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Genetic Manipulation of Leishmania Parasites Facilitates the Exploration of the Polyamine Biosynthetic Pathway as a Potential Therapeutic Target

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Disciplines
Pharmacy and Pharmaceutical Sciences

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Chapter II

Genetic Manipulation of *Leishmania* Parasites Facilitates the Exploration of the Polyamine Biosynthetic Pathway As a Potential Therapeutic Target

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Abstract

Parasites of the genus *Leishmania* cause a variety of devastating and often fatal diseases, ranging from cutaneous ulcerative lesions to fatal visceralizing infections that affect an estimated 12 million people worldwide. Unfortunately, vaccines are not available and the current arsenal of drugs used to treat leishmaniasis is far from ideal. Thus the need for new therapeutic targets and a better understanding of host-parasite interactions is urgent. One biochemical pathway that has been successfully exploited for the treatment of a related parasitic disease, African trypanosomiasis, is the polyamine biosynthetic pathway. In order to elucidate the polyamine biosynthetic pathway and to explore its potential as a therapeutic target in *Leishmania*, we have generated and characterized gene deletion mutants and polyamine enzyme overproducer strains. These studies revealed that the polyamine pathway in *Leishmania* is significantly different from that of the mammalian host and polyamines were found to be essential for parasite proliferation. Infectivity studies in macrophages and mice with gene deletion mutants revealed that at least two polyamine biosynthetic enzymes, ornithine decarboxylase and spermidine synthase, are necessary for *Leishmania donovani* to establish a successful infection. However, arginase gene deletion mutants of *Leishmania mexicana* are still capable of eliciting an infection, albeit at lower levels than wild type parasites. Ongoing studies address whether the disparities in infectivity are due to the loss of specific enzymes within the pathway or to differences between the two *Leishmania* species. Furthermore, the gene deletion mutants are useful tools to investigate the relative

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contribution of host and parasite polyamine biosynthetic enzymes in parasite infectivity. This chapter will summarize how genetic manipulations in *Leishmania* have advanced our understanding of the polyamine pathway and its role in host-parasite interactions.

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**Leishmania Parasites and Their Relevance for Human Health**

Parasites of the genus *Leishmania* cause a variety of devastating and often fatal diseases in humans and domestic animals worldwide. *Leishmania* belong to the order of trypanosomatids, which include *Trypanosoma brucei*, the causative agent of African sleeping sickness, and *Trypanosoma cruzi*, which produces Chagas disease. The spectrum of leishmaniasis ranges from cutaneous ulcerative lesions to fatal visceralizing infections and affects an estimated 12 million people worldwide [1, 2]. Visceral leishmaniasis targets primarily the liver and spleen and is caused by *L. donovani*, *L. infantum* (Africa and Asia), and *L. chagasi* (Central and South America). This type of leishmaniasis is invariably fatal if not treated. Cutaneous leishmaniasis, which produces skin ulceration at the bite site, is caused by about 20 different *Leishmania* species, including *L. mexicana*, *L. major*, and *L. amazonensis*. A third form of the disease, mucocutaneous leishmaniasis, which targets and destroys the mucous membranes of nose and mouth, is caused by *L. braziliensis* in South America. The specific host tissue tropism of the different *Leishmania* species is poorly understood and exceptions occur. For example, *L. chagasi* may lead to cutaneous leishmaniasis in Central America, while dormant *L. donovani* parasites can trigger cutaneous disease years after the visceral form has been successfully treated as observed in post kala-azar dermal leishmaniasis (PKDL) in India and Africa [3, 4].

*Leishmania* has a digenetic life cycle in which the extracellular promastigote form resides in the sand fly vector, whereas the intracellular amastigote form inhabits the phagolysosome of macrophages in the infected host. The flagellated promastigote resides in the gut of the sand fly but will eventually migrate to the proboscis of the insect. Here, metacyclic or infective promastigotes will be regurgitated into the bite site when the sand fly takes a blood meal. *Leishmania* promastigotes are then phagocytosed by host macrophages. Usually, phagosomes fuse with lysosomes to form phagolysosomes, which are a highly effective in killing foreign pathogens. However, *Leishmania* parasites inhibit phagolysosomal maturation and withstand the hostile environment. Promastigotes convert into the smaller, non-flagellated amastigotes, which proliferate inside this host cell compartment. When a sand fly takes a blood meal from an infected host, it ingests parasitized macrophages and the released amastigotes transform back into promastigotes, completing the life cycle.

Due to the absence of effective vaccines, therapeutic treatment has offered the only avenue of defense against leishmaniasis and other parasitic diseases [5-7]. Drug treatment of the different forms of leishmaniasis, however, is far from ideal with toxic side effects and drug resistance limiting the effectiveness of a small number of drugs. The toxic antimonials are still considered the first line of treatment and the only new drug, miltefosine, which is also the only anti-leishmanial medicine that can be administered orally, is teratogenic [8]. Thus, the need for new therapeutic targets and a better understanding of host-parasite interactions is urgent.
The Polyamine Pathway

One pathway that has already been targeted for parasitic diseases is that of polyamine biosynthesis. Difluoromethylornithine (DFMO or eflofnithine), which targets the first and rate-limiting step of this pathway, is a clinically approved treatment for African sleeping sickness caused by T. brucei [9-12], a parasite closely related to Leishmania. Polyamines are essential and ubiquitous cations that are especially important for rapidly proliferating cells such as parasites and cancer cells. The three biologically relevant polyamines, putrescine, spermidine, and spermine have an aliphatic carbon backbone and contain multiple amino groups that are positively charged under physiological conditions (Figure 1). These compounds play critical roles in key cellular processes such as growth, differentiation, and macromolecular biosynthesis. However, despite numerous studies on the functions of polyamines, their roles for cell structure and function are still not fully understood.

