done to understand the different metabolites used by *Leishmania* spp. promastigotes, axenic and/or intracellular amastigotes. These studies have focused in finding differences between these forms regarding carbon sources and metabolism (Kloehn et al., 2016). A metabolomic comparison of *L. (L.)*

mexicana promastigotes versus axenic amastigotes showed a 30-fold decrease in glucose and glutamate uptake by the latter forms (Saunders et al., 2014). Meanwhile, *L. (L.) mexicana* intracellular amastigotes showed a similar phenotype to axenic amastigotes with low glucose and amino acids uptake rates (Saunders et al., 2014). These authors showed that both *L. (L.) mexicana* axenic and intracellular amastigotes use less glucose than promastigotes, also, this "stringent" metabolism is linked to decreased protein and lipid biosynthesis which could be a direct response to differentiation signals. Moreover, a study comparing differential metabolites between *L. (V.) braziliensis* promastigotes and axenic amastigotes identified significantly decreased levels of polyamines, amino acids (Lys, Arg, Val and Gly) and fatty acids in the latter forms (Jara et al., 2017). Since polyamines are related to trypanothione synthesis, its downregulation might be correlated to amastigotes' stress response, while, low amino acids levels are directly related to lower biosynthetic activity and less uptake of amino acids as seen in *L. (L.) mexicana* axenic amastigotes (Jara et al., 2017; Saunders et al., 2014).

4. Axenic amastigotes as suitable model for drug testing

The use of axenic amastigotes as a biological model for primary drug screening in *Leishmania* spp. has been relevant since the 1990s. The first studies tested antifungal compounds such as ketoconazole and terbinafine (Vannier-Santos et al., 1995), organometallic complexes (Mesa-Valle et al., 1996), as well as clinically used compounds for leishmaniasis treatment such as sodium stibogluconate, meglumine antimoniate, pentamidine, paromomycin, amphotericin B (Taylor and Williams, 1991; Callahan et al., 1997). However, one study showed differences in susceptibility to the activity of pentavalent antimony (Sb^V) between axenic and intracellular amastigotes (infecting THP-1 cells) and evidenced the necessity of *in vivo* conversion of Sb^V into trivalent antimony (Sb^{III}), its active form (Sereno et al., 1998). Differences were found in pentamidine IC_{50} values between axenic and intracellular amastigotes infecting THP-1 cells (Sereno et al., 2000). While axenic and intracellular amastigotes infecting RAW 264.7 cells not showed differences in the activity of Sb^V or Glucantime (formulation with meglumine antimoniate) (Carrió et al., 2000). In fact, other studies showed that clinically used compounds, except sodium stibogluconate, present similar effects in axenic and intracellular amastigotes (De Rycker et al., 2013). The evaluation of susceptibility to amphotericin B and miltefosine using axenic amastigotes is widespread since host cellular mechanisms are not involved on the effect of these compounds (Vermeersch et al., 2009).

Several research groups have used axenic amastigotes for drug screening, as summarized in Table 3, which presents a critical analysis of studies that used this form compared to intracellular amastigotes and promastigotes. In general, the use of axenic amastigote presents advantages including *(i)* ease of manipulation; *(ii)* quantification of the drug's effect is fast and inexpensive and *(iii)* decreased use of laboratory animals in cases of experiments using peritoneal murine macrophages or bone marrow-derived cells, as well as amastigotes derived from infected tissues (Sereno et al., 2007; Rodrigues et al., 2015b). Therefore, these assays allows automation and facilitate the evaluation of a large number of compounds, but it encounters several problems including *(i)* compound must be able to cross the host cell membranes to reach the parasitophorous vacuole; *(ii)* culture media used to maintain axenic amastigotes does not replicate the *in vivo* conditions; (*iii*) lack of cross-talk between host-parasite and consequently differences in gene products of axenic and intracellular amastigotes (Callahan et al., 1997; Vermeersch et al., 2009; Rochette et al., 2009; Pescher et al., 2011; De Muylder et al., 2011; De Rycker et al., 2013; Berry et al., 2018). Thus, compounds with host cell-dependent mechanisms of action, as pentavalent antimonials; and that interferes only with host cell metabolism, as naloxonazine, have few or no effects on the axenic forms (De Muylder et al., 2011, 2016).

In addition of drug screening, axenic amastigotes can be useful to improve comprehension of the mechanism of action of new drug candidates (Sereno et al., 1998; Teixeira et al., 2002; Tiuman et al., 2014). Moreover, compounds that have already shown activity in the axenic, but not in intracellular amastigotes, can be evaluated in drug delivery strategies for addressing these compounds to the parasitophorous vacuole while maintaining their effect (De Rycker et al., 2013; Nühs et al., 2015).

Studies that compared axenic and intracellular amastigotes showed that 66% of them not presented similar IC_{50} values (Table 3), including two studies that used a large number of compounds (909-member library of bioactive compounds and 15659 diverse compounds) (De Muylder et al., 2011; De Rycker et al., 2013). Both studies showed a lot of false-negatives results; for example, compounds with action on intracellular amastigotes but that were not selected in tests with axenic amastigotes; as well as, false-positives results, in which compounds did not show activity when tested on intracellular amastigotes (De Muylder et al., 2011; De Rycker et al., 2013). However, among the studies that presented similarities between both amastigote models, one developed a new assay using axenic amastigotes able to predict and identify compounds with leishmanicidal effects on intracellular amastigotes (Nühs et al., 2015). This assay modified experimental conditions including initial cell density, parameters for evaluating drug effect in relation to control, as well as the methodology to count viable cells based on luminescence. It was validated using a panel of 9907 compounds previously tested by the same group (De Rycker et al., 2013; Nühs et al., 2015).

In these studies analyzed, the most used method to calculate IC_{50} values is direct counting using a Neubauer chamber (~38%) (Table 3). The other methods are based in cell dye exclusion test (Trypan Blue and Propidium iodide) or cell metabolism (tetrazolium and resazurin reduction, as well as ATP production). Counting the number of parasites inside macrophages, in intracellular amastigotes assays, demonstrates the cytocidal ability of a compound. While, to only observe non-growth in axenic assays does not mean that this compound will act on the parasite (De Rycker et al., 2012, 2013). Then, to identify new active compounds over axenic amastigotes, the effect observed must to be cytocidal rather than static or growth slowing (De Rycker et al., 2012, 2013; Nühs et al., 2015). The assay that reports only cidal compounds contributes to decrease false-positive rates and allows its use for high-throughput screening (De Rycker et al., 2013; Nühs et al., 2015). However, to achieve these results, is necessary to use a more sensitive methodology to verify cell viability. For instance, the reduction of MTT, MTS and XTT by the parasite cells, into formazan is measured by changes in absorbance using spectrophotometers; resazurin reduction to resorufin is measured by change in fluorescence using a microplate fluorometer (Riss et al., 2016). While methods based on quantitation of ATP, use luminometers and are more sensitive than colorimetric or fluorometric assays (Riss et al., 2016). However, the need for luminometer instruments is a limiting factor in many research laboratories.

5. Remarks and conclusions

The use of promastigotes provides information about the phase of *Leishmania* spp. in the vector insect. Then, biochemical and physiological findings should be evaluated with caution when extrapolated to the amastigote forms. Indubitably, one of the motivations behind the wide use of axenic amastigotes is the advantage regarding the greater number of parasites that can be obtained without the need of *in vitro* or *in vivo* infection assays, which could include the use of laboratory animals. The axenic amastigote model does not reproduce the intracellular environment, therefore, the results obtained with this form will not always coincide with intracellular amastigotes. If this model is the option available to the research group, its use requires several precautions and the results obtained should be discussed within this context.

Throughout this review, it was shown that several research groups obtain axenic amastigotes with protocols adapted in their laboratory,

Table 3
Studies of drug screening with comparison between axenic and intracellular amastigotes.

