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Ornithine decarboxylase or gamma-glutamylcysteine synthetase overexpression protects *Leishmania (Vianna) guyanensis* against antimony

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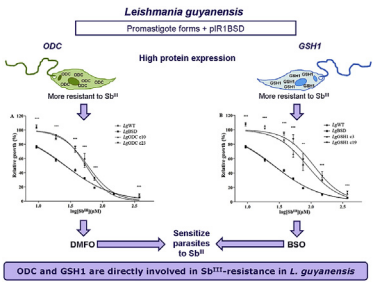
HIGHLIGHTS

- ODC- or GSH1-overexpressing *L. guyanensis* are more resistant to Sb^{III}.
- DFMO and BSO sensitize LgWT and ODC and GSH1-overexpressors to Sb^{III}.
- DFMO and BSO increase the anti-leishmanial effect of Sb^{III}.
- ODC and GSH1 are implicated in Sb^{III}-resistance in New World *Leishmania*.

GRAPHICAL ABSTRACT



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ABSTRACT

Trypanosomatids present a unique mechanism for detoxification of peroxides that is dependent on trypanothione (bisglutathionylspermidine). Ornithine decarboxylase (ODC) and γ-glutamylcysteine synthetase (GSH1) produce molecules that are direct precursors of trypanothione. In this study, *Leishmania guyanensis odc* and *gsh1* overexpressor cell lines were generated to investigate the contribution of these genes to the trivalent antimony (Sb^{III})-resistance phenotype. The ODC- or GSH1-overexpressors parasites presented an increase of two and four-fold in Sb^{III}-resistance index, respectively, when compared with the wild-type line. Pharmacological inhibition of ODC and GSH1 with the specific inhibitors α-difluoromethylornithine (DFMO) and buthionine sulfoximine (BSO), respectively, increased the antileishmanial effect of Sb^{III} in all cell lines. However, the ODC- and GSH1-overexpressor were still more resistant to Sb^{III} than the parental cell line. Together, our data shows that modulation of ODC and GSH1 levels and activity is sufficient to affect *L. guyanensis* susceptibility to Sb^{III}, and confirms a role of these genes in the Sb^{III}-resistance phenotype.

1. Introduction

Leishmaniasis is a complex of diseases caused by 21–30 different species of protozoan parasites belonging to the genus *Leishmania*. This neglected tropical disease is endemic in 98

Abbreviations: Lg, Leishmania guyanensis; ODC, ornithine decarboxylase; GSH1, gamma-glutamylcysteine synthetase; Sb^{III}, trivalent antimony; DFMO, α-difluoromethylornithine; BSO, buthionine sulfoximine.

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countries, especially in Central and South America, Southern Europe, North and East Africa, Middle East, and in the Indian subcontinent (Alvar et al., 2012). Annually, it has been estimated 1.3 million of new cases of the disease and about 30,000 deaths worldwide (WHO, 2016). Leishmaniasis comprises three different main clinical manifestations that range from self-healing cutaneous (CL) and mucocutaneous (MCL) skin lesions to a visceral (VL) form that is lethal if untreated (WHO, 2016). In the New World, *Leishmania (Viannia) guyanensis* causes both CL and MCL (Guerra et al., 2011).

The control of leishmaniasis is based on chemotherapy and there is no human vaccine available to date (Kumar and Engwerda, 2014). Pentavalent antimony-containing compounds such as N-methyl-glucamine (Glucantime®) and sodium stibogluconate (SSG) (Pentostam®) are the main drugs used to treat all forms of the disease for almost eight decades (Mohapatra, 2014). The mechanism of action of this drug is not fully understood. It is known that the pentavalent (Sb^V) form needs to be reduced to the active trivalent form (Sb^{III}), which has leishmanicidal effect against both amastigote and promastigote forms of the parasite (Shaked-Mishan et al., 2001). The antimony has been proposed to inhibit glycolysis, fatty acid oxidation (Berman et al., 1987) and trypanothione reductase, the enzyme responsible for sustaining the parasite' redox homeostasis (Cunningham and Fairlamb, 1995). The cytotoxicity of Sb^{III} has also been associated to alterations in the mitochondrial membrane potential that lead to an increase in the production of reactive oxygen species (ROS) and in the influx of Ca^{++} (Mukherjee et al., 2002; Sudhandiran and Shaha, 2003; Mehta and Shaha, 2006).