The polyamine pathway has been characterized in detail in the mammalian host (Figure 2). The first and rate-limiting enzyme is ornithine decarboxylase (ODC), which catalyzes the conversion of the amino acid ornithine to putrescine. Subsequently, putrescine is metabolized to spermidine by the action of spermidine synthase (SPD). This enzyme catalyzes the addition of an aminopropyl group, which is donated from decarboxylated S-adenosylmethionine. The enzyme S-adenosylmethionine decarboxylase (ADOMETDC) is responsible for the decarboxylation of S-adenosylmethionine, which commits the compound for polyamine biosynthesis. A second aminopropyl group is added to spermidine to produce spermine in a reaction catalyzed by spermine synthase (SPM). In mammals, spermine can be back-converted to spermidine and putrescine by the concerted actions of spermidine/spermine N(1)-acetyltransferase (SSAT) and polyamine oxidase (POA). Spermidine is an essential polyamine that also plays a vital role in a unique modification reaction that is necessary to activate eukaryotic translation initiation factor (eIF5A), a protein involved in translation and RNA metabolism [13, 14]. The enzyme deoxyhypusine synthase (DHS) catalyzes the addition of an aminopropyl group derived from spermidine to a lysine residue of eIF5A to form deoxyhypusine and the enzyme deoxyhypusine hydroxylase (DOHH) completes the reaction by forming hypusine.

![Figure 1. Structures of the common polyamines.](image-url)
The enzyme abbreviations utilized are ARG: arginase; ODC: ornithine decarboxylase; SPD: spermidine synthase; SPM: spermine synthase; ADOMETDC: S-adenosylmethionine decarboxylase; ADC: arginine decarboxylase; AGM: agmatinase; DHS: deoxyhypusine synthase; DOHH: deoxyhypusine hydroxylase. DFMO is difluoromethylornithine, a suicide inhibitor of ODC. The question mark denotes the controversial hypothesis that mammalian cells contain an ADC activity.

Figure 2. The polyamine biosynthetic pathway in the mammalian host.

Evidence from yeast and mammalian cells suggest that the hypusination reaction is indispensable for activation of eIF5A and cell viability [15, 16].

Spermidine is also used in the synthesis of trypanothione, a reaction that occurs only in trypanosomatid parasites. Trypanothione is composed of two molecules of glutathione linked by spermidine and plays a crucial role in fighting oxidative stress in these pathogens [17-21]. Although trypanothione metabolism is an essential and unique pathway for trypanosomatids, this therapeutically relevant pathway has been the subject of several recent review articles [22-25] and will therefore not be discussed further in this chapter.

An alternative pathway for putrescine production exists in plants and some microorganisms where arginine decarboxylase (ADC) converts the amino acid arginine to agmatine, which is further metabolized to putrescine by agmatinase (AGM) or agmatine deiminase and N-carbamoylputrescine hydrolase. While the presence of AGM in mammalian cells has been described and is generally recognized, the existence of an ADC is disputed [26-28].

Because polyamines are a validated target in the related African trypanosomes, we used genetic methods to elucidate this pathway in Leishmania with the goal to evaluate its potential as a therapeutic target.

**Genetic techniques Available for the Manipulation of Leishmania parasites**

Genetic manipulations can be performed in Leishmania and facilitate the characterization of metabolic processes and the identification of proteins that are important for parasite
proliferation and infectivity. The most significant tool is the ability to generate gene deletion mutants via targeted gene replacement. *Leishmania* are diploid organisms that readily undergo homologous recombination and knockout strategies are hence straightforward. Gene deletion studies are an example of reverse genetic analysis where the outcome or phenotype of a targeted mutation is examined. The more classical method, forward genetic analysis, attempts to discover the genetic basis of an observed phenotype.

In order to generate gene deletion mutants, constructs containing a drug resistance cassette flanked by upstream and downstream sequences of the target gene need to be created (Figure 3). Linearized DNA constructs are then purified and introduced into the parasite by electroporation. Transfected parasites are subsequently plated on semi-solid agar plates in the presence of the selective drug and colonies are picked to achieve the genetic homogeneity of a clonal cell line. Because *Leishmania* are diploid organisms, a second round of targeted gene replacement is necessary to create knockout parasites. Loss of heterozygosity can also be induced, however, a negative selection scheme is necessary to stimulate the loss of the second gene copy. Southern blotting and/or polymerase chain reactions (PCR) are utilized to characterize the resulting genotype and to verify that the anticipated homologous recombination event occurred in both heterozygous and homozygous parasites.

The deletion of an essential gene is only possible if the mutants can be rescued by nutrient supplementation or other salvage strategies. For example, the removal of polyamine biosynthetic pathway genes from *Leishmania* is possible because the auxotrophic mutants survive in the presence of exogenous polyamines. Knockouts of essential genes cannot be performed if rescue strategies are unavailable.

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**Figure 3. Targeted gene replacement.**

*^A* Replacement constructs are introduced into parasites via electroporation. *^B* The target gene is replaced with the drug resistance cassette when homologous recombination occurs. *^C* Parasites are plated on semi-solid agar plates containing the selective drug and colonies are picked. *^D* Colonies are expanded in liquid media. The genotype is confirmed by Southern blotting or PCR analysis. The process is subsequently repeated to replace the second gene with a different drug resistance marker.
However, the ability to generate chromosomal gene deletions in the presence, but not absence, of episomes that express the targeted gene is one way to demonstrate that a gene is indispensable [29, 30].