Compound	Method for assessing drugs effect	Difference between AA and P?	Difference between AA and IA?	Reference
Ketoconazole and terbinafine	• Direct counting by Neubauer chamber: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	No	Vannier-Santos et al., 1995
Six antileishmanial agents	• Direct counting by Neubauer chamber: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	No	Callahan et al., 1997
Meglumine [Sb(V)]	• Detection of viable cells by MTT assay: **P and AA** • Direct counting of infected THP-1 cells: **IA**	Yes	Yes	Sereno et al., 1998
Pentamidine and amphotericin B	• Detection of viable cells by MTT assay: **P and AA** • Direct counting of infected THP-1 cells: **IA**	Yes	Yes	Sereno et al., 2000
Pentavalent antimony	• Detection of viable cells by acid phosphatase activity: **P and AA** • Direct counting of infected RAW 264.7 cells: **IA**	Yes	No	Carrió et al., 2000
Aromatic dications	Detection of viable cells by tetrazolium reduction: **AA** • Direct counting of infected J774.G8 cells: **IA**	Not tested	Yes	Brendle et al., 2002
3-aryl-5-thiocyanatomethyl-1,2,4-oxadiazoles	• Detection of viable cells by tetrazolium reduction: **AA** • Direct counting of infected J774.G8 cells: **IA**	Not tested	Yes	Cottrell et al., 2004
Spirolactone iridoids	Detection of viable cells by MTT assay: **P and AA** Direct counting of infected peritoneal murine macrophage: **IA**	Yes	Yes	(Castillo et al., 2007)
Cisplatin	• Detection of viable cells by flow cytometry analysis: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	No	Tavares et al., 2007
Meglumine antimoniate	• Direct counting by Neubauer chamber: **AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Not tested	Yes	Morais-Teixeira et al., 2008
Copaiba oils	• Direct counting by Neubauer chamber: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	No	Santos et al., 2008
Antileishmanial reference drugs	• Detection of viable cells by resazurin assay: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	Yes	Vermeersch et al., 2009
Essential oil	• Direct counting by Neubauer chamber: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	Yes	Santin et al., 2009
Eupomatenoid-5	• Direct counting by Neubauer chamber: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	Yes	Vendrametto et al., 2010
Imidothiocarbamates and imidoselenocarbamates	• Detection of viable cells by flow cytometry analysis: **P and AA** • Detection of infected THP-1 cells by flow cytometry analysis: **IA**	Yes	Yes	Moreno et al., 2011
Selenocyanates and diselenides	• Detection of viable cells by trypan blue assay: **P and AA** • Detection of infected THP-1 cells by flow cytometry analysis: **IA**	Yes	Yes	Plano et al., 2011
Agaricus blazei Murill water extract	• Detection of viable cells by MTT assay: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	Yes	Valadares et al., 2011
909-member library of bioactive compounds	Detection of viable cells by CellTiter assay: **P and AA** Direct counting of infected THP-1 cells: **IA**	Yes	Yes	De Muylder et al., 2011
Imidazole- or pyrazole-based benzo[g]phthalazine derivatives	• Direct counting by Neubauer chamber: **P and AA**	Yes	Yes	Sánchez-Moreno et al., 2012

(*continued on next page*)

Table 3 (*continued*)

Compound	Method for assessing drugs effect	Difference between AA and P?	Difference between AA and IA?	Reference
	• Direct counting of infected J774.2 cells: **IA**			
4-Acetoxydolastane diterpene	• Direct counting by Neubauer chamber: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	Yes	Dos Santos et al., 2011
Beta-carboline-3-carboxamide derivatives	• Direct counting by Neubauer chamber: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	No	Pedroso et al., 2011
Chromene-2-thione Analogues	• Detection of viable cells by MTT assay: **P and AA** • Direct counting of infected J774A.1 cells: **IA**	Yes	Yes	Verma et al., 2012
Terpenoid derivatives	• Direct counting by Neubauer chamber: **P and AA** • Direct counting of infected J774.2 cells: **IA**	Yes	No	Ramírez-Macías et al., 2012a
Dihydrochalcones and benzoic acid derivatives	• Detection of viable cells by MTT assay: **AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Not tested	Yes	Cabanillas et al., 2012
Flavonoids	• Direct counting by Neubauer chamber: **P and AA** • Direct counting of infected J774.2 cells: **IA**	Yes	Yes	Ramírez-Macías et al., 2012b
Benzaldehyde thiosemicarbazone derived	• Direct counting by Neubauer chamber: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	No	Britta et al., 2012
15,659 diverse compounds	• Detection of viable cells by resazurin assay: **AA** • Detection of Leishmania-GFP in infected THP-1 cells: **IA**	Not tested	Yes	De Rycker et al., 2013
Parthenolide	• Direct counting by Neubauer chamber: **AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Not tested	Yes	Tiuman et al., 2014
N-butyl-[1-(4-methoxy)phenyl-9H-β-carboline]-3-carboxamide	• Direct counting by Neubauer chamber: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	No	Stefanello et al., 2014
4-Nitrobenzaldehydethiosemicarbazone	• Direct counting by Neubauer chamber: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	No	Britta et al., 2014
Essential oil	• Detection of viable cells by MTT assay: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	No	Rodrigues et al., 2015a
Strychnobiflavone flavonoid	• Detection of viable cells by MTT assay: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	Yes	Lage et al., 2015
~10,000 DOS compounds	• Detection of viable cells by BacTiter-Glo assay: **AA** • Detection of Leishmania-GFP in infected THP-1 cells: **IA**	Not tested	No	Nühs et al., 2015
2-Amino-thiophene derivatives	• Detection of viable cells by MTT assay: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	Yes	Rodrigues et al., 2015b
Piperolactam A	• Direct counting by Neubauer chamber: **AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Not tested	No	Bhattacharya et al., 2016
Naloxonazine	• No described: **P and AA** • Detection of Leishmania-GFP in infected THP-1 cells: **IA**	Yes	Yes	De Muylder et al., 2016
Naphthoquinone derivate	• Detection of viable cells by MTT assay: **P and AA** • Direct counting of infected peritoneal murine macrophage: **IA**	Yes	No	Mendonça et al., 2018
Compounds active against kinetoplastids	• Detection of parasites expressing NanoLuc-PEST enzyme: **AA and IA**	Not tested	Yes	Berry et al., 2018

(*continued on next page*)

Table 3 (*continued*)

Compound	Method for assessing drugs effect	Difference between AA and P?	Difference between AA and IA?	Reference
8-Aryl-6-chloro-3-nitro-2-(phenylsulfonylmethyl) imidazo[1,2-a]pyridines	• Detection of viable cells by MTT assay: **P** • Detection of viable cells by luciferase assay: **AA** • Direct counting of infected THP-1 cells: **IA**	Yes	Yes	Fersing et al., 2018
2-Aryl-quinazolin-4(3H)-ones	• No described: **P, AA, IA**	Yes	Yes	Romero et al., 2019
Methyl gallate	• Detection of viable cells by MTT assay: **P and AA** • Direct counting of infected J774A.1 cells: **IA**	No	Yes	Dias et al., 2020

AA: axenic amastigotes; IA: intracellular amastigotes; P: promastigotes; MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide).

which was exemplified here for *L. (V.) braziliensis* (Table 1). Variations in the amastigogenesis conditions impairs a critical comparison between the results obtained. In general, such protocols follow the minimum conditions expected for amastigogenesis in the intracellular environment, such as temperature changes. However, in several cases, a set of other physicochemical and nutritional factors, that are essential to the amastigote form, are not analyzed. It is important to bear in mind that inconsistencies related to different protocols can possibly induce a stimulus that is not experienced during *in vivo* amastigogenesis, causing distinct gene and metabolic expression. Despite the difficulty of applying a standard protocol, the goal should be a consensus to support fundamental steps for induction methods, which must follow the logic behind parasite' experiences between its environments (invertebrate and vertebrate hosts).

