Antimony resistance and gene expression in *Leishmania*; Spotlight on molecular and proteomic aspects

Rajamanthrilage Kasun Madusanka¹,², Nadira D. Karunaweera¹*, Hermali Silva¹, Angamuthu Selvapandiyan²*

¹Department of Parasitology, Faculty of Medicine, University of Colombo, No. 25, Kynsey Road, Colombo 8, Sri Lanka.
²Department of Molecular Medicine, School of Interdisciplinary Sciences and Technology, Jamia Hamdard, New Delhi, 110062, India.

* Authors for correspondence: Nadira D. Karunaweera, nadira@parasit.cmb.ac.lk; Angamuthu Selvapandiyan, selvapandiyan@jamiahamdard.ac.in

ORCID List
Rajamanthrilage Kasun Madusanka- 0000-0002-8793-0862
Nadira D. Karunaweera- 0000-0003-3985-1817
Hermali Silva- 0000-0003-2288-1294
Angamuthu Selvapandiyan- 0000-0002-2194-1534
Abstract

Leishmaniasis is a vector-borne parasitic disease caused by *Leishmania* parasites with a spectrum of clinical manifestations, ranging from skin lesions to severe visceral complications. Treatment of this infection has been extremely challenging with the concurrent emergence of drug resistance. Presumably, the early diagnosis of Sb-resistant cases and effective treatment strategies are of greater importance to combat clinical unresponsiveness. The differential gene expression and the discrepancies of protein functions are believed to contribute towards the appearance of two distinct phenotypes; resistant and sensitive, where the current diagnostic tools fail to differentiate between them. The identification of the gene expression patterns and molecular mechanisms coupled with Sb resistance can be leveraged to prompt diagnosis and select the most effective treatment methods. Unfortunately, the collective understanding of this subject lags behind. The present study attempts to use comparative expression of Sb resistance-associated genes in the resistant and sensitive *Leishmania*, as the basis with a comprehensive approach to disclose their relative abundance in clinical or *in vitro* selected isolates to gain an understanding of the molecular mechanisms of antimony response/resistance. Depending on the prevailing data, we suggest that the analysis of resistance gene expression would verify the Sb resistance or susceptibility only to a certain extent, however, none of the individual expression patterns of the studied genes was diagnostic as a biomarker of Sb response of *Leishmania*. The findings highlighted will be useful in bridging the knowledge gap and discovering innovative diagnostic tools and novel therapeutic targets.

**Keywords:** Antimonial, Antimony resistance, Gene expression, *Leishmania*, Leishmaniasis, Poor drug response, Resistance biomarkers, Treatment
Introduction

Leishmaniasis is a complex infectious disease caused by unicellular parasites of the genus *Leishmania* and has become a huge burden on many of the undeveloped and developing tropical countries worldwide (Desjeux, 1996; World Health Organisation, 2023). The microbial promastigote stage of this parasite is transmitted to the mammalian hosts, including humans, by *Phlebotomus* or *Lutzomyia* sand flies (Burza et al., 2018; Killick-Kendrick, 1999). Leishmaniasis causes approximately 1-2 million cases and more than 20,000 deaths annually while 350 million people are at risk (World Health Organisation, 2010; Alvar et al., 2012; *PAHO/WHO Leishmaniasis Fact Sheet*, 2017; World Health Organisation, 2023). Impoverished people and dearth of healthcare facilities have become the major instigators of the disease, which have exacerbated the current risks of acquiring this disease to a significant level (Burza et al., 2018; Selvapandiyan et al., 2019; World Health Organisation, 2023).

There are different clinical manifestations of leishmaniasis viz. visceral (VL), cutaneous (CL), and mucosal (MCL), whereas the most life-threatening and commonly reported cases are VL and CL forms respectively (Burza et al., 2018; *PAHO/WHO Leishmaniasis Fact Sheet*, 2017). VL, which is abundant in Africa, Brazil and India, causes severe damage to the reticuloendothelial system with dissemination of parasites and more than 95% of cases ending up fatally, if left untreated (World Health Organisation, 2023; Aronson et al., 2017). About 95% of CL occur in the Americas, Asia and the Mediterranean basin and the majority of mucocutaneous cases are reported in countries such as Ethiopia, Bolivia, Peru, and Brazil (Burza et al., 2018). Thus, the widespread nature and the potential risk of the outbreak of this disease necessitate expeditious disease control measures and prevention campaigns along with rapid diagnosis, increased public awareness and effective treatment strategies.