An important control experiment is to generate complemented or add-back strains, where the deleted gene is re-introduced into the knockout strain either on an episome or as a chromosomal integration [31-38]. Comparison of the knockout cell line and the complemented strain ensure that the observed phenotypic changes are indeed caused by the gene deletion event and not by other sporadic mutations or adaptations that might have occurred.

The opposite of gene deletion, the introduction of genes into Leishmania parasites, is also possible. Constructs containing endogenous genes can be introduced to generate overproducer strains and the phenotypic characterization of the resultant mutant can provide valuable information about the target gene. Furthermore, genes from different species can be transfected to create transgenic parasites. Endogenous or exogenous genes can be introduced as part of an episome or stably integrated into the chromosome. Advantages of these expression systems range from the introduction of reporter genes to facilitate imaging of fusion proteins or whole organisms in in vivo infections [39-41], expression and purification of recombinant proteins [42-44], and functional studies that help to identify genes involved in drug sensitivity, protism, virulence or the host immune response [45-48].

It is important to note that the cultivation of Leishmania parasites is straightforward. Extracellular parasites can be maintained as the promastigote (insect) form indefinitely and in large quantities in liquid culture medium. Amastigotes can also be cultured as an extracellular or axenic form by utilizing acidic media and higher temperatures, which mimic the host phagolysosomal environment. Although axenic amastigotes are only a model system and not true intracellular amastigotes, the advantage is that parasite metabolism can be nutritionally and biochemically characterized without the contamination of host components. Furthermore, infectivity studies in vitro in macrophages as well as in vivo in mice and hamsters are now routinely performed.

The Polyamine Pathway in Promastigotes

We initiated our analysis of the polyamine pathway in Leishmania by generating gene deletion mutants in L. donovani promastigotes. Three knockout lines, LdAodc, LdAspd, and LdΔadometdc, were created [32, 33, 35]. In addition, complemented control cell lines were generated that contained copies of the deleted genes on episomes; LdAodc [ODC], LdAspd [SPD], LdΔadometdc [ADOMETDC]. The knockout mutants were not able to produce putrescine (LdAodc) or spermidine (LdAodc, LdAspd, LdΔadometdc) confirming that no alternative pathways for polyamine production are present in Leishmania parasites. As expected, the genetically manipulated promastigotes require polyamine supplementation for survival. The LdAodc parasites are able to grow in spermidine; however, much higher concentrations of spermidine compared to putrescine are required [32].

Thus, spermidine is sufficient and essential for promastigote proliferation; but putrescine is necessary for optimal proliferation. This observation suggests that putrescine is not merely a precursor for spermidine synthesis but plays an additional and important role in Leishmania.
Polyamine pool analysis of parasites revealed that spermine is not being produced by the parasite (Figure 4) and a spermine synthase gene is not present in the annotated genome databases of *Leishmania* species. This is an unexpected discovery, as spermine is postulated to be a crucial metabolite in mammalian cells [49].

![Polyamine biosynthesis and transport](image)

**Transporters for arginine (AAP3) and for putrescine/spermidine (POT1) have been identified on the molecular level. Ornithine transport has been characterized. Individual transporters for putrescine and spermidine have been proposed. Parasite ARG is located in the glycosome, necessitating import of arginine into the glycosome and export of ornithine from the glycosome to the cytosol. ODC, SPD, and ADOMETDC (not shown) are located in the cytosol.**

**Figure 4. Polyamine biosynthesis and transport in *Leishmania* promastigotes.**

Analysis of the knockout parasites also revealed that the back-conversion pathway present in mammalian cells is lacking in *Leishmania* (Figure 4). Spermidine cannot be converted to putrescine. Similarly, spermine, although taken up by parasites, is not further metabolized or utilized [32]. Furthermore, the half-life of the leishmanial ODC and ADOMETDC proteins are much longer than that of their human counterparts [35, 50]. This is a relevant observation since the selectivity of the suicide inhibitor DFMO for *T. brucei* is based on the fact that ODC is a stable enzyme in the parasite but is rapidly turned over in the human host; DFMO binds to human and parasite ODC with similar affinities [51] but the human protein is re-synthesized continuously.

More recently, investigations into the hypusine pathway have been initiated in protozoan pathogens [29, 52-56]. *Leishmania* parasites contain two putative *DHS* genes (*DHSL20, DHS34*) and one putative *DOHH* gene [29, 52]. Biochemical characterization of the proteins encoded by the two putative *DHS* genes revealed that one protein (*DHSL20*) was not functional as a DHS but the other protein, *DHS34*, expressed the expected catalytic activity [29]. Unfortunately, gene deletion mutants of *DHS* or *DOHH* cannot be generated if the enzymes are essential, because rescue by supplementing modified eIF5A is not possible. As expected, a *DHS* null mutant could not be produced. However, chromosomal knockouts were generated in cell lines that carried an episome containing the *DHS* coding sequence [29].
circumstantial evidence implies that DHS is an essential enzyme in *Leishmania*. Interestingly, the sequence of the functional and dispensable DHS protein is strikingly different from the human DHS, with unique insertions and an overall low identity of 32.8%, evoking the possibility that selective inhibitors may be identified.

Taken together, the characterization of the gene deletion mutants in the promastigote stage of *Leishmania* validates the polyamine pathway as a prospective therapeutic target. The biosynthetic enzymes are necessary for the synthesis of the essential polyamines and the pathway is substantially different from the human polyamine pathway.

These disparities include the absence of SPM and the lack of a back-conversion pathway in *Leishmania*, substantial sequence differences in the sequence of at least some of the polyamine enzymes compared to the human counterparts, and the varied half-lives of the human and parasite ODC and ADOMETDC proteins.