In general, the studies characterize the axenic amastigotes through morphology, looking for parasite roundness with absence of an apparent flagellum, often using them at different times of culture, as exemplified in the case of *L. (V.) braziliensis* (Table 1). Evaluation by complementary methodologies is necessary to better characterization the amastigotes obtained in axenic cultures. Thus, the definition of biomarkers, typically of intracellular amastigotes, could assist in the accuracy of induction methods and provide greater robustness to the results obtained. As shown in the omic findings (Table 2, Fig. 1), some genes and/or proteins are potent candidates that could be used as biomarkers for this characterization. Despite the need of further studies in this field, the results so far indicate that these biomarkers are often species-specific. In addition, this fact highlights the singularities of each species and, consequently, the need of protocols respecting the diversity of each *Leishmania* spp. For example, in *L. (L.) infantum*, which is the species used in the majority of omics publications discussed here, A2, Amastin, SHERP, HASPB, TRyS genes have an increased expression in both intracellular and axenic amastigotes. In other species, the number of publications is smaller; however, it is possible to highlight the presence of TXN and STI1 proteins in both amastigote forms of *L. (L.) mexicana* and *L. (L.) donovani*, respectively.

The axenic amastigote model has been explored to understand cellular structure, metabolic pathways, and signaling networks of *Leishmania* spp. In addition, they are extensively applied in drug screening (Table 3). In general, the results obtained in these studies are not in agreement with those found in intracellular amastigotes (Table 3). Thus, it is recommended to use more sensitive methodologies to verify cell viability in axenic amastigotes, such as ATP measurement. This methodology is more costly due to expensive reagents and the need for a luminometer instrument; yet, it is essential for greater accuracy of results and comparison between axenic and intracellular amastigotes.

The knowledge accumulated for more than 30 years over axenic amastigotes is an unequivocal achievement for the understanding of *Leishmania* spp. biology. In fact, the increased number of publications on this topic brings new information; however, some problems are evidenced regarding the diversity of induction protocols and absence of controls besides morphology analysis. In the specific case of drug tests, the need to reassess the methodologies used is emphasized. Data gathered here must be taken into account for adequate use of axenic amastigotes.

Funding

This study was financed in part by the Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brasil (CNPq: 301744/2019-0), Rio Network of Innovation in nanosystems for the health - Nanohealth/FAPERJ **(**E-26/010.000983/2019**)** and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES: Finance Code 001). Geovane Dias-Lopes and Franklin Souza-Silva are research fellows at CAPES. Carlos R. Alves are research fellows at CNPq.

Author Contributions

Each author made substantial contributions to the conception and design of this work. All the authors have approved the submitted version.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

References

Akopyants, N.S., Matlib, R.S., Bukanova, E.N., Smeds, M.R., Brownstein, B.H., Stormo, G. D., Beverley, S.M., 2004. Expression profiling using random genomic DNA microarrays identifies differentially expressed genes associated with three major developmental stages of the protozoan parasite Leishmania major. Mol. Biochem. Parasitol. 136 (1), 71–86. https://doi.org/10.1016/j.molbiopara.2004.03.002.

Alcolea, P.J., Alonso, A., Gómez, M.J., Sánchez-Gorostiaga, A., Moreno-Paz, M., González-Pastor, E., Toraño, A., Parro, V., Larraga, V., 2010. Temperature increase prevails over acidification in gene expression modulation of amastigote differentiation in Leishmania infantum. BMC Genomics 11 (1), 31. https://doi.org/10.1186/1471-2164-11-31.

Almeida, R., Gilmartin, B.J., McCann, S.H., Norrish, A., Ivens, A.C., Lawson, D., Levick, M.P., Smith, D.F., Dyall, S.D., Vetrie, D., Freeman, T.C., Coulson, R.M., Sampaio, I., Schneider, H., Blackwell, J.M., 2004. Expression profiling of the Leishmania life cycle: CDNA arrays identify developmentally regulated genes present but not annotated in the genome. Mol. Biochem. Parasitol. 136 (1), 87–100. https://doi.org/10.1016/j.molbiopara.2004.03.004.

Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., et al., 2012. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7 (5), e35671. https://doi.org/10.1371/journal.pone.0035671.

Balanco, J.M.D.F., Pral, E.M.F., Da Silva, S., Bijovsky, A.T., Mortara, R.A., Alfieri, S.C., 1998. Axenic cultivation and partial characterization of Leishmania braziliensis amastigote-like stages. Parasitology 116 (2), 103–113. https://doi.org/10.1017/s003118209700214x.

Basu, M., Gupta, P., Dutta, A., Jana, K., Ukil, A., 2020. Increased host ATP efflux and its conversion to extracellular adenosine is crucial for establishing Leishmania infection. J. Cell Sci. 133 (7) https://doi.org/10.1242/jcs.239939.

Bates, P.A., Robertson, C.D., Tetley, L., Coombs, G.H., 1992. Axenic cultivation and characterization of Leishmania mexicana amastigote-like forms. Parasitology 105 (2), 193–202. https://doi.org/10.1017/S0031182000074102.

Bates, P.A., Rogers, M.E., 2004. New insights into the developmental biology and transmission mechanisms of Leishmania. Curr. Mol. Med. 4 (6), 601–609. https://doi.org/10.2174/1566524043360285.

Bente, M., Harder, S., Wiesgigl, M., Heukeshoven, J., Gelhaus, C., Krause, E., Clos, J., Bruchhaus, I., 2003. Developmentally induced changes of the proteome in the protozoan parasite Leishmania donovani. Proteomics 3 (9), 1811–1829. https://doi.org/10.1002/pmic.200300462.

Berry, S.L., Hameed, H., Thomason, A., Maciej-Hulme, M.L., Abou-Akkada, S.S., Horrocks, P., Price, H.P., 2018. Development of NanoLuc-PEST expressing Leishmania mexicana as a new drug discovery tool for axenic-and intramacrophage-based assays. PLoS Negl. Trop. Dis. 12 (7), e0006639 https://doi.org/10.1371/journal.pntd.0006639.

Bhattacharya, P., Mondal, S., Basak, S., Das, P., Saha, A., Bera, T., 2016. In vitro susceptibilities of wild and drug resistant Leishmania donovani amastigotes to piperolactam A loaded hydroxypropyl-β-cyclodextrin nanoparticles. Acta Trop. 158, 97–106. https://doi.org/10.1016/j.actatropica.2016.02.017.

Brendle, J.J., Outlaw, A., Kumar, A., Boykin, D.W., Patrick, D.A., Tidwell, R.R., Werbovetz, K.A., 2002. Antileishmanial activities of several classes of aromatic dications. Antimicrob. Agents Chemother. 46 (3), 797–807. https://doi.org/10.1128/aac.46.3.797-807.2002.

Britta, E.A., Scariot, D.B., Falzirolli, H., Ueda-Nakamura, T., Silva, C.C., Dias Filho, B.P., Borsali, L., Nakamura, C.V., 2014. Cell death and ultrastructural alterations in Leishmania amazonensis caused by new compound 4-Nitrobenzaldehyde thiosemicarbazone derived from S-limonene. BMC Microbiol. 14, 236. https://doi.org/10.1186/s12866-014-0236-0.

Britta, E.A., Silva, A.P.B., Ueda-Nakamura, T., Dias-Filho, B.P., Silva, C.C., Sernaglia, R. L., Nakamura, C.V., 2012. Benzaldehyde thiosemicarbazone derived from limonene complexed with copper induced mitochondrial dysfunction in Leishmania amazonensis. PLoS One 7 (8), e41440. https://doi.org/10.1371/journal.pone.0041440.

