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Leishmania amazonensis: Increase in ecto-ATPase activity and parasite burden of vinblastine-resistant protozoa

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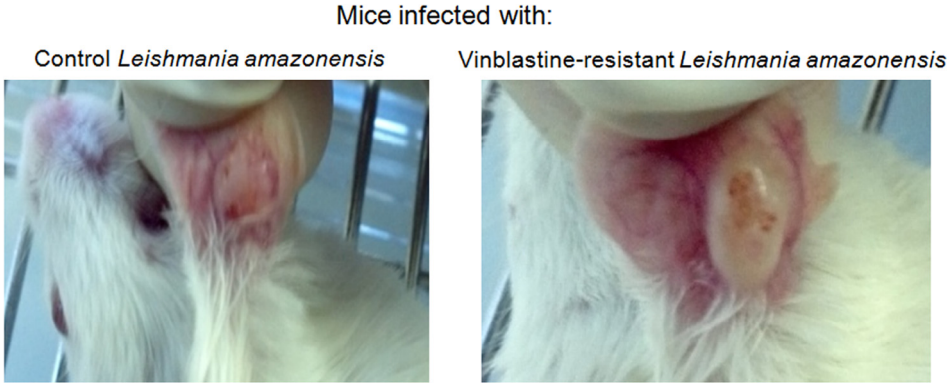
HIGHLIGHTS

- The vinblastine-resistant parasites show a higher ecto-ATPase activity.
- *Leishmania* infection severity is associated to higher ecto-ATPase activity.
- Resistant parasites selected by prolonged drug exposure show aberrant morphology.

GRAPHICAL ABSTRACT



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ABSTRACT

Leishmania amazonensis is a protozoan parasite that induces mucocutaneous and diffuse cutaneous lesions upon infection. An important component in treatment failure is the emergence of drug-resistant parasites. It is necessary to clarify the mechanism of resistance that occurs in these parasites to develop effective drugs for leishmaniasis treatment. Promastigote forms of *L. amazonensis* were selected by gradually increasing concentrations of vinblastine and were maintained under continuous drug pressure (resistant cells). Vinblastine-resistant *L. amazonensis* proliferated similarly to control parasites. However, resistant cells showed changes in the cell shape, irregular flagella and a decrease in rhodamine 123 accumulation, which are factors associated with the development of resistance, suggesting the MDR phenotype. The Mg-dependent-ecto-ATPase, an enzyme located on cell surface of *Leishmania* parasites, is involved in the acquisition of purine and participates in the adhesion and infectivity process. We compared control and resistant *L. amazonensis* ecto-enzymatic activities. The control and resistant *Leishmania* ecto-ATPase activities were 16.0 ± 1.5 nmol Pi $\times h^{-1} \times 10^{-7}$ cells and 40.0 ± 4.4 nmol Pi $\times h^{-1} \times 10^{-7}$cells, respectively. Interestingly, the activity of other ecto-enzymes present on the *L. amazonensis* cell surface, the ecto-5′ and 3′-nucleotidases and ecto-phosphatase, did not increase. The level of ecto-ATPase modulation is related to the degree of resistance of the cell. Cells resistant to 10 μM and 60 μM of vinblastine have ecto-ATPase activities of 22.7 ± 0.4 nmol Pi $\times h^{-1} \times 10^{-7}$ cells and 33.8 ± 0.8 nmol Pi $\times h^{-1} \times 10^{-7}$cells, respectively. *In vivo* experiments showed that both lesion size and parasite burden in mice infected with resistant parasites are greater than those of *L. amazonensis* control cells. Furthermore, our data established a

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relationship between the increase in ecto-ATPase activity and greater infectivity and severity of the disease caused by vinblastine-resistant *L. amazonensis* promastigotes. Taken together, these data suggest that ecto-enzymes could be potential therapeutic targets in the struggle against the spread of leishmaniasis, a neglected world-wide public health problem.

1. Introduction

Leishmaniasis is endemic in 88 countries, mainly in tropical and subtropical areas, and it is caused by obligate intra-macrophage protozoa belonging to the genus *Leishmania* (Lopes et al., 2010). *Leishmania* parasites exist as motile flagellate promastigotes that live in the digestive tract of blood-sucking sandflies. Vertebrate hosts are infected with promastigotes of the parasite via the bite of a sandfly. These promastigote forms rapidly differentiate into non-flagellated amastigotes within mononuclear phagocytes in the vertebrate host. The clinical manifestations of leishmaniasis (visceral, cutaneous, mucocutaneous and diffuse cutaneous) depend on parasite species, host genetic factors and immune response (Coelho et al., 2008).

Leishmania amazonensis is prevalent in many regions of Brazil and induces mucocutaneous and diffuse cutaneous lesions upon infection (Grimaldi and Tesh, 1993). Despite the considerable progress made in the study of the biochemistry, physiology and molecular biology of *Leishmania* parasites, there are no effective vaccines or vector control programs, and isolated drugs and combined chemotherapies are therefore the most widely used tools against leishmaniasis. However, treating this disease has remained highly problematic due to the side effects of conventional drugs and parasite drug resistance (Croft et al., 2006; Leandro and Campino, 2003).