### Effect of Polyamine Analogues on Promastigote Proliferation

Several groups have examined the effects of polyamine analogues on *Leishmania* proliferation and infectivity. DFMO is toxic to *Leishmania* promastigotes, axenic amastigotes and intracellular amastigotes in macrophages [36, 57-60]. Furthermore, the drug inhibits parasite proliferation in mice and hamsters [61-64].

Several other ODC inhibitors have been evaluated: 3-aminooxy-1-aminopropane (APA), 1,4-diamino-2-butanone (DAB), and gamma-guanidinoxypropylamine (GAPA) inhibit proliferation of promastigotes and amastigotes, and furthermore APA and DAB have shown to reduce macrophage infectivity substantially [65-67].

#### Table 1. Amino acid sequence comparison of *Leishmania* and mammalian polyamine biosynthetic enzymes

<table>
<thead>
<tr>
<th><em>Leishmania</em> protein</th>
<th>Sequence identity to human counterpart</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. mexicana</em> ARG</td>
<td>38.5%</td>
<td>Roberts et al. 2004</td>
</tr>
<tr>
<td><em>L. donovani</em> ODC</td>
<td>40%(to murine counterpart)</td>
<td>Hanson et al. 1992</td>
</tr>
<tr>
<td><em>L. donovani</em> SPD</td>
<td>56%</td>
<td>Roberts et al. 2001</td>
</tr>
<tr>
<td><em>L. donovani</em> ADOMETDC</td>
<td>30%</td>
<td>Roberts et al. 2002</td>
</tr>
<tr>
<td><em>L. donovani</em> DHS34</td>
<td>32.8%</td>
<td>Chawla et al. 2010</td>
</tr>
<tr>
<td><em>L. donovani</em> DOHH</td>
<td>40.6%</td>
<td>Chawla et al. 2012</td>
</tr>
</tbody>
</table>

DAB and GAPA inhibit putrescine uptake [65, 67], and DAB causes mitochondrial damage [67], indicating that putrescine may be particularly important for mitochondrial function or structure. Moreover, DFMO, APA and DAB are not toxic to macrophages at concentrations that profoundly affect the ability of the parasite to grow [36, 66]. Bis (benzyl) polyamine analogues, known inhibitors of both ODC and ADOMETDC enzyme activity, lower intracellular polyamine levels *in vitro* [68], and administration of N,N-bis(3-
[(phenylmethyl) amino] propyl)-1,7-diaminoheptane (MDL27695) suppresses \textit{L. donovani} liver burden in mice by 50\% [63] or 99\% [69]. Recently, the structure of the leishmanial ODC bound to APA has been modeled according to the solved crystal structure of the human ODC – APA complex [70]. The structure, kinetic properties, and half-life of the leishmanial ODC are substantially different from the human counterpart [50, 70] and the structural information may prove useful for the design or computer-based screening of novel ODC inhibitors.

**Overproducer Strains As Tools to Investigate the Mode of Action of Polyamine Analogues**

To test whether polyamine analogues indeed target enzymes of the polyamine pathway, a cell based screening assay has been developed that takes advantage of polyamine enzyme overproducing strains. The genetically manipulated pathogens are used as tools to investigate the mode of action of cytotoxic analogs in \textit{Leishmania} based on the prediction that enzyme overproducer strains will be more resistant to a drug that specifically targets the overproduced protein. Wild type parasites were transfected with episomal constructs containing the ODC, SPD, or ADOMETDC gene. The episomal constructs also contained drug resistance markers that allowed selection of transfected parasites. Furthermore, increased drug concentrations facilitated the overexpression of the polyamine biosynthetic proteins, presumably due to the high copy number of the episomes. Wild type and overproducer strains were then incubated in serial inhibitor dilutions and the EC [50] values were compared. These experiments confirmed that DFMO, 5-([Z]-4-amino-2-butenyl) methylamino)-5-deoxyadenosine (AbeAdo or MDL73811), and n-butyamine target ODC, ADOMETDC, and SPD, respectively, as had previously been proposed [71-73]. Conversely, pentamidine, berenil, and methylglyoxal bis (guanylhydrazone) (mitoguazone or MGBG) did not kill parasites via polyamine enzyme inhibition. These compounds have previously been speculated to be toxic solely due to inhibition of the polyamine biosynthetic enzymes but other cellular targets have also been proposed [74-80]. The overproducer strains were utilized for a pilot screen of 25 compounds and we demonstrated that this system offers a rapid cell-based screen for assessing whether synthetic polyamine analogs exert their toxicity predominantly by targeting the polyamine biosynthetic enzymes in \textit{Leishmania}. Ultimately, the drug resistance induced by amplification of target genes and resulting overproduction of the encoded protein offers a general strategy for evaluating the mode of action of therapeutic agents.

**Relevance of the Polyamine Biosynthetic Pathway for \textit{Leishmania} Infectivity**

Although studies in promastigotes were promising, the ultimate validation of the polyamine pathway as a medical target requires the evaluation of the infective amastigote stage of the parasite. A long debated but unsolved question remained whether amastigotes were capable of salvaging polyamines from the phagolysosomal compartment in macrophages. On the one hand, short term infectivity studies in mice and hamsters revealed
substantial growth inhibition of intracellular parasites with administration of DFMO, although parasites were not eradicated [61-64]. On the other hand, the phagolysosome has been postulated to be rich in nutrients, including polyamines [81-84], and polyamine uptake has been demonstrated in both the promastigote and amastigotes form [85-88]. Thus, the prevailing belief has been that Leishmania parasites reside in a polyamine rich environment and that the polyamine biosynthetic pathway would therefore not be a promising therapeutic target. Gene deletion mutants present an ideal tool to solve this controversy since these organisms are not able to synthesize endogenous polyamines and thus depend on salvage of polyamines for proliferation.

