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L-Asparaginase of *Leishmania donovani*: Metabolic target and its role in Amphotericin B resistance

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ABSTRACT

Emergence of Amphotericin B (AmB) resistant *Leishmania donovani* has posed major therapeutic challenge against the parasite. Consequently, combination therapy aimed at multiple molecular targets, based on proteome wise network analysis has been recommended. In this regard we had earlier identified and proposed L-asparaginase of *Leishmania donovani* (LdAI) as a crucial metabolic target. Here we report that both LdAI overexpressing axenic amastigote and promastigote forms of *L. donovani* survives better when challenged with AmB as compared to wild type strain. Conversely, qRT-PCR analysis showed an upregulation of LdAI in both forms upon AmB treatment. Our data demonstrates the importance of LdAI in imparting immediate protective response to the parasite upon AmB treatment. In the absence of structural and functional information, we modeled LdAI and validated its solution structure through small angle X-ray scattering (SAXS) analysis. We identified its specific inhibitors through ligand and structure-based approach and characterized their effects on enzymatic properties (K_m, V_{max}, K_{cat}) of LdAI. We show that in presence of two of the inhibitors L1 and L2, the survival of *L. donovani* is compromised whereas overexpression of LdAI in these cells restores viability. Taken together, our results conclusively prove that LdAI is a crucial metabolic enzyme conferring early counter measure against AmB treatment by *Leishmania*.

1. Introduction

Leishmaniasis, caused by protozoan parasite *Leishmania* manifests into three major clinical forms, cutaneous, mucocutaneous and visceral which is usually but not exclusively species specific (Desjeux, 2004). Considering all forms, the disease is prevalent in 98 countries with estimated 1.3 million new cases worldwide (Alvar et al., 2012). However, the major death toll (20000–30000 deaths per year) results mainly from visceral leishmaniasis (Kala-azar) caused by *Leishmania donovani* and is predominantly endemic in the East Africa and Indian subcontinent (Alvar et al., 2012). Due to high morbidity rates and rapidly developing drug resistance, the parasite causes socio-economic loss and accounts to ninth largest disease burden among all infectious diseases (Chakravarty and Sundar, 2010; Croft et al., 2006; Alvar et al., 2012). Shortcomings of current line therapies for the disease pose challenges in developing novel anti-microbial only in leishmaniasis but also other parasites infecting humans. This calls for exploring newer and potent drug targets with clear understanding of the molecular basis of drug resistance (Chandra and Puri, 2015, Gilleard and Beech, 2007; Mateos-Gonzalez et al., 2015; Venkatesan and Borrmann, 2015; Saunders et al., 2013). *Leishmania* exhibits a digenetic life cycle as promastigotes in insects and amastigotes in mammals (Tsigankov et al., 2014). Upon infecting the host this protozoan resides inside the hostile environment of macrophages and differentially expresses wide array of proteins for survival (Naderer and Mcconville, 2011, Morales et al., 2008, 2010; Casgrain et al., 2016; Cull et al., 2014). Enzymes involved in energy metabolism and defense strategies such as suppression and evasion of host immune responses are primarily implicated in survival mechanisms (Mcconville, 2016; Mcconville and Naderer, 2011).

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Asparagine/glutamine related metabolic pathways in *Leishmania* had been subject of recent interest to understand their role in maintaining cellular homeostasis (Faria et al., 2016; Nowicki and Cazzulo, 2008; Manhas et al., 2014). For instance, aspartate uptake has been proved crucial in TCA cycle anaplerosis of *Leishmania mexicana* and glutamate as metabolic precursor for other pathways (Saunders et al., 2011; Nowicki and Cazzulo, 2008). More recently, through reconstructed energy metabolism network of *Leishmania infantum,* glutamate biosynthesis has been implicated as a crucial target against the parasite (Faria et al., 2016; Moreno et al., 2014). In many related pathogens such as *Salmonella typhi, Helicobacter pylori* etc. also, which need to survive under harsh acidic conditions inside host, Asn/Gln metabolism has been proved to be important for cellular survival, host invasion, mediation of virulence and host immunity (Scotti et al., 2010; Kullas et al., 2012; Shibayama et al., 2011). Interestingly, *Leishmania* manages to carry its digenetic life cycle by maintaining a neutral intracellular pH, in spite of acidic extracellular environment posed by the host macrophage (Zilberstein et al., 1991). Naturally, any tinkering with this pH difference across parasite's membrane will be detrimental for its survival. In this line, exposure to membrane altering agents such as polyene antibiotic Amphotericin B (AmB) has been successful in treating this parasite. AmB interacts with ergosterol within the membrane, altering its permeability through the formation of non-aqueous and aqueous pores resulting in positive K^+/H^+ gradient (Palacios and Serrano, 1978; Jiang et al., 1994; Herec et al., 2005; Cohen, 2010; Saha et al., 1986; Luque-Ortega et al., 2003). This in turn follows a caspase-3 dependent apoptosis of the pathogen (Saha et al., 1986; Cohen, 2010). Additionally, this antibiotic is known to exhibit immunomodulatory effects and foster parasitic clearance by stimulating the transcription and production of proinflammatory cytokines like TNF-α, IL-1β, MCP-1, MIP-1β, nitric oxide, prostaglandins and intercellular adhesion molecule-1 from murine and human immune cells (Mesa-Arango et al., 2012; Sau et al., 2003). This occurs via signaling molecules as Toll like receptor (TLR)-2, Bruton's tyrosine kinase (Btk) and phospholipase C (PLC) (Arning et al., 1995; Matsuo et al., 2006; Bellocchio et al., 2005; Mihu et al., 2014). Further AmB mediates induction of free radicals which in turn is linked to protein kinase C (PKC) (Mukherjee et al., 2010). Interestingly, the immediate countermeasures adopted by *L. donovani* to reduce pH imbalance inflicted by AmB have not been extensively studied. One defensive mechanism could be the over-expression of surface P-type K^+, H^+-ATPase, normally expressed in parasites for expelling protons by nutrient mediated symport (Jiang et al., 1994; Anderson and Mukkada, 1994). However, studies on AmB resistance by Brotherton et al. proposed against over-expression of K^+, H^+-ATPase, as this coutermeasure could result in unnecessary depletion of ATP in the parasite, already in stressed state (Brotherton et al., 2014). Therefore, to maintain a neutral intracellular environment in such conditions, an ATP independent mechanism should be ideally operating to neutralize H^+ gradient.