A number of different treatment strategies like chemotherapy, thermotherapy and cryotherapy are currently being used to treat leishmaniasis globally, but antimony (Sb) is the
mainstay of treatment (Guerin et al., 2002; Silva et al., 2021; Madusanka et al., 2022). Sodium stibogluconate (SSG, Pentostam) and meglumine antimoniate (MA, Glucantime) are the two major medicaments of pentavalent Sb (SbV) based drugs in use (Guerin et al., 2002; Haldar et al., 2011). The most effective dosage of Sb(V) is 20 mg/kg/day for 20-28 days and the injections may cause localised pain (Madusanka et al., 2022). Initially, antimonials were tremendously successful, however, the responsiveness has dwindled over the decades of use and its current therapeutic prospects appear dim (Silva et al., 2021). The increased drug unresponsiveness in leishmaniasis is attributed to the inappropriate use of drug regimens resulting in progressive drug tolerance in parasites (Sundar et al., 1994). Both the metal-containing-drug activity and emergence of resistance in Leishmania are closely associated with the trypanothione-based thiol metabolism (Krauth-Siegel and Comini, 2008; Monte-Neto et al., 2011; Mukhopadhyay et al., 1996; Ouellette et al., 2004), and the resistance has been particularly linked to increased Sb detoxification and sequestration (Dumetz et al., 2018; Gazanion et al., 2016; Moreira et al., 2013). Apart from that plethora of genes, protein functions and metabolic pathways are interconnected with the arousal of Sb unresponsiveness, which is of greatest concern for its epidemiology and threatens to undermine disease control efforts. The differential gene expression and genetic modifications are of paramount importance for Leishmania in bringing about drug resistance, and such discrepancies are informative in predicting possible drug responses (Adaui et al., 2011; Biyani et al., 2011; Carter et al., 2006; Ghosh et al., 2020, 2022; Kumar et al., 2010; Oliaee et al., 2018; Torres et al., 2010). Often, the Sb resistance is accompanied by the transcriptional modifications of a certain set of genes that collaboratively interfere with the therapeutic effect of Sb detoxification through the incorporation of its active form into conjugates and diminishing the intracellular Sb build up (Haimeur et al., 2000). For example, Patino et al. demonstrated the presence of more than 800 differentially expressed genes in Sb-resistant and sensitive Leishmania (Patino
et al., 2019). Moreover, a proteomic study quantitatively evaluated the Sb-resistant and susceptible isolates of *L. donovani*, whose genes were differentially expressed in relation to stress-related pathways, intracellular survival and other key metabolic pathways (Biyani *et al.*, 2011). Apart from that studies have reported the differential gene expression in Sb resistance (Andrade *et al.*, 2020; Das *et al.*, 2015; Walker *et al.*, 2012).

Although there are a multitude of findings published on Sb resistance-related gene expression, indicating both parallel and contradictory observations, an overall discussion based on findings of individual studies is warranted to determine the collective scientific significance. In this review, we strived to summarise the variations of Sb resistance-related gene expressions in *Leishmania* with reference to their relative abundances in terms of mRNA or protein level fluctuations. Furthermore, this study will disclose intriguing areas related to the battle against Sb resistance, which would help in navigating future research towards more productive discoveries in disease control and prevention of leishmaniasis.

**Molecular basis of Sb effect and resistance**

For more than six decades antimony was the first line of treatment against all forms of leishmaniasis that showed high efficacy (Haldar *et al.*, 2011; Negera *et al.*, 2012). According to the pro-drug model, the Sb(V) reduces to its active trivalent state (Sb(III)) by trypanothione (T(SH)₂), the most effective intracellular thiol in *Leishmania* parasites (Dos Santos Ferreira *et al.*, 2003), either within the host cell (López *et al.*, 2015), prior to importation in to the parasite or within the parasite itself (Denton *et al.*, 2004; Haldar *et al.*, 2011; Shaked-Mishant *et al.*, 2001; Zhou *et al.*, 2004). Moreover, host macrophage thiols like GSH and glycycysteine also are known to achieve non-enzymatic Sb reduction (Dos Santos Ferreira *et al.*, 2003). Antimonials have been found to enter the parasite cells via phosphate transporters (Rosen, 2002). Most notably, this molecular reduction followed by the production of more toxic Sb(III),
which exerts a lethal effect on *Leishmania*, is stage-specific as it predominantly takes place in the amastigotes compared to the promastigotes, which in turn elucidates the comparatively higher Sb(V) susceptibility of amastigotes (Callahan *et al*., 1994; Ephros *et al*., 1999; Goyard *et al*., 2003; Shaked-Mishant *et al*., 2001). The exact mechanism of the therapeutic action is an enigma and it is believed that Sb induces parasite cell apoptosis (Fig. 1) through genomic DNA degradation, accumulation of reactive oxygen species (ROS) and nitric oxide, diminishing mitochondrial potential, and increasing intracellular Ca\(^{2+}\) (Basu *et al*., 2006; Garg and Goyal, 2015; Lee *et al*., 2002; Moreira *et al*., 2011; Sereno *et al*., 2001; Sudhandiran and Shaha, 2003; Vergnes *et al*., 2007). These impose a lethal stress on parasites by inhibiting macromolecular synthesis and energy metabolism to diminish their vital metabolic pathways together with the interruption of glycolysis and fatty acid oxidation, which ultimately lead to the death of parasites (Berman *et al*., 1985; Herman *et al*., 1987).