When peritoneal macrophages were infected with wild type and LdAodc parasites, a substantial reduction in pathogen per macrophage were observed 72 hours after infection [36]. These results were mirrored in murine infectivity studies that revealed a profound reduction in parasite number in both liver and spleen after 4 weeks of infection. The average number of parasites per gram liver was \( \sim 10^5 \) in mice infected with wild type L. donovani but only \( \sim 10^2 \) parasites in mice infected with LdAodc cell lines [36]. Administration of putrescine to the drinking water of the infected mice partially restored the loss of infectivity of LdAodc parasites, demonstrating that the reduced intracellular survival was indeed due to the deletion of ODC and lack of endogenous putrescine synthesis [64]. Similar results were obtained when LdAspd parasites were used in murine infectivity studies [38]. However, a slightly higher number of LdAspd parasites were recovered from the livers of infected mice (\( \sim 10^3 \) organisms per gram liver) compared to LdAodc (\( \sim 10^2 \) organisms per gram liver). The cause for this disparity in infectivity phenotype between the LdAodc and LdAspd parasites is unclear but could be due to differences in either the amount of available putrescine versus spermidine in the phagolysosomal compartment or differential uptake abilities of intracellular amastigotes. These infectivity studies demonstrated that ODC and SPD are essential for a robust infection and that insufficient amounts of polyamines are present in the phagolysosomal compartment. Because L. donovani parasites cannot salvage sufficient amounts of putrescine or spermidine, the polyamine biosynthetic enzymes ODC, SPD, and ADOMETDC are potential targets for therapeutic treatment of visceral leishmaniasis.

**The Role of Parasite Arginase for Promastigote Proliferation**

In Leishmania, arginase (ARG) converts the essential amino acid arginine to ornithine, which is then directly funneled into polyamine biosynthesis [89]. ARG gene deletion mutants have been generated in L. mexicana, L. major, and L. amazonensis [89-91]; all three are agents of cutaneous leishmaniasis. Studies in the promastigote stage of the parasite determined that the sole essential role of ARG is the synthesis of ornithine for polyamine biosynthesis and that the gene deletion mutants required ornithine or putrescine supplementation for survival [89-91]. These studies in the promastigote stage of the parasite establish the parasite ARG as an essential enzyme for polyamine biosynthesis.

Intriguingly, the leishmanial ARG sequence contains a SKL motif, which directs the enzyme to the glycosomal compartment, while the remaining polyamine biosynthetic enzymes are localized to the cytosol [89]. The glycosome, an essential cellular organelle
unique to *Leishmania* and similar parasites, is evolutionary related to the peroxisomes of mammalian cells. In *Leishmania*, glycosome biogenesis is essential and the organelle houses several crucial metabolic and biosynthetic pathways; however, it is unknown why these pathways are restricted to the glycosome [92, 93]. To investigate the importance of the glycosomal localization for ARG function, a SKL deletion mutant was expressed in the *L. mexicana Darg* background [89]. Phenotypic characterization confirmed that the mutant protein was expressed at similar levels as the wild type protein and that it was mislocalized to the cytosol. Proliferation studies revealed that the glycosomal localization of ARG was not necessary for its function in *L. mexicana* promastigotes [89]. Similar experiments in *L. amazonensis*, however, found that a mislocalized argAΔskl protein was expressed at only very low levels in the cytosol, indicating that proper localization is significant in *L. amazonensis* promastigotes [90]. However, the promastigotes were able to survive without ornithine or putrescine supplementation, presumably due to residual arginase activity of the argAΔskl protein [90].

Another intriguing observation is that although ornithine is the direct product of the ARG reaction, much higher concentrations of exogenous ornithine (>1mM) than putrescine (5–10 μM) are necessary to support optimal proliferation of *L. mexicana Darg* promastigotes [89]. Similar discrepancies between ornithine and putrescine requirements were observed in *L. major Darg* promastigotes [91]. Uptake assays established that transport capabilities are similar for ornithine and putrescine in *L. mexicana* and thus do not account for the disparity in nutrient requirements [89]. It is feasible, that ODC is the rate limiting enzyme for polyamine biosynthesis and the finding that *L. mexicana* parasites contain much higher levels of intracellular ornithine (~130 nmol/10^7 parasites) than putrescine (~2.5 nmol/10^7 parasites) supports this conjecture [89]. The observation that parasites contain elevated pools of ornithine also offers a hypothesis for the compartmentalization of ARG to the glycosome. Since the parasites generate a high amount of ornithine, cytosolic arginine would be rapidly depleted and not available for protein synthesis if ARG were localized to the cytosol. The experiments described above were performed in arginine rich culture media, which may explain why parasites proliferated well despite a mislocalized argAΔskl enzyme.

The *Leishmania* genome also contains a putative *AGM* gene (*L. mexicana* CBZ27141.1); however, extensive genetic and biochemical experiments have demonstrated that this gene does not encode a functional AGM or ARG, and targeted gene deletion studies revealed that the gene product is not an essential protein (Riley, Roberts, and Ullman, personal observations). Interestingly, an ortholog of this putative *AGM* sequence is also present in the genomes of the other two trypanosomatids, *T. brucei* and *T. cruzi*. The ADC/AGM alternative pathway for putrescine production clearly does not exist in *Leishmania* as *Δode* null mutants are not viable without putrescine supplementation [32].