Cabanillas, B.J., Le Lamer, A.C., Castillo, D., Arevalo, J., Estevez, Y., Rojas, R., Valadeau, C., Bourdy, G., Sauvain, M., Fabre, N., 2012. Dihydrochalcones and benzoic acid derivatives from Piper dennisii. Planta Med. 78 (9), 914–918. https://doi.org/10.1055/s-0031-1298459.

Callahan, H.L., Portal, A.C., Devereaux, R., Grogl, M.A.X., 1997. An axenic amastigote system for drug screening. Antimicrob. Agents Chemother. 41 (4), 818–822. https://doi.org/10.1128/AAC.41.4.818.

Campbell, D.A., Thomas, S., Sturm, N.R., 2003. Transcription in kinetoplastid protozoa: why be normal? Microb Infect 5 (13), 1231–1240. https://doi.org/10.1016/j.micinf.2003.09.005.

Cao, D.P., Chen, D.L., Chen, J.P., Liao, L., Yang, B.B., Hu, X.S., 2012. Axenic culture and identification of amastigotes from Sichuan human strain of Chinese Leishmania isolates. Vet. Parasitol. 183 (3–4), 353–355. https://doi.org/10.1016/j.vetpar.2011.07.044.

Carrió, J., De Colmenares, M., Riera, C., Gallego, M., Arboix, M., Portus, M., 2000. Leishmania infantum: stage-specific activity of pentavalent antimony related with the assay conditions. Exp. Parasitol. 95 (3), 209–214. https://doi.org/10.1006/expr.2000.4537.

Castillo, D., Arevalo, J., Herrera, F., Ruiz, C., Rojas, R., Rengifo, E., Vaisberg, A., Lock, O., Lemesre, J.L., Gornitzka, H., Sauvain, M., 2007. Spirolactone iridoids might be responsible for the antileishmanial activity of a Peruvian traditional remedy made with Himatanthus sucuuba (Apocynaceae). J. Ethnopharmacol. 112 (2), 410–414. https://doi.org/10.1016/j.jep.2007.03.025.

Chanmol, W., Jariyapan, N., Somboon, P., Bates, M.D., Bates, P.A., 2019. Axenic amastigote cultivation and in vitro development of Leishmania orientalis. Parasitol. Res. 118 (6), 1885–1897. https://doi.org/10.1007/s00436-019-06311-z.

Clayton, C.E., 2016. Gene expression in kinetoplastids. Curr. Opin. Microbiol. 32, 46–51. https://doi.org/10.1016/j.mib.2016.04.018.

Cohen-Freue, G., Holzer, T.R., Forney, J.D., McMaster, W.R., 2007. Global gene expression in Leishmania. Int. J. Parasitol. 37 (10), 1077–1086. https://doi.org/10.1016/j.ijpara.2007.04.011.

Cottrell, D.M., Capers, J., Salem, M.M., DeLuca-Fradley, K., Croft, S.L., Werbovetz, K.A., 2004. Antikinetoplastid activity of 3-aryl-5-thiocyanatomethyl-1, 2, 4-oxadiazoles. Bioorg. Med. Chem. 12 (11), 2815–2824. https://doi.org/10.1016/j.bmc.2004.03.054.

Cupolillo, E., Brahim, L.R., Toaldo, C.B., de Oliveira-Neto, M.P., de Brito, M.E.F., Falqueto, A., Naiff, M.F., Grimaldi, G., 2003. Genetic polymorphism and molecular epidemiology of Leishmania (Viannia) braziliensis from different hosts and geographic areas in Brazil. J. Clin. Microbiol. 41 (7), 3126–3132. https://doi.org/10.1128/jcm.41.7.3126-3132.2003.

Cysne-Finkelstein, L., Temporal, R.M., Alves, F.A., Leon, L.L., 1998. Leishmania amazonensis: long-term cultivation of axenic amastigotes is associated to metacyclogenesis of promastigotes. Exp. Parasitol. 89 (1), 58–62. https://doi.org/10.1006/expr.1998.4276.

Dayakar, A., Chandrasekaran, S., kumar Prajapati, V., Veronica, J., Sundar, S., Maurya, R., 2012. A rapid method to assess the stage differentiation in Leishmania donovani by flow cytometry. Exp. Parasitol. 132 (4), 495–500. https://doi.org/10.1016/j.exppara.2012.09.006.

da Silva, I.A., Morato, C.I., Quixabeira, V.B.L., Pereira, L.I.D.A., Dorta, M.L., de Oliveira, M.A.P., Horta, M.F., Ribeiro-Dias, F., 2015. In vitro metacyclogenesis of Leishmania (Viannia) braziliensis and Leishmania (Leishmania) amazonensis clinical field isolates, as evaluated by morphology, complement resistance, and infectivity to human macrophages. Biomed. Res. Int. 2015, 393049 https://doi.org/10.1155/2015/393049.

De Muylder, G., Ang, K.K., Chen, S., Arkin, M.R., Engel, J.C., McKerrow, J.H., 2011. A screen against Leishmania intracellular amastigotes: comparison to a promastigote screen and identification of a host cell-specific hit. PLoS Negl. Trop. Dis. 5 (7), e1253. https://doi.org/10.1371/journal.pntd.0001253.

De Muylder, G., Vanhollebeke, B., Caljon, G., Wolfe, A.R., McKerrow, J., Dujardin, J.C., 2016. Naloxonazine, an amastigote-specific compound, affects Leishmania parasites through modulation of host-encoded functions. PLoS Negl. Trop. Dis. 10 (12), e0005234 https://doi.org/10.1371/journal.pntd.0005234.

De Rycker, M., Hallyburton, I., Thomas, J., Campbell, L., Wyllie, S., Joshi, D., Cameron, S., Gilbert, I.H., Wyatt, P.G., Frearson, J.A., Fairlamb, A.H., Gray, D.W., 2013. Comparison of a high-throughput high-content intracellular Leishmania donovani assay with an axenic amastigote assay. Antimicrob. Agents Chemother. 57 (7), 2913–2922. https://doi.org/10.1128/AAC.02398-12.

De Rycker, M., O'Neill, S., Joshi, D., Campbell, L., Gray, D.W., Fairlamb, A.H., 2012. A static-cidal assay for Trypanosoma brucei to aid hit prioritization for progression into drug discovery programmes. PLoS Negl. Trop. Dis. 6, e1932. https://doi.org/10.1371/journal.pntd.0001932.

Dias, C.N., Nunes, T.A.L., de Sousa, J.M.S., Costa, L.H., Rodrigues, R.R.L., Araújo, A.J., Filho, J.D.B.M., da Silva, M.V., Oliveira, M.R., Carvalho, F.A.A., Rodrigues, K.A.F., 2020. Methyl gallate: Selective antileishmanial activity correlates with host-cell directed effects. Chem. Biol. Interact. 320, 109026 https://doi.org/10.1016/j.cbi.2020.109026.

Diaz, J.R., Ramírez, C.A., Nocua, P.A., Guzman, F., Requena, J.M., Puerta, C.J., 2018. Dipeptidyl peptidase 3, a novel protease from Leishmania braziliensis. PLoS One 13 (1), e0190618. https://doi.org/10.1371/journal.pone.0190618.

Santos, Dos, O., A., Britta, E.A., Bianco, E.M., Ueda-Nakamura, T., Dias Filho, B.P., Pereira, R.C., Nakamura, C.V., 2011. 4-Acetoxydolastane diterpene from the Brazilian brown alga Canistrocarpus cervicornis as antileishmanial agent. Mar Drugs 9 (11), 2369–2383. https://doi.org/10.3390/md9112369.

Dostálová, A., Volf, P., 2012. Leishmania development in sand flies: parasite-vector interactions overview. Parasit. Vectors 5 (1), 1–12. https://doi.org/10.1186/1756-3305-5-276.