**Sb resistance triggered gene expression.**

The exact molecular mechanisms and biochemistry of Sb resistance in *Leishmania* still remain ambiguous and yet to be expounded (Fernandez-Prada *et al*., 2018). However, the mostly argued mechanism of Sb resistance is linked to the increased Sb(III) detoxification and sequestration (Ashutosh *et al*., 2007; Garg and Goyal, 2015; Gazanion *et al*., 2016) which subsequently result in reduced Sb accumulation within the parasites (Fig. 2) (Ouellette *et al*., 2004; Ouellette and Papadopoulou, 1993). Furthermore, the formation of Sb-trypanothione conjugates in the presence of excess trypanothione and its rapid extrusion are largely exploited for Sb resistance in *Leishmania* (Mukhopadhyay *et al*., 1996; Rai *et al*., 2013). There was a consistently high thiol levels in Sb-resistant and genetically different clinical isolates of *Leishmania* (Khanra *et al*., 2022). Antimony resistance in *Leishmania* is markedly associated with the expression of
proteins related to antimony reduction and transport and, thiol synthesis (Table 1) (Khanra et al., 2022). The recent publications revealed 844 and 803 differentially expressed genes between Sb(SSG) resistant and sensitive L. braziliensis and L. panamensis respectively, with over 100 genes showing \( \geq 2 \)-fold change in resistant lines of each strain (Patino et al., 2019). Sb resistant overexpression of transcripts assigned in the gene ontology (GO) categories such as ubiquitination, host–parasite interaction, protein phosphorylation, microtubule-based movement, and cellular process and the downregulated processes were rRNA processing, ribosome biogenesis, ribonucleoprotein complex, nucleosome assembly and translation (Andrade et al., 2020). In addition, most of the differentially expressed proteins in SAG-sensitive isolate AG83-S versus SAG-resistant GE1-R were related to translation and metabolic enzymes (Biyani et al., 2011).

The copy number variation (CNV) is among the crucial molecular mechanisms deployed by Leishmania to increase the transcript levels of resistance genes. CNV involves the duplication of either specific genomic region or complete chromosomes, as intra or extrachromosomal elements (Leprohon et al., 2015; Papadopoulou et al., 2016; Ullman, 1995). Moreover, the gene amplification that occurs in a part at the amplicon or intrachromosomal level, also has been observed in differential gene expression, which leads to metal-drug resistance in Leishmania including Sb stress (Grondin et al., 1997; Leprohon et al., 2009; Mukherjee et al., 2013). The presence of single nucleotide polymorphisms (SNP) in genes encoding functional and structural proteins related to Sb resistance is known to regulate Leishmania resistance towards chemotherapy (Coelho et al., 2012; Downing et al., 2011; Rastrojo et al., 2018). The presence of three SNPs in serine acetyltransferase (SAT), the protein involved in cysteine synthesis, exhibited increased Sb resistance in L. infantum mutants having impaired ABC transporters (Douanne et al., 2020). Further, a SNP that occurred in protein kinase in L. infantum has influence on Sb resistance (Brotherton et al., 2013). The resistance
phenotype of *Leishmania* is a final product of various cellular mechanisms including, gene overexpression accompanied by preadaptations like structural and functional modulations.

**ATP-binding cassette (ABC) transporter (MRPA)**

The ABC transporter superfamily consists of functionally Sb-resistant proteins of *Leishmania* that have exerted an Sb detoxification potential via direct membrane efflux of Sb to the extracellular milieu (Douanne et al., 2020; El Fadili et al., 2005; Singh et al., 2014). Multidrug-resistant protein A (MRPA, also known as ABCC3) is one of the ABC transporters, formerly identified as a P-glycoprotein (P-gp) (Ouellette et al., 1998), and has been reported to be a *Leishmania* intracellular protein found in membrane vesicles near the flagellar pocket, at the sites of endo- and exocytosis of the parasite (Fig. 1) (Ashutosh et al., 2007; El Fadili et al., 2005). Previous literature has amply demonstrated the inevitable role of MRPA in Sb resistance via intracellular sequestration of Sb-thiol conjugates into vesicles (Gazanion et al., 2016; Moreira et al., 2013; Mukherjee et al., 2007; Singh et al., 2014). In addition, MRPA expression resulted in increased drug resistance in *L. donovani* in relation to altered fluidity in the cell membranes and decreased drug accumulation (Bhandari et al., 2014). Previously MRPA was suggested as one of the two prediction models in determining the Sb treatment failure that could predict the treatment outcome with high accuracy (Torres et al., 2013). Hence, the MRPA expression could be a crucial characteristic of Sb resistance (Fig. 2). Interestingly, *L. donovani* was able to develop sodium stibogluconate (SSG) resistance even under arsenic (As) stress, because both Sb and As pressure could trigger the same overexpression of the ABC transporter (Perry et al., 2013).