Vinblastine is used *in vitro* to develop resistant *Leishmania*, although it is not the drug of choice for the treatment of leishmaniasis (Gueiros-Filho et al., 1995). This drug is an antimicrotubule agent that blocks cell division, interfering with the function of the mitotic spindle and blocking the cells at the metaphase/anaphase transition during mitosis (Little and Seehaus, 1988). In *Leishmania*, there are three classes of microtubules, the flagellar, mitotic and subpellicular microtubules, and these are involved in locomotion, cell division and cell shape, respectively. Moreover, the microtubule monomer tubulin is the major protein of the insect stage of *Leishmania mexicana* (Fong and Chang, 1981). Classically, vinca alkaloids were the earliest antimicrotubule agents to be used in the clinic as antimitotics and are microtubule-depolymerizing agents (Jayanarayan and Dey, 2002). However, there is insufficient evidence in kinetoplastid parasites to conclusively state and classify the mechanisms of resistance to antimicrotubule agents as yet.

Members of the ATP-binding cassette (ABC) transmembrane protein family, including P-glycoprotein (PGP), have been characterized in *Leishmania* spp. (Callahan and Beverley, 1991; Chow et al., 1993; Henderson et al., 1992; Ouellette et al., 1990). Having 42 ABC family member proteins, *Leishmania* is the protozoan parasite with the largest ABC data set (Sauvage et al., 2009). Overexpression of drug target enzymes and the amplification of genes encoding PGP-like molecules in *Leishmania* have been associated with the development of drug resistance (Gueiros-Filho et al., 1995; Sauvage et al., 2009). These genes share high sequence homology with the mammalian MDR genes and were found to be overexpressed in vinblastine-selected multi-resistant *Leishmania donovani*, *Leishmania enriettii* and *L. amazonensis* developed *in vitro* (Chow et al., 1993; Gueiros-Filho et al., 1992, 1995; Henderson et al., 1992). The mechanisms by which *Leishmania* spp. acquire drug-resistance are subject of intense research, and drug-resistant strains of *Leishmania* have been generated *in vitro* by stepwise exposure to increasing concentrations of vinblastine (Gueiros-Filho et al., 1995) and other drugs (Borst and Ouellette, 1995) to serve as models for resistance studies.

The ecto-nucleotidases are located on the plasma membrane surface, where they hydrolyze extracellular nucleotides, and have been described in different cell types (Knowles, 2011; Meyer-Fernandes, 2002; Zimmermann, 2000, 2012). Early reports have described the ecto-ATPase activities of different protozoan parasites, such as *Toxoplasma gondii* (Nakaar et al., 1998), *Leishmania tropica* (Meyer-Fernandes et al., 1997), *L. amazonensis* (de Almeida Marques-da-Silva et al., 2008; Pinheiro et al., 2006), *Leishmania braziliensis* (Rezende-Soares et al., 2010), *Entamoeba histolytica* (Barros et al., 2000), *Trypanosoma cruzi* (Bernardes et al., 2000; Bisaggio et al., 2003; Fietto et al., 2004; Meyer-Fernandes et al., 2004), *Trypanosoma rangeli* (Fonseca et al., 2006) and *Trypanosoma brucei* (de Souza Leite et al., 2007). In this work, we demonstrated that *L. amazonensis* promastigotes resistant to 100 μM vinblastine showed increased levels of ecto-ATPase activity but not of other ecto-enzyme activities. We also observed that vinblastine-resistant parasites displayed an atypical shape, and a greater disease severity was demonstrated in *in vivo* experiments.

2. Materials and methods

2.1. Microorganisms

The Mhom/Br/75/Josefa strain of *L. amazonensis* was used throughout this study. It was isolated from a human case of diffuse cutaneous leishmaniasis in Brazil by Dr. Cuba-Cuba (Universidade de Brasília, Brazil) and has been maintained in our laboratory since then, both in axenic culture and by hamster footpad inoculation. Promastigote populations were maintained in Warren's medium supplemented with 10% heat-inactivated fetal bovine serum at 22 °C (Warren, 1960) in the presence or absence of 100 μM vinblastine. Three days after inoculation, cells were collected by centrifugation, washed twice, and kept in buffer A (116.0 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, 50.0 mM Hepes–Tris buffer, pH 7.2). Cellular viability was assessed before and after incubations by motility and Trypan blue dye exclusion (Pinheiro et al., 2007). Viability was not affected by the conditions employed here.

2.2. Establishment of L. amazonensis vinblastine-resistant cell lines

L. amazonensis promastigotes were selected by gradually increasing the vinblastine concentration, as described (Coderre et al., 1983; Gueiros-Filho et al., 1995). The cells were supplemented with a higher concentration of vinblastine every two weeks, beginning with 10 μM vinblastine and ending when promastigotes were able to grow in 100 μM vinblastine. These cells were maintained under drug pressure for the remaining studies.

2.3. Circularity and projected surface area calculations

Control and resistant *L. amazonensis* strains were washed and processed as described in 2.1 and photographed directly, without fixation, on a Zeiss Axioplan 2 microscope. Cells were outlined free-hand with ImageJ software, and circularity and area parameters were evaluated (n = 26 for each sample). The circularity of the cells was automatically calculated by ImageJ 1.46 r, using the formula $4\pi\times(\text{area}/\text{perimeter}^2)$. A circularity factor of 1.0 indicates a perfect circle, and as this value approaches 0.0, it indicates an increasingly elongated polygon. The measurement of cell area refers to the area