Duarte, M.C., Pimenta, D.C., Menezes-Souza, D., Magalhães, R.D., Diniz, J.L., Costa, L.E., Chávez-Fumagalli, M.A., Lage, P.S., Bartholomeu, D.C., Alves, M.J.M., Fernandes, A. P., Soto, M., Tavares, C.A.P., Gonçalves, D.U., Rocha, M.O.C., Coelho, E.A.F., 2015. Proteins selected in Leishmania (Viannia) braziliensis by an immunoproteomic approach with potential serodiagnosis applications for tegumentary leishmaniasis. Clin. Vaccine Immunol. 22 (11), 1187–1196. https://doi.org/10.1128/CVI.00465-15.

Eperon, S., McMahon-Pratt, D., 1989. Extracellular cultivation and morphological characterization of amastigote-like forms of Leishmania panamensis and L. braziliensis. J. Protozool. 36 (5), 502–510. https://doi.org/10.1111/j.1550-7408.1989.tb01086.x.

Fakhry, Y.E., Ouellette, M., Papadopoulou, B., 2002. A proteomic approach to identify developmentally regulated proteins in Leishmania infantum. Proteomics 2 (8), 1007–1017. https://doi.org/10.1002/1615-9861(200208)2:8<1007::AID-PROT1007>3.0.CO;2-G.

Fersing, C., Boudot, C., Pedron, J., Hutter, S., Primas, N., Castera-Ducros, C., Bourgeade-Delmas, S., Sournia-Saquet, A., Moreau, A., Cohen, A., Stigliani, J.L., Pratviel, G., Crozet, M.D., Wylle, S., Fairlamb, A., Valentin, A., Rathelot, P., Azas, N., Courtioux, B., Verhaeghe, P., Vanelle, P., 2018. 8-Aryl-6-chloro-3-nitro-2-(phenylsulfonylmethyl) imidazo [1, 2-a] pyridines as potent antitrypanosomatid molecules bioactivated by type 1 nitroreductases. Eur. J. Med. Chem. 157, 115–126. https://doi.org/10.1016/j.ejmech.2018.07.064.

Fiebig, M., Kelly, S., Gluenz, E., 2015. Comparative life cycle transcriptomics revises Leishmania mexicana genome annotation and links a chromosome duplication with parasitism of vertebrates. PLoS Pathog. 11 (10), e1005186 https://doi.org/10.1371/journal.ppat.1005186.

Figarella, K., Uzcategui, N.L., Zhou, Y., LeFurgey, A., Ouellette, M., Bhattacharjee, H., Mukhopadhyay, R., 2007. Biochemical characterization of Leishmania major aquaglyceroporin LmAQP1: possible role in volume regulation and osmotaxis. Mol. Microbiol. 65 (4), 1006–1017. https://doi.org/10.1111/j.1365-2958.2007.05845.x.

Gomes, C.B., Silva, F.S., Charret, K.D.S., Pereira, B.A.S., Finkelstein, L.C., Santos-de-Souza, R., de Castro Côrtes, L.M., Pereira, M.C.S., Rodrigues de Oliveira, F.O., Alves, C.R., 2017. Increasing in cysteine proteinase B expression and enzymatic activity during in vitro differentiation of Leishmania (Viannia) braziliensis: first evidence of modulation during morphological transition. Biochimie 133, 28–36. https://doi.org/10.1016/j.biochi.2016.11.015.

Gossage, S.M., Rogers, M.E., Bates, P.A., 2003. Two separate growth phases during the development of Leishmania in sand flies: implications for understanding the life cycle. Int. J. Parasitol. 33 (10), 1027–1034. https://doi.org/10.1016/s0020-7519(03)00142-5.

Gupta, N., Goyal, N., Rastogi, A.K., 2001. In vitro cultivation and characterization of axenic amastigotes of Leishmania. Trends Parasitol. 17 (3), 150–153. https://doi.org/10.1016/s1471-4922(00)01811-0.

Handman, E., Bullen, D.V., 2002. Interaction of Leishmania with the host macrophage. Trends Parasitol. 18 (8), 332–334. https://doi.org/10.1016/s1471-4922(02)02352-8.

Hermoso, T., Pérez, J., Cornivelli, L., Hernandez, A.G., 1994. Extracellular phosphorylation in Leishmania major and Leishmania mexicana during heat shock transformation. Acta Trop. 56 (1), 1–6. https://doi.org/10.1016/0001-706X(94)90034-5.

Herrera, D.S.M., Rodríguez, L.E.C., Castellanos, C.C.R., Hernández, M.H.R., 2020. Identification and sub-cellular localization of a NAD transporter in Leishmania braziliensis (LbNDT1). Heliyon 6 (7), e04331. https://doi.org/10.1016/j.heliyon.2020.e04331.

Herricks, J.R., Hotez, P.J., Wanga, V., Coffeng, L.E., Haagsma, J.A., Basáñez, M.G., et al., 2017. The global burden of disease study 2013: What does it mean for the NTDs? PloS Negl. Trop. Dis. 11 (8), e0005424 https://doi.org/10.1371/journal.pntd.0005424.

Holzer, T.R., McMaster, W.R., Forney, J.D., 2006. Expression profiling by whole-genome interspecies microarray hybridization reveals differential gene expression in procyclic promastigotes, lesion-derived amastigotes, and axenic amastigotes in Leishmania mexicana. Mol. Biochem. Parasitol. 146 (2), 198–218. https://doi.org/10.1016/j.molbiopara.2005.12.009.

Iantorno, S.A., Durrant, C., Khan, A., Sanders, M.J., Beverley, S.M., Warren, W.C., Berriman, M., Sacks, D.L., Cotton, J.A., Grigg, M.E., 2017. Gene expression in Leishmania is regulated predominantly by gene dosage. MBio 8 (5). https://doi.org/10.1128/mBio.01393-17 e01393-17.

Iyer, J.P., Kaprakkaden, A., Choudhary, M.L., Shaha, C., 2008. Crucial role of cytosolic tryparedoxin peroxidase in Leishmania donovani survival, drug response and virulence. Mol. Microbiol. 68 (2), 372–391. https://doi.org/10.1111/j.1365-2958.2008.06154.x.

Jara, M., Berg, M., Caljon, G., de Muylder, G., Cuypers, B., Castillo, D., Arevalo, J., 2017. Macromolecular biosynthetic parameters and metabolic profile in different life stages of Leishmania braziliensis: amastigotes as a functionally less active stage. PLoS One 12 (7), e0180532. https://doi.org/10.1371/journal.pone.0180532.

Junior, L.F., da Fonseca Pires, S., Burchmore, R., McGill, S., Weidt, S., Ruiz, J.C., Guimarães, F.G., Chapeourouge, A., Perales, J., de Andrade, H.M., 2021. Proteomic analysis reveals differentially abundant proteins probably involved in the virulence of amastigote and promastigote forms of Leishmania infantum. Parasitol. Res. 120 (2), 679–692. https://doi.org/10.1007/s00436-020-07020-8.

Kaul, P., Malla, N., Kaur, S., Mahajan, R.C., Ganguly, N.K., 2000. Evaluation of a 200-kDa amastigote-specific antigen of L. donovani by enzyme-linked immunosorbent assay (ELISA) for the diagnosis of visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 94 (2), 173–175. https://doi.org/10.1016/s0035-9203(00)90264-5.

Kaye, P., Scott, P., 2011. Leishmaniasis: complexity at the host–pathogen interface. Nat. Rev. Microbiol. 9 (8), 604–615. https://doi.org/10.1038/nrmicro2608.

Kloehn, J., Blume, M., Cobbold, S., Saunders, E., Dagley, M., McConville, M., 2016. Using metabolomics to dissect host–parasite interactions. Curr. Opin. Microbiol. 32, 59–65. https://doi.org/10.1016/j.mib.2016.04.019.

Krauth-Siegel, R.L., Meiering, S.K., Schmidt, H., 2003. The parasite-specific trypanothione metabolism of trypanosoma and leishmania. Biol. Chem. 384 (4), 539–549. https://doi.org/10.1515/BC.2003.062.