**The Role of Host and Parasite Arginase for Infectivity**

Mammalian host cells contain two types of ARG: type I, which is cytosolic and expressed at high levels in the liver, and type II, which is located in the mitochondria [94]. In macrophages arginine is a key amino acid for two competing pathways: arginine can be
converted to ornithine by the action of ARG or alternatively to the potent anti-leishmanial agent nitric oxide by the inducible nitric oxide synthase (iNOS) [95, 96]. Murine infectivity models have documented an increased expression of ARG I in susceptible Balb/c mice associated with a TH-2 cell response and increased parasite proliferation [95, 97-101]. Conversely, the expression of iNOS has been correlated with a TH-1 cell response and decreased parasite loads in the Leishmania resistant CBA mouse model [97, 102, 103]. Numerous experiments have now correlated increased ARG I activity with augmented parasite loads [96, 97, 100]. In addition, pharmaceutical inhibition of host ARG has been found to reduce parasite numbers in macrophages and mice [97, 98, 100]. Thus, the mammalian ARG has been firmly established as a key factor for Leishmania infections [97-101, 104-108]. It is not completely understood how increased levels of host ARG I cause or contribute to disease exacerbation. One effect of higher ARG activity may be a depletion of arginine levels, which could reduce the production of nitric oxide. A local reduction of arginine has also been shown to impair the development of T cells leading to suppression of the immune response and increased parasitemia [109-111]. Furthermore, it has been speculated that increased ARG activity enhances the levels of host polyamines, which may be salvaged by intracellular parasites and thus increase their proliferation [96, 100, 101].

To determine the role of parasite ARG for infectivity, in vitro and in vivo infectivity studies have been performed with all three Δarg strains (L. mexicana, L. major, and L. amazonensis Δarg) [37, 90, 91, 112]. In vitro infectivity studies established that both L. mexicana wild type and Δarg parasites were able to scavenge ornithine and putrescine from the phagolysosome of macrophages incubated in supplemented media. However, investigations in BALB/c mice demonstrated that infectivity with L. mexicana Δarg was markedly reduced compared to wild type parasites. [37] Similarly, the L. major Δarg and L. amazonensis Δarg deficient strains exhibited a reduced infectivity phenotype in mice [90, 91, 112]. The diminished infectivity of the L. mexicana Δarg parasites appeared to correlate with an increased potential to produce nitric oxide by macrophages infected with L. mexicana Δarg parasites compared to macrophages infected with wild type parasites [37]. Furthermore, no difference in infectivity was observed between L. mexicana wild type and Δarg parasites in iNOS-deficient macrophages. One possible explanation for the observation that L. mexicana Δarg parasites are less infective is that the ARG deficient parasites cannot use arginine for polyamine production, and consequently have a reduced uptake and need of host arginine. This, in turn, would leave the host cell with more arginine available for the synthesis of nitric oxide. Such a conjecture is also plausible because studies have demonstrated that the level of the L. donovani LdAAP3 arginine transporter and thus, arginine uptake, is regulated by intracellular arginine levels [113]. In addition, it is likely that arginine levels in macrophages are limited since they are governed solely by availability and uptake from the plasma. Indeed, macrophages that lack the arginine transporter mCAT2B exhibit reduced levels of both polyamines and nitric oxide [96, 114]. Thus, intracellular amastigotes and the host compete for a limited arginine pool and the balance between parasite and host metabolism of arginine may be crucial in determining the outcome of leishmanial infections.

Intriguingly, a correlation between reduced infectivity and increase nitric oxide production was not found in macrophages infected with L. major Δarg, suggesting that differences in the various cutaneous Leishmania species and their interactions with the mammalian host exist. Overall, a rational explanation for the reduced infectivity of the different cutaneous Δarg Leishmania strains is that although ornithine or putrescine can be
scavenged from the phagolysosome, endogenous biosynthesis produces more of these essential nutrients.

Taken together, these studies suggest important roles for both host and parasite arginase for optimal parasite infections. Thus, although inhibition of parasite arginase alone would not be a sufficient therapeutic strategy, the dual inhibition of host and parasite arginase may provide a novel treatment strategy. Inhibition of both enzymes would prevent polyamine biosynthesis and may furthermore stimulate host defense mechanisms like nitric oxide production. The amino acid sequence of the *Leishmania* and human ARG enzyme is ~38% identical and the kinetic and biochemical characteristics of the recombinant enzymes are similar [115]. A preliminary screen identified inhibitors that target both recombinant enzymes [115], supporting the concept that dual inhibition of human and parasite enzyme may be possible.

**Polyamine Uptake by *Leishmania* Parasites**

Both promastigotes and amastigotes are able to synthesize polyamines *de novo* [32, 36] and have the ability to transport polyamines [85-88]. Studies in *L. donovani* and *L. mexicana* promastigotes found multiple polyamine transport systems, which suggests that different transporters exist for putrescine and spermidine uptake [85, 88]. The gene of the first eukaryotic polyamine transporter was cloned from *L. major* (LmPOT1) [87]. In order to functionally characterize the putative transporter, the gene was expressed in *Xenopus laevis* oocytes and furthermore transfected into the related parasite *T. brucei*, which has poor endogenous putrescine transport.

The LmPOT1 transporter has high affinity for both putrescine and spermidine. However, while LmPOT1 is expressed in *L. major* promastigotes, the transporter could not be detected in amastigotes. A comparison between *L. mexicana* promastigotes and amastigotes also suggest that disparate transport systems exist in the two different developmental stages of the parasite [85].

Studies moreover revealed that at least putrescine uptake is regulated. Inhibition of ODC by DFMO resulted in increased putrescine uptake in promastigotes and conversely, elevated concentration of putrescine in the media resulted in decreased putrescine uptake [86, 88]. It is well established that intracellular levels of polyamines are intricately regulated in mammalian cells and it appears that the same may be true for *Leishmania* parasites. The observation that polyamine enzyme overproducer strains only modestly increase intracellular polyamine levels despite profoundly elevated enzyme levels also argues that polyamine pools are controlled in the parasite [46].