Extrachromosomal amplification within circular amplicons of MRPA has been extensively studied in different *Leishmania* species (Leprohon et al., 2009; Moreira et al., 2013; Mukherjee et al., 2007). Likewise, the adaptive gene amplification of MRPA observed
in *L. infantum* during *in vitro* Sb(III) selection corroborates its significance in Sb tolerance (Ubeda *et al.*, 2014). More importantly, MRPA amplification confers the first line of defence against Sb(III) stress in *Leishmania*, providing the driving force for the inception of underlying molecular adaptations upon an infection and signalling pathways (Dumetz *et al.*, 2018). Therefore, its expression level during drug pressure could be a determinant of the parasites’ destiny as well as providing strong insights upon the subsequent development of Sb resistance. Beyond Sb transportation, an indirect correlation was explored between *Leishmania* MRPA expression and cellular redox homeostasis that was affected by Glucose-6-phosphate dehydrogenase (G6PDH) and trypanothione reductase interaction upon metalloid exposure including Sb (Ghosh *et al.*, 2017). Dumets et al. demonstrated the importance of overexpression of the H locus, which harbours the MRPA gene, over M locus and increases the Sb resistance in 3-fold (Dumetz *et al.*, 2018).

MRPA was expressed in *Leishmania* with a direct correlation to Sb-resistant phenotype and it was widely expressed in most of the parasite and clinical forms of leishmaniasis (Barrera *et al.*, 2017; Fekrisoofiabadi *et al.*, 2019; Mukherjee *et al.*, 2007). For example, the MRPA expression was found to be markedly augmented in Sb-resistant Indian isolates of *L. donovani* and *L. tropica* respectively, compared to the sensitive counterparts (Khanra *et al.*, 2022). Furthermore, two independent methods viz cDNA-AFLP approach and qPCR analysis demonstrated approximately similarly augmented expressions (2-3- fold) of MRPA in Sb-resistant clinical anthropochronic cutaneous leishmaniasis ACL isolates of *L. tropica* compared to the sensitive counterparts (Kazemi-Rad *et al.*, 2013; Mohebali *et al.*, 2019). An antibody assay recognised high MRPA levels in *L. guyanensis*, and *L. amazonensis* resistant lines, but the detection was not successful for the sensitive lines, which further demonstrated differential MRPA expression between two phenotypes (Moreira *et al.*, 2013). Moreover, a full genome microarray hybridisation in *L. amazonensis* showed a robust (5 fold) MRPA expression in Sb-
resistant parasites compared to its wild-type (Monte-Neto et al., 2011). Interestingly, the obstruction of MRPA expression conferred increased drug susceptibilities in *Leishmania*, for instance, the MRPA null mutants of *L. infantum* promastigotes exhibited drastic declines (20 fold) in their IC50 values against Sb(III), whereas the corresponding amastigotes showed increased sensitivity to Sb(V) compared to their wild type (Douanne et al., 2020). Appropriately, following the selection of Sb(III) resistance, the transcript levels of sensitive parasites of a study were elevated reaching a 1.5-3.0-fold expression as same as the resistant parasites (Dumetz et al., 2018). This observation affirms that the selective drug pressure is able to provoke Sb resistance in *Leishmania* through MRPA-mediated mechanisms, as a preadaptation of parasites to harsh conditions, thus, it is salient to indicate that the overexpression of this protein may enable the parasites to withstand or weaken the Sb therapeutic effect as a successful counter mechanism. In addition, studies have reported adaptive expression of MRPA in an Sb concentration dependant manner, whereas an initial elevation of the copy number was seen in all the Sb(III) resistant mutants and it was gradually decreased to the wild-type level in the subsequent several passages in the absence of drug pressure (Haimeur et al., 2000). This would further inform the drug pressure-induced overexpression and the significance of MRPA-mediated pathways to achieve Sb resistance in certain instances.