In the last 25 years, many cases of treatment failure and resistance to antimonials were reported in several countries including Brazil (Oliveira-Neto et al., 1997), Bolivia (Bermúdez et al., 2006) Colombia (Palacios et al., 2001), India (Sundar, 2001), Iran (Sarkari et al., 2016) and Peru (Arevalo et al., 2007). The most extreme situation takes place in Bihar (India) where 50–65% of the patients are unresponsive to Sb^V-treatment (Sundar, 2001). Parasites use a range of mechanisms to acquire resistance to these drugs such as increase in drug efflux/sequestration or decrease in drug uptake, lower rate of drug activation/reduction and gene amplification (Beverley, 1991; Haldar et al., 2011). Several reports link the level and activity of enzymes from the thiol-redox metabolism of *Leishmania* spp. to antimony resistance (Guimond et al., 2003; Mukherjee et al., 2007; Rai et al., 2013). The central molecule of this metabolism is based the low molecular mass dithiol trypanothione (bisglutathionylspermidine), which provides reducing power for several cellular functions including the protection against reactive oxygen species (Olin-Sandoval et al., 2010; Manta et al., 2013). The two building blocks of trypanothione, glutathione and spermidine are provided by the glutathione- and polyamine-biosynthetic pathway, respectively (Manta et al., 2013). γ-Glutamylcysteine synthetase (GSH1) is the first enzyme of the glutathione pathway that produces γ-glutamylcysteine, a direct precursor of glutathione (Meister and Anderson, 1983). An increase of GSH1 mRNA levels have been reported in some *L. tarentolae* samples with *in vitro*-induced resistance to antimony (Guimond et al., 2003) and some Sb^V-resistant *L. donovani* field isolates (Mukherjee et al., 2007; Singh et al., 2014). Amplification of *gsh1* gene was also observed in Sb^V-resistant *L. donovani* field isolates (Mukherjee et al., 2007; Singh et al., 2014) and in *L. tarentolae* mutants selected for resistance to arsenite (As^{III}) or Sb^{III} (Grondin et al., 1997; Haimeur et al., 1999, 2000).

Ornithine decarboxylase (ODC) converts L-ornithine in putrescine that will be transformed in spermidine by spermidine synthase (Müller et al., 2001). Polyamines contribute to parasite growth and in the As^{III}/antimony-resistance in *Leishmania* parasites (Haimeur et al., 1999; Singh et al., 2007; Birkholtz et al., 2011). Gene amplification and increase in the ODC levels were observed in Sb^V-resistant *L. donovani* field isolates (Mukherjee et al., 2007; Rai et al., 2013) and in *L. tarentolae* mutants selected for resistance to As^{III} (Haimeur et al., 1999).

Despite these evidences, the potential role of ODC and GSH1 enzymes in Sb^{III} resistance for the New World *Leishmania* species has not yet been addressed in detail. In the present study we used a genetic and pharmacological approach to dissect the contribution of ODC and GSH1 enzymes from *L. (V.) guyanensis* (a New World *Leishmania* species) to Sb^{III}-resistance.

2. Material and methods

2.1. Parasites

Promastigotes forms of *Leishmania (Viannia) guyanensis* (IUMB/BR/1985/M9945) were grown at 26 °C in M199 medium (Liarte and Murta, 2010). All assays were performed using parasites in the logarithmic growth phase.

2.2. Cloning, expression and purification of recombinant *L. guyanensis GSH1*

A 2067 bp fragment corresponding to the complete open reading frame (ORF) of the *gsh1* gene (*LbrM.18.1700*) was amplified from *L. guyanensis* genomic DNA using primers: GSH1pQE-31 forward: 5′-CGCGGATCCGATGGGTCTCTTGACAACTGG-3′ and GSH1pQE-31 reverse: 5′-CGCAAGCTTTTATGCGCTGCTCTCCTGTT-3′. The underlined sequences correspond to *Bam*HI and *Hind*III restriction sites, respectively, to facilitate cloning. PCR amplification was carried as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. The PCR amplicons of *gsh1* were digested with restriction enzymes and then inserted into the corresponding sites of the pQE-31 (Qiagen, Valencia, CA, USA). *Escherichia coli* M15 strain transformed with the pQE31-GSH1 construct were cultured for 12 h in the presence of 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG—Promega, Madison, WI, U.S.A.) at 30 °C, harvested and lysed. The His-tagged recombinant GSH1 was purified using nickel-nitrilotriacetic acid (Ni-NTA) coupled to sepharose (Qiagen) columns. The recombinant protein was used to generate polyclonal antiserum in New Zealand white rabbits according to an immunization protocol previously described (Murta et al., 2006).

2.3. Generation of ODC and GSH1 overexpressor cells from *L. guyanensis*

Fragments of 1911 and 2067 bp corresponding to *odc* (*LbrM.12.0300*) and *gsh1* (*LbrM.18.1700*) encoding regions, respectively, were amplified with *Pfx* DNA polymerase (Invitrogen) from *L. guyanensis* genomic DNA using the forward primer: 5′-TAGATCT**CCACC**ATGATGAAGATGGTTACCGCC-3′ and the reverse primer: 5′-TTAGATCTCTATAAGGCAATAGAGCTCACC-3′ to amplify *odc* and the forward primer 5′-TGGATCC**CCACC**ATGGGTCTCTTGACAACTGG-3′ and the reverse primer: 5′-ATGGATCCTTATGCGCTGCTCTCCTGTTTCTCAGC-3′ to amplify *gsh1*. Bold letters indicate the Kosak sequence and the underlined sequences correspond to *Bgl*II and *Bam*HI restriction sites of *odc* and *gsh1* genes, respectively. The *odc* and *gsh*1 amplicons were cloned into pGEM T-Easy™ vector (Invitrogen) and subsequently submitted to DNA sequencing. The constructs containing *odc* or *gsh1* were digested with *Bgl*II and *Bam*HI, respectively, and the fragments released were introduced into the dephosphorylated pIR1-BSD expression vector, kindly provided by Dr. Stephen Beverley (Washington