Lage, P.S., Chávez-Fumagalli, M.A., Mesquita, J.T., Mata, L.M., Fernandes, S.O., Cardoso, V.N., Soto, M., Tavares, C.A.P., Leite, J.P.V., Tempone, A.G., Coelho, E.A., 2015. Antileishmanial activity and evaluation of the mechanism of action of strychnobiflavone flavonoid isolated from Strychnos pseudoquina against Leishmania infantum. Parasitol. Res. 114 (12), 4625–4635. https://doi.org/10.1007/s00436-015-4708-4.

Lainson, R., Shaw, J.J., 1987. Evolution, classification and geographical distribution. In: Peters W, Killick-Kendrick R, editors. The Leishmaniases in Biology and Medicine. Volume 1. Academic Press; London: pp. 1–120.

Lambertz, U., Ovando, M.E.O., Vasconcelos, E.J., Unrau, P.J., Myler, P.J., Reiner, N.E., 2015. Small RNAs derived from tRNAs and rRNAs are highly enriched in exosomes from both old and new world Leishmania providing evidence for conserved exosomal RNA Packaging. BMC Genomics 16 (1), 151. https://doi.org/10.1186/s12864-015-1260-7.

McNicoll, F., Drummelsmith, J., Müller, M., Madore, E., Boilard, N., Ouellette, M., Papadopoulou, B., 2006. A combined proteomic and transcriptomic approach to the study of stage differentiation in Leishmania infantum. Proteomics 6 (12), 3567–3581. https://doi.org/10.1002/pmic.200500853.

Mendonça, D.V.C., Lage, D.P., Calixto, S.L., Ottoni, F.M., Tavares, G.D.S.V., Ludolf, F., Chávez-Fumagalli, M.A., Schneider, M.S., Duarte, M.C., Tavares, C.A.P., Alves, R.J., Coimbra, E.S., Coelho, E.A.F., 2018. Antileishmanial activity of a naphthoquinone derivate against promastigote and amastigote stages of Leishmania infantum and Leishmania amazonensis and its mechanism of action against L. amazonensis species. Parasitol. Res. 117 (2), 391–403. https://doi.org/10.1007/s00436-017-5713-6.

Mesa-Valle, C.M., Moraleda-Lindez, V., Craciunescu, D., Osuna, A., 1996. Antileishmanial action of organometallic complexes of Pt (II) and Rh (I). Mem. Inst. Oswaldo Cruz. 91 (5), 625–633. https://doi.org/10.1590/S0074-02761996000500015.

Morais-Teixeira, E.D., de Carvalho, A.S., Costa, J., Duarte, S.L., Mendonça, J.S., Boechat, N., Rabello, A., 2008. In vitro and in vivo activity of meglumine antimoniate produced at Farmanguinhos-Fiocruz, Brazil, against Leishmania (Leishmania) amazonensis, L (L.) chagasi and L (Viannia) braziliensis. Mem. Inst. Oswaldo Cruz. 103 (4), 358–362. https://doi.org/10.1590/S0074-02762008000400008.

Morales, M.A., Watanabe, R., Dacher, M., Chafey, P., Osorio y Fortéa, J., Scott, D.A., Beverley, S.M., Ommen, G., Clos, J., Hem, S., Lenormand, P., Rousselle, J.-C., Namane, A., Späth, G.F., 2010. Phosphoproteome dynamics reveal heat-shock protein complexes specific to the Leishmania donovani infectious stage. Proc Nat. Acad. Sci. U.S.A. 107 (18), 8381–8386. https://doi.org/10.1073/pnas.0914768107.

Morales, M.A., Watanabe, R., Laurent, C., Lenormand, P., Rousselle, J.-C., Namane, A., Späth, G.F., 2008. Phosphoproteomic analysis of Leishmania donovani pro- and amastigote stages. Proteomics 8 (2), 350–363. https://doi.org/10.1002/pmic.200700697.

Moreno, D., Plano, D., Baquedano, Y., Jiménez-Ruiz, A., Palop, J.A., Sanmartín, C., 2011. Antileishmanial activity of imidothiocarbamates and imidoselenocarbamates. Parasitol. Res. 108 (1), 233–239. https://doi.org/10.1007/s00436-010-2073-x.

Nasereddin, A., Schweynoch, C., Schonian, G., Jaffe, C.L., 2010. Characterization of Leishmania (Leishmania) tropica axenic amastigotes. Acta Trop. 113 (1), 72–79. https://doi.org/10.1016/j.actatropica.2009.09.009.

Niño, A., Camacho, M., 2005. Leishmania (Viannia) braziliensis growth in vitro culture relies more on folic acid availability than Leihsmania (Leishmania) amazonensis. Mem. Inst. Oswaldo Cruz. 100 (3), 309–310. https://doi.org/10.1590/S0074-02762005000300017.

Nugent, P.G., Karsani, S.A., Wait, R., Tempero, J., Smith, D.F., 2004. Proteomic analysis of Leishmania mexicana differentiation. Mol. Biochem. Parasitol. 136 (1), 51–62. https://doi.org/10.1016/j.molbiopara.2004.02.009.

Nühs, A., De Rycker, M., Manthri, S., Comer, E., Scherer, C.A., Schreiber, S.L., Ioset, J.R., Gray, D.W., 2015. Development and validation of a novel Leishmania donovani screening cascade for high-throughput screening using a novel axenic assay with high predictivity of leishmanicidal intracellular activity. PLoS Negl. Trop. Dis. 9 (9), e0004094 https://doi.org/10.1371/journal.pntd.0004094.

Okwor, I., Uzonna, J., 2016. Social and economic burden of human leishmaniasis. Am. J. Trop. Med. Hyg. 94 (3), 489–493. https://doi.org/10.4269/ajtmh.15-0408.

Pedroso, R.B., Tonin, L.T.D., Ueda-Nakamura, T., Filho, B.D., Sarragiotto, M.H., Nakamura, C.V., 2011. Beta-carboline-3-carboxamide derivatives as promising antileishmanial agents. Ann. Trop. Med. Parasitol. 105 (8), 549–557. https://doi.org/10.1179/2047773211Y.0000000005.

Pescher, P., Blisnick, T., Bastin, P., Späth, G.F., 2011. Quantitative proteome profiling informs on phenotypic traits that adapt Leishmania donovani for axenic and intracellular proliferation. Cell. Microbiol. 13 (7), 978–991. https://doi.org/10.1111/j.1462-5822.2011.01593.x.

Plano, D., Baquedano, Y., Moreno-Mateos, D., Font, M., Jiménez-Ruiz, A., Palop, J.A., Sanmartín, C., 2011. Selenocyanates and diselenides: a new class of potent antileishmanial agents. Eur. J. Med. Chem. 46 (8), 3315–3323. https://doi.org/10.1016/j.ejmech.2011.04.054.

Puentes, F., Diaz, D., Hoya, R.D., Gutíerrez, J.A., Lozano, J.M., Patarroyo, M.E., Moreno, A., 2000. Cultivation and characterization of stable Leishmania guyanensis complex axenic amastigotes derived from infected U937 cells. Am. J. Trop. Med. Hyg. 63 (1), 102–110. https://doi.org/10.4269/ajtmh.2000.63.102.

Rainey, P.M., Spithill, T.W., McMahon-Pratt, D., Pan, A.A., 1991. Biochemical and molecular characterization of Leishmania pifanoi amastigotes in continuous axenic culture. Mol. Biochem. Parasitol. 49 (1), 111–118. https://doi.org/10.1016/0166-6851(91)90134-r.