Contrariwise, MRPA-independent resistance mechanisms were also possible in which it was not ubiquitously upregulated in all the Sb resistant isolates of a study (Moreira et al., 1998; Mukherjee et al., 2007). Accordingly, MRPA-independent Sb resistance was accompanied by unchanged mRNA levels in resistant *Leishmania*, whereas it was sometimes considered as a protein without an important role in Sb transportation (Dos Reis et al., 2017) or non-essential transcript for Sb resistance, however, the disruption of this protein triggered Sb hypersensitivity in both amastigotes and promastigotes of *L. infantum* (Douanne et al., 2020).
2020). The presence of high resistance index was found to be essential for the upregulation of MRPA, whilst the energy dependent Sb resistance pathway of resistant mutants was not relied on the upregulation of this gene (Dos Reis et al., 2017; Rai et al., 2013). In a study related to L. donovani, Kumar et al. observed upregulation of MRPA in six resistant and one sensitive isolate with no significant elevation of its expression in another four resistant isolates of the same species (Kumar et al., 2012). Moreover, the MRPA level of L. panamensis was augmented only in vitro adapted Sb-resistant strains and no significant difference was observed in clinically resistant lines (Barrera et al., 2017). This was further corroborated by the studies that have reported non or negligible amplification of MRPA in glucantime-resistant clinical Leishmania isolates (Gómez Pérez et al., 2016; Moreira et al., 1998; Ullman et al., 1989). In addition, an MRPA amplification was observed only in three out of four SAG-resistant isolates and the rest did not show any sign of amplification (Mukherjee et al., 2007). Therefore, there must be multiple factors behind the MRPA expression under Sb exposure that can modulate its expression. Accordingly, MRPA was a candidate marker for drug resistance with 69% accuracy as a prediction model in determining the treatment outcome of clinical Sb-treatment upon L. braziliensis (CL) (Torres et al., 2013).

Of note, many studies have surveyed paradoxical results about MRPA expression linked to its expression in Sb-resistant and sensitive parasites which are not satisfactorily resolved yet. Although MRPA seemed to have wide expression in Leishmania, species-specific discrepancies of the expression levels were also possible in Sb resistance. For example, a quantitative RT-PCR analysis showed no differential expression in Sb resistant L. infantum, in spite of 2-fold increased mRNA expression in Sb(III) resistant isolates of L. guyanensis, L. braziliensis and L. amazonensis compared to the susceptible lines (Moreira et al., 2013). Moreover, increased MRPA levels were seen (1.5-25.2-fold) in Sb(III) resistant L. braziliensis and Sb(V) unresponsive L. tropica along with simultaneous expression in respective responsive
lines, so it diminishes the possibility of the functional relevance of MRPA in resistance phenotypes (Oliaee et al., 2018; Rugani et al., 2019). On the other hand, Victoria et al. demonstrated that Sitamaquine can successfully circumvent the Sb resistance caused by MRPA expression (Pérez-Victoria et al., 2011) and the MRPA-mediated Sb resistance was reverted by the buthionine sulfoximine, a glutathione biosynthesis-specific inhibitor (El Fadili et al., 2005). Hence, the obstruction of the interaction between MRPA and glutathione may be an effective approach for drug design. (Fekrisoofiabadi et al., 2019).

Moreover, Callahan et al. demonstrated an oxidative state dependant selective Sb resistance in L. major, where the MRPA expression could manifest resistance to Sb(III) and not against Sb(V), albeit with clear evidence for the intracellular conversion of Sb(V) to its reduced form during its mode of action in the parasites (Callahan and Beverley, 1991; Dos Santos Ferreira et al., 2003). Moreover, significant MRPA expression was observed in promastigotes under increased Sb(III) stress compared to the intracellular amastigotes exposed to Sb(V) (Fernandez-Prada et al., 2018; Gazanion et al., 2016). Accordingly, the amastigotes must have evolved pathways to confer drug protection via MRPA-independent mechanisms as well. Collectively, the MRPA expression in Sb resistance is not a consistent event, which is broadly affected by multiple factors.

**Aquaglyceroporin (AQP1)**

Aquaglyceroporins are a subcategory of aquaporins that primarily involve water and glycerol transportation in mammalian cells (Mukhopadhyay et al., 2014; Verkman, 2008). In *Leishmania*, AQP1 has been implicated as a protein that imports Sb(III) into the cells and its decreased expression has been broadly discussed attributed to Sb resistance in many studies (Fig. 2A) (Gómez Pérez et al., 2016; Gourbal et al., 2004). It is predominantly found in the flagellums of the promastigote stage, which is then relocated to the parasite surface eventually
after post-translational phosphorylation by the MAPK (G. Mandal et al., 2012; Sharma et al., 2015). Since the AQP1 expression is highly associated with the Sb accumulation in Leishmania, reduced expression or the perturbation of its gene expression has been extensively reported in relation to Sb resistance (Gourbal et al., 2004; Khanra et al., 2022; Marquis et al., 2005; Mohebali et al., 2019; Mukherjee et al., 2013; Sharma et al., 2015).