While polyamine uptake is not critical for *Leishmania*, the import of the amino acid arginine is essential. Arginine is vital for both polyamine and protein synthesis. An arginine transporter, AAP3, has been identified and characterized in *L. donovani* and *L. amazonensis* [113, 116]. In promastigotes, intracellular arginine pools regulate the amount of *Leishmania* AAP3 arginine transporter and thus arginine uptake [113, 116]. It is likely that arginine uptake is also regulated in amastigotes and that both parasite and host cell compete for intracellular arginine [37].
Interaction of Host and Parasite Polyamine Pathways in *Leishmania* infections

Infectivity studies with gene deletion mutants can be used to address whether host polyamine metabolism influences parasite polyamine homeostasis. *LdΔode* parasites exhibit dramatically reduced infectivity phenotypes in macrophages and mice [36]. Since amastigotes are able to transport putrescine, the logical explanation is that insufficient amounts of host putrescine are available.

Such a conjecture is not unreasonable because host polyamine metabolism is intricately regulated and putrescine levels are maintained at constant levels. Thus, it appears that *L. donovani* parasites do not benefit from host putrescine pools but depend on endogenous putrescine biosynthesis. The *LdΔspd* parasites also show a severely reduced infectivity phenotype but to a lesser degree than *LdΔode* [38].

It should be noted, that spermidine is the essential and sufficient polyamine in promastigotes [32]. Thus, the observation that intracellular *LdΔode* amastigotes are more incapacitated than *LdΔspd* amastigotes is somewhat surprising. Possible explanations include that less putrescine than spermidine is present in the phagolysosome or that *L. donovani* amastigotes have unequal uptake capabilities for the two polyamines. In contrast, *Leishmania* deficient in ARG exhibit only a moderate loss in infectivity compared to the severely reduced infectivity of *LdΔode* and *LdΔspd* parasites. All three ARG deficient strains that were generated, *L. mexicana*, *L. major*, and *L.amazonensis* Δarg, were still able to establish infections [37, 90, 91, 112].

The differences in infectivity phenotypes can be rationalized by the assumption that ornithine is present in higher quantities than putrescine or spermidine in the phagolysosome or that ornithine uptake in amastigotes is much more robust than uptake of the polyamines. One should recall thought that Δarg promastigotes require much higher concentration of exogenous ornithine than putrescine to allow optimal growth [89, 91], an observation that contradicts the hypothesis that amastigotes would be more successful in scavenging ornithine than putrescine.

Furthermore, it needs to be emphasized that the Δode and Δspd parasites have been generated in *L. donovani*, a visceralizing strain, whereas the Δarg deletions were introduced into *Leishmania* species that cause a cutaneous disease. It is feasible that the ornithine and polyamine milieu of macrophages of the skin is different from that of macrophages residing in liver and spleen.

The generation and direct comparison of Δarg, Δode, Δspd mutants within the same species is necessary to solve this conundrum. Nevertheless, a model of host and parasite polyamine metabolism and salvage has been constructed that can be used as a basis for further investigations (Figure 5).

This model speculates that high amounts of ornithine are available for parasite uptake but only limited amounts of putrescine and spermidine are present in the phagolysosome. Although the augmented host ARG activity in *Leishmania* infections has always been speculated to produce increased amounts of polyamines for parasite salvage, ornithine may actually be more important than polyamines for parasite scavenging and enhanced proliferation.
Comparison of Host-Parasite Interactions Among Trypanosomatid Parasites

The polyamine pathway has been investigated in several other protozoan parasites, including the trypanosomatids *T. brucei* and *T. cruzi*. Similar genetic manipulations have been performed to elucidate the metabolic pathway in *T. brucei*. In addition to classical knockout studies, the technique of RNA interference (RNAi) has been utilized [117-120]. RNAi has the advantage of allowing inducible knockdown of target gene expression and thus facilitates the study of essential genes. However, a drawback of RNAi is that sometimes the knockdown is achieved at only partial levels and residual enzyme activity may be present. It is of interest to note that most *Leishmania* species have lost the protein repertoire necessary for the RNA silencing machinery.

Investigations in *T. brucei* and *T. cruzi* have discovered a unique regulatory protein — prozyme — that is required for ADOMETDC activation [118, 121-123]. This regulatory mechanism is distinct from that of mammalian cells. Prozyme has not been studied in *Leishmania*, but the gene encoding this protein is present in the genome database of several *Leishmania* species and it is thus likely that the *Leishmania* ADOMETDC also requires heterodimerization with prozyme for full activation.

![Polyamine metabolism diagram](image)

The polyamine biosynthetic enzymes of *Leishmania* amastigotes and the host polyamine biosynthetic enzymes are displayed. Arginine uptake by amastigotes is essential and indicated with a solid black arrow. Salvage of ornithine is postulated to be efficient and indicated with a solid grey arrow. Uptake of putrescine and spermidine is likely to be minimal and thus indicated with hashed arrows.

Figure 5. A model of host and parasite polyamine metabolism and parasite scavenging.
T. brucei parasites contain a polyamine biosynthetic pathway similar to *Leishmania* consisting of ARG, ODC, SPD and ADOMETDC [117-120, 124-126]. However, in contrast to *Leishmania*, T. brucei parasites are extracellular pathogens that are present in the host's blood stream. Polyamine levels in blood are low and thus T. brucei is dependent on endogenous polyamine biosynthesis [118]. The lack of salvageable polyamines offers an explanation for the efficacy of the ODC inhibitor DFMO in the treatment of African sleeping sickness.