Ramírez-Macías, I., Marín, C., Chahboun, R., Olmo, F., Messouri, I., Huertas, O., Rosales, M.J., Gutierrez-Sánchez, R., Alvarez-Manzaneda, E., Sánchez-Moreno, M., 2012a. In vitro evaluation of new terpenoid derivatives against Leishmania infantum and Leishmania braziliensis. Mem. Inst. Oswaldo Cruz. 107 (3), 370–376. https://doi.org/10.1590/S0074-02762012000300012.

Ramírez-Macías, I., Marín, C., Díaz, J.G., Rosales, M.J., Gutiérrez-Sánchez, R., Sánchez-Moreno, M., 2012b. Leishmanicidal activity of nine novel flavonoids from Delphinium staphisagria. Sci. World J. 2012, 203646 https://doi.org/10.1100/2012/203646.

Riss, T.L., Moravec, R.A., Niles, A.L., Duellman, S., Benink, H.A., Worzella, T.J., Minor, L., 2016. Cell viability assays. In Assay Guidance Manual [Internet]. Eli Lilly & Company and the National Center for Advancing Translational Sciences.

Rochette, A., Raymond, F., Corbeil, J., Ouellette, M., Papadopoulou, B., 2009. Whole-genome comparative RNA expression profiling of axenic and intracellular amastigote forms of Leishmania infantum. Mol. Biochem. Parasitol. 165 (1), 32–47. https://doi.org/10.1016/j.molbiopara.2008.12.012.

Rodrigues, K.A.F., Amorim, L.V., Dias, C.N., Moraes, D.F.C., Carneiro, S.M.P., de Amorim Carvalho, F.A., 2015a. Syzygium cumini (L.) Skeels essential oil and its major constituent α-pinene exhibit anti-Leishmania activity through immunomodulation in vitro. J. Ethnopharmacol. 160, 32–40. https://doi.org/10.1016/j.jep.2014.11.024.

Rodrigues, K.A.F., Dias, C.N.S., Néris, P.L.N., Rocha, J.C., Scotti, M.T., Scotti, L., Mascarenhas, S.R., Veras, R.C., de Medeiros, I.A., Keesen, T.S.L., Oliveira, T.B., Lima, M.C.A., Balliano, T.L., Aquino, T.M., Moura, R.O., Junior, F.J.B.M., Oliveira, M.R., 2015b. 2-Amino-thiophene derivatives present antileishmanial activity mediated by apoptosis and immunomodulation in vitro. Eur. J. Med. Chem. 106, 1–14. https://doi.org/10.1016/j.ejmech.2015.10.011.

Romero, A.H., López, S.E., Rodríguez, N., Oviedo, H., 2018. Antileishmanial activity, structure–activity relationship of series of 2-(trifluoromethyl) benzo [b][1, 8] naphthyridin-4 (1H)-ones. Arch. Pharm. 351 (8), 1800094 https://doi.org/10.1002/ardp.201800094.

Romero, A.H., Rodríguez, N., Oviedo, H., 2019. 2-Aryl-quinazolin-4 (3H)-ones as an inhibitor of leishmania folate pathway: in vitro biological evaluation, mechanism studies and molecular docking. Bioorg. Chem. 83, 145–153. https://doi.org/10.1016/j.bioorg.2018.10.028.

Sacks, D.L., Perkins, P.V., 1984. Identification of an infective stage of Leishmania promastigotes. Science 223 (4643), 1417–1419. https://doi.org/10.1126/science.6701528.

Sacks, D.L., Pimenta, P.F., McConville, M.J., Schneider, P., Turco, S.J., 1995. Stage-specific binding of Leishmania donovani to the sand fly vector midgut is regulated by conformational changes in the abundant surface lipophosphoglycan. J. Exp. Med. 181 (2), 685–697. https://doi.org/10.1084/jem.181.2.685.

Sádlová, J., Price, H.P., Smith, B.A., Votýpka, J., Volf, P., Smith, D.F., 2010. The stage-regulated HASPB and SHERP proteins are essential for differentiation of the protozoan parasite Leishmania major in its sand fly vector, Phlebotomus papatasi. Cell. Microbiol. 12 (12), 1765–1779. https://doi.org/10.1111/j.1462-5822.2010.01507.x.

Salotra, P., Sreenivas, G., Nasim, A.A., Raju, B.S., Ramesh, V., 2002. Evaluation of enzyme-linked immunosorbent assay for diagnosis of post-kala-azar dermal leishmaniasis with crude or recombinant k39 antigen. Clin. Diagn. Lab. Immunol. 9 (2), 370–373. https://doi.org/10.1128/cdli.9.2.370-373.2002.

Sánchez-Moreno, M., Gómez-Contreras, F., Navarro, P., Marín, C., Ramírez-Macías, I., Olmo, F., Sanz, A.M., Campayo, L., Cano, C., Yunta, M.J., 2012. In vitro leishmanicidal activity of imidazole-or pyrazole-based benzo [g] phthalazine