AQP1 expression increased the Sb(III) accumulation in Leishmania compared to the untreated control (Sharma et al., 2015), therefore, the suppression of its transcripts is much preferred by the resistant Leishmania and vice versa (Fig. 2) (Douanne et al., 2020; Kazemi-Rad et al., 2013). Accordingly, single allele disruption or subtelomeric deletion of AQP1 resulted in drastically reduced Sb accumulation in Leishmania accompanied by prompt Sb-resistance (Gourbal et al., 2004; Monte-Neto et al., 2015). Furthermore, terminally deleted mutants of AQP1 could restore their Sb(III) resistance following the episomal transfection of the gene through which the IC50 of the mutants (by 20-50 folds) subsequently dropped in a rigorous decline (Mukherjee et al., 2013). The AQP1 expression in Sb-resistant isolates of ACL showed suppression than that of the sensitive strains (Kazemi-Rad et al., 2013) and more significantly, a negative correlation was seen between AQP1 expression and the IC50 or time taken to cure ACL lesion of the responsive cases of natural isolates (Khanra et al., 2022; Oliaee et al., 2018). Similarly, the decreased expression of AQP1 in the Sb-resistant clinical isolates of L. donovani and L. tropica was further affirmed by a negative correlation between IC50 and AQP1 expression (Khanra et al., 2022; Mohebali et al., 2019). Leishmania parasites of CL or PKDL had more robust antimonial accumulation than that of the VL, and were more Sb sensitive, which was rendered so by the elevated expression and mRNA stability of AQP1 (G. Mandal et al., 2015; Mishra et al., 2013). A downregulation of AQP1 was seen in majority of Sb(III) resistant VL and PKDL derived L. donovani isolates albeit with several exceptions (Mandal et al., 2010). Not only that, an atypical form of tegumentary leishmaniasis caused by
*L. braziliensis* showed an outstanding 65-fold downregulation of AQP1 in their clinical isolates than that of the reference strain (Rugani *et al.*, 2019). In addition, there was a comparable AQP1 expression in the Sb treatment failure isolates of *L. major* which was found to be 58.71-fold less than that of the treatment responsive isolates (Sharma *et al.*, 2015). More importantly, the two Sb transporters, MRPA and PRP1 found to have increased their expression in clinically resistant parasites along with simultaneous suppression of AQP1 transcripts, with an emphasis on the increased Sb detoxification plus decreased influx (Khanra *et al.*, 2022). Apart from that, the AQP1 expression in Sb(V) resistant *L. donovani* isolated from Nepal and the AQP1 copy number derived from chromosome 31 in the resistant mutants of *L. major* was found to be lower than that of their sensitive strains (Decuypere *et al.*, 2005). Interestingly, the transfection of AQP1 followed by its increased expression in *L. tarentolae, L. major, L. infantum* developed hypersensitivity to metalloids such as As(III) and Sb(III) (Gourbal *et al.*, 2004). Another study also revealed supportive evidence of similar hypersensitivity in *L. major* isolates of CL patients and secondarily, their resistance emerged with the deletion, inactivation through mutation and reduced expression of APQ1 (Eslami *et al.*, 2021). Conversely, *in vitro* transfection failed to enhance Sb susceptibility of resistant promastigote lines as well as sensitive lines compared to their respective parent strains (Mandal *et al.*, 2010). *L. donovani* clinical isolates of SAG-resistant and sensitive parasites showed marked down and up regulations respectively, whereas the expression difference was more prominent between the amastigote lines than the respective promastigotes. The Sb(V) resistant *L. donovani* isolated from Nepal exhibited 6-7 fold significantly lower AQP1 expression than in the sensitive strains (Decuypere *et al.*, 2005).

There are many disputes among research findings about the AQP1 expression in drug-resistant *Leishmania*. For instance, Maharjan *et al.* suggested that the downregulation of AQP1 was just one of the Sb-resistant mechanisms in *Leishmania* and not all the resistant ones consistently downregulate it (Maharjan *et al.*, 2008). Further, it was ascertained by the high
AQP1 copy number observed in the resistant parasites compared to the sensitives, which was not in line with the reduced import of Sb(III) (Maharjan et al., 2008). It was also suggested that AQP1 is not an essential protein for the survival of Leishmania (Plourde et al., 2015). In vitro selected Sb-resistant mutant L. braziliensis, L. infantum, and L. guyanensis did not show a significant difference in AQP1 mRNA level compared to the control, in agreement with the absence of its function in Sb transportation in non-natural resistant mutants (Dos Reis et al., 2017; Moreira et al., 2013; Torres et al., 2010). Interestingly, a study on L. panamensis revealed decreased AQP1 levels in vitro-adapted Sb-resistant strains and no significant difference was observed in the clinically resistant lines (Barrera et al., 2017). Therefore, growing evidence has suggested the possibility of APQ1 neutral Sb resistance and therefore, it has also hinted at the prevalence of many critical cellular functions of these transcripts, other than the Sb influx.