We hypothesize that the L-asparaginase (LdAI) of *Leishmania donovani* may be a crucial metabolic enzyme rendering immediate protection or defense to parasite on exposure to AmB. Our hypothesis is based on the fact that ammonia (NH^{4+}), released by the action of LdAI amidohydrolase on Asn/Gln, could be involved in counteracting the acid influx (acid stress) mediated by AmB induced membrane pore formation. Our hypothesis is supported by studies on a similar L-asparaginase of *Mycobacterium tuberculosis* where the ammonia released aids in its survival inside acidic environment of human alveolar macrophages (Gouzy et al., 2014). Likewise, *E. coli* exploits similar survival mechanism to resist environmental acidic stress by enzymatic release of ammonia through glutaminase (Lu et al., 2013).

Our hypothesis was validated when LdAI transfected strains of both promastigote and axenic amastigotes of *Leishmania donovani* showed increased tolerance and better survival against AmB compared to wild type strains. Retrospectively, LdAI upregulation was observed in both forms upon AmB treatment, supporting the fact that LdAI is conferring survival advantage to these AmB challenged parasites. We further characterized LdAI structurally and functionally. Earlier we had modeled the structure of this enzyme and proposed potential inhibitors, L1 and L2 (Singh et al., 2015). In this study we report that these inhibitors (albeit at high concentrations but non-toxic to humans) could effectively retard the growth of *Leishmania* indicating necessity of LdAI to the parasite. Overall this study on LdAI provides direct experimental evidence of its metabolic importance in *L. donovani* survival which had been overlooked in earlier genome wide network analysis. Although pathways for Asp/Glu production may exhibit redundancy, nonetheless the findings reveal new and additional functional measure taken by the parasite in counteracting drug effects.

2. Materials and methods

2.1. Construction of LdAI expression vectors and its transfection in L. donovani

The LdAI gene was PCR amplified from *L. donovani* genome using primer pairs 5′ TTTTAAGCTTACAATGGAAGCGGGAATAGAGG 3′ and 5′ TTTTCTCGAGTCACAGCTTCGCATGCACTTCG 3′ containing restriction sites *Hin*dIII and *Xho*I (underlined), respectively. The amplified product was cloned into pLpneo2 for expression in *Leishmania*. Positive clones were verified by sequencing. *L. donovani* AG83 cells were transfected with clone or empty vector using electroporation following the high-voltage protocol described by Robinson and Beverley (Beverley and Clayton, 1993). Transfectants were selected and maintained in the presence of G418 (100 μg/ml). RT PCR was used to verify the transcription of the cloned gene (as discussed in Section 2.3.2).

2.2. Survival of wild type and LdAI over-expressing parasites upon treatment with Amphotericin B and inhibitors L1 and L2

L. donovani wild type AG83 (WT) and pLP2-LdAI transfected promastigotes (WT-LdAI) were maintained in M199 medium (pH 5.5) containing 10% FBS as described. The late exponential phase parasites were appropriately diluted (2×10^6 promastigotes/ml) and treated with varying concentrations of Amphotericin B and previously screened inhibitors L1 and L2, prepared in M199 medium lacking FBS. Similarly, for testing chemosusceptiblity of wild type (WT) and LdAI transfected amastigotes (WT-LdAI) to AmB, axenically grown amastigotes were used as established in protocol by Sereno D et al. (Sereno and Lemesre, 1997). Parasite viability was evaluated using the quantitative colorimetric MTT assay where OD was measured at 570 nm by using micro plate reader. IC_{50} was defined as the concentration that reduced parasite survival by 50%. The results are expressed as % survival with mean and standard error from three independent experiments. IC_{50} of each compound and AmB was calculated by fitting the data into Dose-response (log (inhibitor) vs. normalized response) equation using GraphPad Prism 5:

$$Y = \frac{100}{1 + 10^{(X - LogIC50)}}$$

where IC_{50} is the concentration that gives response intermediate between the maximum and the minimum.