derivatives against Leishmania infantum and Leishmania braziliensis species. J. Antimicrob. Chemother. 67 (2), 387–397. https://doi.org/10.1093/jac/dkr480.
Santarém, N., Cunha, J., Silvestre, R., Silva, C., Moreira, D., Ouellette, M., Cordeiro-Da-Silva, A., 2014. The impact of distinct culture media in Leishmania infantum biology and infectivity. Parasitology 141 (2), 192–205. https://doi.org/10.1017/S0031182013001388.
Santin, M.R., Santos, dD.os, O., A., Nakamura, C.V., Dias Filho, B.P., Ferreira, I.C.P., Ueda-Nakamura, T., 2009. In vitro activity of the essential oil of Cymbopogon citratus and its major component (citral) on Leishmania amazonensis. Parasitol. Res. 105 (6), 1489–1496. https://doi.org/10.1007/s00436-009-1578-7.
Santos, A.O., Ueda-Nakamura, T., Dias Filho, B.P., Junior, V.F.V., Pinto, A.C., Nakamura, C.V., 2008. Effect of Brazilian copaiba oils on Leishmania amazonensis. J. Ethnopharmacol. 120 (2), 204–208. https://doi.org/10.1016/j.jep.2008.08.007.
Saunders, E.C., Ng, W.W., Kloehn, J., Chambers, J.M., Ng, M., McConville, M.J., 2014. Induction of a stringent metabolic response in intracellular stages of leishmania mexicana leads to increased dependence on mitochondrial metabolism. PLoS Pathog. 10 (1), e1003888 https://doi.org/10.1371/journal.ppat.1003888.
Saxena, A., Lahav, T., Holland, N., Aggarwal, G., Anupama, A., Huang, Y., Volpin, H., Myler, P.J., Zilberstein, D., 2007. Analysis of the Leishmania donovani transcriptome reveals an ordered progression of transient and permanent changes in gene expression during differentiation. Mol. Biochem. Parasitol. 152 (1), 53–65. https://doi.org/10.1016/j.molbiopara.2006.11.011.
Schwartzman, J.D., Pearson, R.D., 1985. The interaction of Leishmania donovani promastigotes and human fibroblasts in vitro. Am. J. Trop. Med. Hyg. 34 (5), 850–855. https://doi.org/10.4269/ajtmh.1985.34.850.
Sereno, D., Cavaleyra, M., Zemzoumi, K., Maquaire, S., Ouaissi, A., Lemesre, J.L., 1998. Axenically grown amastigotes of Leishmania infantum used as an in vitro model to investigate the pentavalent antimony mode of action. Antimicrob. Agents Chemother. 42 (12), 3097–3102. https://doi.org/10.1128/AAC.42.12.3097.
Sereno, D., Da Silva, A.C., Mathieu-Daude, F., Ouaissi, A., 2007. Advances and perspectives in Leishmania cell based drug-screening procedures. Parasitol. Int. 56 (1), 3–7. https://doi.org/10.1016/j.parint.2006.09.001.
Sereno, D., Holzmuller, P., Lemesre, J.L., 2000. Efficacy of second line drugs on antimonyl-resistant amastigotes of Leishmania infantum. Acta Trop. 74 (1), 25–31. https://doi.org/10.1016/s0001-706x(99)00048-0.
Siripattanapipong, S., Boontanom, P., Leelayoova, S., Mungthin, M., Tan-Ariya, P., 2019. In vitro growth characteristics and morphological differentiation of Leishmania martiniquensis promastigotes in different culture media. Acta Trop. 197, 105039 https://doi.org/10.1016/j.actatropica.2019.05.030.
Srividya, G., Duncan, R., Sharma, P., Raju, B.V.S., Nakhasi, H.L., Salotra, P., 2007. Transcriptome analysis during the process of in vitro differentiation of Leishmania donovani using genomic microarrays. Parasitology 134 (11), 1527–1539. https://doi.org/10.1017/S003118200700296X.
Stefanello, T.F., Panice, M.R., Ueda-Nakamura, T., Sarragiotto, M.H., Auzély-Velty, R., Nakamura, C.V., 2014. N-Butyl-[1-(4-methoxy) phenyl-9H-β-carboline]-3-carboxamide prevents cytokinesis in Leishmania amazonensis. Antimicrob. Agents Chemother. 58 (12), 7112–7120. https://doi.org/10.1128/AAC.03340-14.
Tavares, J., Ouaissi, M., Ouaissi, A., Cordeiro-da-Silva, A., 2007. Characterization of the anti-Leishmania effect induced by cisplatin, an anticancer drug. Acta Trop. 103 (2), 133–141. https://doi.org/10.1016/j.actatropica.2007.05.017.
Taylor, D.R., Williams, G.T., 1991. Differentiation and limited proliferation of isolated Leishmania mexicana amastigotes at 27°C. Acta Trop. 50 (2), 141–150. https://doi.org/10.1016/0001-706X(91)90007-7.
Teixeira, M., de Jesus Santos, R., Sampaio, R., Pontes-de-Carvalho, L., dos-Santos, W.L., 2002. A simple and reproducible method to obtain large numbers of axenic amastigotes of different Leishmania species. Parasitol. Res. 88 (11), 963–968. https://doi.org/10.1007/s00436-002-0695-3.
Tiuman, T.S., Ueda-Nakamura, T., Alonso, A., Nakamura, C.V., 2014. Cell death in amastigote forms of Leishmania amazonensis induced by parthenolide. BMC Microbiol. 14 (1), 152. https://doi.org/10.1186/1471-2180-14-152.
Tsigankov, P., Gherardini, P.F., Helmer-Citterich, M., Späth, G.F., Myler, P.J., Zilberstein, D., 2014. Regulation dynamics of Leishmania differentiation: Deconvoluting signals and identifying phosphorylation trends. Mol. Cell. Proteomics 13 (7), 1787–1799. https://doi.org/10.1074/mcp.M114.037705.
Ubeda, J.M., Légaré, D., Raymond, F., Ouameur, A.A., Boisvert, S., Rigault, P., Corbeil, J., Tremblay, M.J., Olivier, M., Papadopoulou, B., Ouellette, M., 2008. Modulation of gene expression in drug resistant Leishmania is associated with gene amplification, gene deletion and chromosome aneuploidy. Genome Biol. 9 (7), R115. https://doi.org/10.1186/gb-2008-9-7-r115.
Valadares, D.G., Duarte, M.C., Oliveira, J.S., Chávez-Fumagalli, M.A., Martins, V.T., Costa, L.E., Leite, J.P.V., Santoro, M.M., Régis, W.C.B., Tavares, C.A.P., Coelho, E.A., 2011. Leishmanicidal activity of the Agaricus blazei Murill in different Leishmania species. Parasitol. Int. 60 (4), 357–363. https://doi.org/10.1016/j.parint.2011.06.001.
Vannier-Santos, M.A., Urbina, J.A., Martiny, A., Neves, A., De Souza, W., 1995. Alterations induced by the antifungal compounds ketoconazole and terbinafine in Leishmania. J. Eukaryot. Microbiol. 42 (4), 337–346. https://doi.org/10.1111/j.1550-7408.1995.tb01591.x.
Vargas-Inchaustegui, D.A., Xin, L., Soong, L., 2008. Leishmania braziliensis infection induces dendritic cell activation, ISG15 transcription, and the generation of protective immune responses. J. Immunol. 180 (11), 7537–7545. https://doi.org/10.4049/jimmunol.180.11.7537.
Vendrametto, M.C., Dos Santos, A.O., Nakamura, C.V., Dias Filho, B.P., Cortez, D.A.G., Ueda-Nakamura, T., 2010. Evaluation of antileishmanial activity of eupomatenoid-5, a compound isolated from leaves of Piper regnellii var. pallescens. Parasitol. Int. 59 (2), 154–158. https://doi.org/10.1016/j.parint.2009.12.009.
Verma, R.K., Prajapati, V.K., Verma, G.K., Chakraborty, D., Sundar, S., Rai, M., Dubey, V. K., Singh, M.S., 2012. Molecular docking and in vitro antileishmanial evaluation of chromene-2-thione analogues. ACS Med. Chem. Lett. 3 (3), 243–247. https://doi.org/10.1021/ml200280r.
Vermeersch, M., da Luz, R.I., Toté, K., Timmermans, J.P., Cos, P., Maes, L., 2009. In vitro susceptibilities of Leishmania donovani promastigote and amastigote stages to antileishmanial reference drugs: practical relevance of stage-specific differences. Antimicrob. Agents Chemother. 53 (9), 3855–3859. https://doi.org/10.1128/AAC.00548-09.
Walker, J., Vasquez, J.-J., Gomez, M.A., Drummelsmith, J., Burchmore, R., Girard, I., Ouellette, M., 2006. Identification of developmentally-regulated proteins in Leishmania panamensis by proteome profiling of promastigotes and axenic amastigotes. Mol. Biochem. Parasitol. 147 (1), 64–73. https://doi.org/10.1016/j.molbiopara.2006.01.008.
WHO Expert Committee on the Control of the Leishmaniases & World Health Organization. (2010). Control of the leishmaniases: report of a meeting of the WHO Expert Commitee on the Control of Leishmaniases, Geneva, 22-26 March 2010. World Health Organization.
Williams, R.A., Tetley, L., Mottram, J.C., Coombs, G.H., 2006. Cysteine peptidases CPA and CPB are vital for autophagy and differentiation in Leishmania mexicana. Mol. Microbiol. 61 (3), 655–674. https://doi.org/10.1111/j.1365-2958.2006.05274.x.
Zhang, W.W., Charest, H., Ghedin, E., Matlashewski, G., 1996. Identification and overexpression of the A2 amastigote-specific protein in Leishmania donovani. Mol. Biochem. Parasitol. 78 (1–2), 79–90. https://doi.org/10.1016/s0166-6851(96)02612-6.
Zhang, W.W., Matlashewski, G., 2001. Characterization of the A2-A2rel gene cluster in Leishmania donovani: Involvement of A2 in visceralization during infection. Mol. Microbiol. 39 (4), 935–948. https://doi.org/10.1046/j.1365-2958.2001.02286.x.
Zilberstein D., 2020. In Vitro Culture for Differentiation Simulation of Leishmania spp. In: Michels P., Ginger M., Zilberstein D. (eds) Trypanosomatids. Methods in Molecular Biology, vol 2116. Humana, New York, NY. 10.1007/978-1-0716-0294-2_3.
Zilberstein, D., Koren, R.N., 2019. Host-Free Systems for Differentiation of Axenic Leishmania. In: Leishmania. Humana Press, New York. Methods Mol. Biol 1971, 1-8. 10.1007/978-1-4939-9210-2_1.
Zilberstein, D., Shapira, M., 1994. The role of pH and temperature in the development of Leishmania parasites. Annu. Rev. Microbiol. 48, 449–471. https://doi.org/10.1146/annurev.mi.48.100194.002313.