In agreement with the wide array of functions achieved by the APQ1, a handful of reports indicate its upregulation without affecting the inherited Sb resistance and noticeably. Suggesting an alternative mechanism of Sb resistance. L. infantum amastigotes with Sb(III) resistance, had increased AQP1 expression, which was reverted to the wild-type in the presence of drug pressure (Marquis et al., 2005). Further, L. major parasites isolated from non-healing cases showed increased AQP1 expression (Alijani et al., 2019; Eslami et al., 2016). In a study aiming to investigate the biomarkers of Sb resistance, L. donovani showed a marked AQP1 upregulation in all the selected clinically Sb sensitive isolates, in comparison to significant down-regulation observed in only 30% of resistant ones, whereas others showed similar expression to the wild-type (Kumar et al., 2012). According to the available evidence on AQP1 expression, it may be a multifunctional protein in Leishmania, which is also significantly involved in Sb resistance. Therefore, many elaborated studies are warranted to clearly understand the network of those functions.
Gamma-glutamylcysteine synthetase (γ-GCS)

Gamma-Glutamylcysteine synthetase (γ-GCS, L-glutamate: L-cysteine γ-ligase) catalyses the rate-limiting step of the glutathione (GSH) biosynthesis that leads to the trypanothione overexpression (Haimeur et al., 2000; Lu, 2001; Mukhopadhyay et al., 1996). During its mode of action, firstly, γ-GCS triggers the covalent bond formation between glutamate (Glu) and cysteine (Cys) to synthesise gamma-glutamylcysteine, which in turn binds with glycine (Gly) resulting in GSH formation (Fig. 1) (Olin-Sandoval et al., 2012). Accordingly, the activity of this protein is controlled by the intracellular GSH levels and the non-allosteric feedback as well as the transcriptional and translational factors (Lu, 2001). Moreover, the γ-GCS overexpression was considered to confer increased virulence, cell viability and drug resistance in parasites (González-Chávez et al., 2019; Pérez-Rosado et al., 2002).

The γ-GCS has been reported to be upregulated in Sb resistant Leishmania and implicated as a protein that triggers Sb-detoxification pathways (Fig. 2A) (Grondin et al., 1997; Mukherjee et al., 2007). For example, an elevated γ-GCS expression was observed in therapeutic failure in L. guyanensis in all the in vitro growth phases of the promastigote (Torres et al., 2010). Its expression in Sb-resistant L. major derived from CL patients was 20 times higher than that of the sensitive and it was suggested to be a possible biomarker in the identification of clinical resistance (Ghbakhloo et al., 2016). Fittingly, there was a positive correlation between γ-GCS expression and the IC50 values of Sb-resistant clinical kala-azar isolates of L. tropica and L. donovani with severalfold overexpression (Khanra et al., 2022). The γ-GCS was not merely associated with developing Sb resistance, however, its depleted expression was associated with adverse effects on parasites, for instance, the downregulation of these transcripts rendered decreased parasite oxidative defence that made the parasites more susceptible to drug effect, which was in line with the reported upregulation and downregulation
in the majority of resistant isolates and the sensitive ones respectively (Fig. 2). The RNA expression level of γ-GCS was 2.1 times higher in clinical Sb-resistant isolates of *L. tropica* compared to the sensitive isolates, but it was upregulated only in 70% of resistant isolates, whereas 75% of sensitive isolates experienced downregulations (Kumar *et al.*, 2012). Additionally, γ-GCS expression was dependent on the host organ and the type of *Leishmania* strain that informs about the influence of environmental factors that could govern its expression (Carter *et al.*, 2006). Apart from that, γ-GCS expression has been attributed to rapid wound healing in ACL that was ascertained by a negative correlation seen between γ-GCS expression and the time taken to cure lesions of the responsive cases of field isolates (Oliaee *et al.*, 2018). Hence, this implicates the functional relevance of γ-GCS expression with a possible relation to GSH-dependent pathways to accelerate the healing process.