The polyamine pathway in *T. cruzi* is more austere than that of the other trypanosomatids; they are not able to synthesize putrescine from amino acid precursors [127-130]. Only two other eukaryotes are known to be incapable of *de novo* polyamine biosynthesis: *Toxoplasma gondii* and *Cryptosporidium parvum* [131, 132].

*T. cruzi* parasites lack both ARG and ODC and although an ADC activity was initially reported [133, 134], it is now generally accepted that the alternative ADC/AGM pathway does not exist in *T. cruzi*. It has been speculated that the trypanosomatids have lost their ODC gene followed by horizontal gene transfer of the ODC sequence from a vertebrate host into *T. brucei* [135]. Support for this hypothesis comes from phylogenetic analyses, which show the *T. brucei* ODC clustered closer to the ODC genes from vertebrates than protozoans. Because of the lack of *de novo* putrescine synthesis, *T. cruzi* parasites are absolutely dependent on putrescine scavenge from the host. Like *Leishmania*, *T. cruzi* is an intracellular parasite taken up by phagocytosis. However, *T. cruzi* parasites escape the phagosome to reside in the host cell cytosol. Thus, the pathogen has access to presumably more abundant polyamine pools compared to *Leishmania* parasites, which live in phagolysosomes.

In summary, the interactions between host and parasite that are necessary to maintain polyamine homeostasis in the pathogen are strikingly different among the three trypanosomatid parasites (Figure 6).

*Leishmania* parasites reside inside the phagolysosome and although they appear to rely on endogenous polyamine biosynthesis (*L. donovani*) they are capable of at least limited ornithine salvage (*L. mexicana, L. major, L. amazonensis*). *T. cruzi* parasites inhabit the cytosol and absolutely depend on putrescine salvage because they are not able to synthesize polyamines *de novo*. Finally, *T. brucei* parasites are extracellular pathogens that live in the bloodstream - a polyamine poor milieu - and rely on endogenous polyamine biosynthesis.

**Conclusion**

Genetic techniques, foremost the ability to generate gene deletion mutants, have facilitated the elucidation of the polyamine biosynthetic pathway in *Leishmania* parasites. Furthermore, the gene deletion mutants are invaluable tools to investigate the relevance of polyamine biosynthesis for parasite infectivity and the interplay between host and parasite polyamine pathways.

In addition, the ability to generate overproducer strains can be utilized for the development of cell-based screening assays that test the mode of action of inhibitory compounds. In this manner, the process of genetic manipulation has been applied to validate the polyamine pathway in *Leishmania* parasites as a potential therapeutic target.
Genetic Manipulation of *Leishmania* Parasites ...

**A** *T. cruzi* amastigotes reside free in the host cell cytosol

**B** *Leishmania* amastigotes proliferate in phagolysosomes inside the host cell

**C** *T. brucei* are extracellular parasites that live in the bloodstream

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*T. cruzi* amastigotes evade the phagosome to reside free in the cytosol and thus have access to the host polyamines putrescine (○) and spermidine (■). Because they are incapable of de novo putrescine synthesis they depend on polyamine salvage. *Leishmania* amastigotes proliferate in phagolysosomal compartments. They are able to synthesize polyamines and although they can salvage ornithine (★), polyamine uptake appears to be limited. *T. brucei* parasites are extracellular pathogens and rely on endogenous polyamine synthesis because polyamine levels in blood are minute.

Figure 6: Comparison of host-parasite interactions among trypanosomatid parasites.

Substantial differences between the polyamine pathways of host and parasite have been revealed. *Leishmania* pathogens lack a back-conversion pathway, do not express spermine synthase, and the polyamine biosynthetic enzymes are more stable than their human counterparts.

An alternative pathway of putrescine production via ADC and AGM is not present in *Leishmania*, and the putative AGM that is present in the *Leishmania* genomic databases does not function as such. Hence, the *Leishmania* polyamine pathway consists of only four enzymes: ARG, ODC, SPD and ADOMETDC. The hypusination pathway that utilizes spermidine for the modification and activation of eIF5A is present in *Leishmania* and both enzymes, DHS and DOHH, have recently been described. Sequence comparisons between the host and parasite enzymes reveal that similarities are moderate, and especially low for DHS and ADOMETDC, which may offer targets for selective inhibitor development. Infectivity studies with *L. donovani* gene deletion mutants revealed that the *LdΔodc* parasites show profoundly reduced infectivity phenotypes compared to wild type parasites and validated ODC as a therapeutic target. Similarly, *LdΔspd* parasites exhibited severely decreased infectivity, although not as severely impaired as observed in the *LdΔodc* cell line. These
observations challenged the conventional belief that polyamines are abundant in the phagolysosomal compartment.

Conversely, Δarg strains that were generated in *L. mexicana*, *L. major* and *L. amazonenesis* show only moderate reduction in infectivity. These observations suggest that ornithine levels are much higher than putrescine and spermidine pools in the phagolysosome. Studies suggesting that an increased host ARG activity is crucial for the exacerbation of leishmaniasis and that host polyamine synthesis is tightly regulated support this conjecture. However, it should be noted that *L. donovani* is a visceralizing strain whereas *L. mexicana*, *L. major* and *L. amazonenesis* cause cutaneous leishmaniasis. Thus, the discrepancies in infectivity phenotypes may also be due to differences in the *Leishmania* species and/or host cell type (macrophages of skin versus liver and spleen).

In conclusion, studies with genetically manipulated *Leishmania* strains revealed that the enzymes of the polyamine biosynthetic pathway, especially ODC and SPD, are potential therapeutic targets. Furthermore, the dual inhibition of host and parasite polyamine synthesis, particularly targeting the host ARG, may have a synergistic effect and is worthwhile to explore.

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