There were also discrepancies in γ-GCS expression levels in *Leishmania* in relation to Sb stress and parasite defence. The resistant *Leishmania* isolates including the resistant standards were neutral in γ-GCS expression, while the sensitive parasites showed inconsistencies of expression having either upregulated (2.32-fold), downregulated (<0.6-fold) or unaltered (Mohebali *et al.*, 2019). Rai *et al.* suggested that γ-GCS is not consistently expressed, not involved in naturally Sb-resistant *Leishmania* or it is having a role only in highly resistant parasites (Rai *et al.*, 2013). Furthermore, a pronounced downregulation was seen in Sb-resistant *L. donovani* (Decuypere *et al.*, 2005, 2008). Even though γ-GCS has been studied as an inducer of thiol biosynthesis, also γ-GCS-independent thiol elevations have been characterised in natural Sb-resistant *L. donovani*. Furthermore, the γ-GCS amplification found to have negligible in those parasites, showing that it was not directly involved in thiol synthesis of that particular strain (Mittal *et al.*, 2007). Based on the current evidence, thiol production may not have solely dependent on the γ-GCS activity, which may sometimes enable its differences in expression without interfering Sb-resistance, thus minimising the likelihood of
this protein to be a potential expression marker of Sb resistance.

**Mitogen activated protein kinase (MAPK)**

Mitogen-activated protein kinases (MAPK) are primarily involved in the phosphorylation of other proteins and are associated with cellular stress response, proliferation, infectivity, differentiation, and apoptosis (Hindley and Kolch, 2002; Wiese, 1998). There are around 17 different MAPK proteins in *Leishmania*. MAPK3 and MAPK9 are exclusively expressed in the promastigote stage and are involved in flagellum maintenance (Bengs *et al*., 2005), whereas MAPK1 and MAPK2 are implicated in Sb resistance (Sharma *et al*., 2015). The Sb resistance achieved through MAPK was found to have been associated with the modulation of aquaglyceroporin activity (AQP) (Mandal *et al*., 2012). Furthermore, the co-expression of MAPK with AQP1 increases Sb(III) uptake and drug sensitivity in *L. major* (G. Mandal *et al*., 2012). Therefore, MAPK must have at least an indirect effect on the Sb transportation mechanisms of *Leishmania* cells (Fig. 1). Metal-based drugs like Sb(III) induce ROS production leading to subsequent cell apoptosis interconnected with activation of MAPK signalling cascade (Garg and Goyal, 2015; Leonard *et al*., 2004; Mann *et al*., 2006), which is why favourable downregulation of MAPK could be a promising adaptation to avert Sb cytotoxicity.

A several fold decreased expression of MAPK1 was observed in Sb-resistant *L. donovani* compared to the sensitive reference that showed a slight increase, which is suggestive of the possible involvement of MAPK1 in triggering cell death pathways upon Sb exposure (Ashutosh *et al*., 2012). Furthermore, observation of reduced protein level in those resistant strains further validated the aforementioned downregulation, and besides, the MAPK overexpression enabled cells to have 2-3 fold increased susceptibility to both Sb(V) and Sb(III) than the cells transfected with the empty vectors (Ashutosh *et al*., 2012). In addition, RT-PCR
assay revealed a differential expression of MAPK with a suppression of its transcript levels in Sb-resistant *L. major* and *L. tropica* clinical isolates compared to the respective sensitive parasites (Kazemi-Rad et al., 2013; Sharma et al., 2015). The deletion of MAPK2 in *L. major* resulted in reduced uptake of Sb(III) and slower healing (Mandal et al., 2012) because of increased parasite viability following less Sb toxicity. Contrarily, MAPK transcripts were more abundantly expressed in 90% of SAG-resistant *L. donovani* clinical isolates together with one of the sensitive lines (Kumar et al., 2012). Altogether, most of the time MAPK can show decreased expression as a preadaptation to Sb resistance, but the inconsistency of its abundance could be a negative factor for the suitability of this protein as a biological Sb marker. However, the positive regulation of MAPK accompanied by AQP1 mediated Sb accumulation would be an attractive phenomenon for drug designing (Fig. 2B) (Mandal et al., 2012)

**Ornithine decarboxylase (ODC)**

ODC is the rate-limiting protein of the polyamine biosynthetic pathway, which is important for cell growth and proliferation, and its expression is mostly mediated by gene amplification (Haimeur et al., 1999; Ilari et al., 2015). It is involved in spermidine biosynthesis as the final product (Fig. 1), and studies have revealed pronounced amplification attributed to both the Sb and As resistance in *Leishmania* (Fonseca et al., 2017; Haimeur et al., 1999). Thus, the elevated ODC may functionally assist the parasites to alleviate the antiproliferative effect caused by metalloid drugs.

There was an overexpression of ODC in Sb-resistant *L. donovani* compared to the sensitive line, however, it was not expressed on extrachromosomal circles. Apart from that it was further validated by the protein level overexpression in all the resistant isolates (Mukherjee et al., 2007). Both the promastigotes and the amastigotes of *L. donovani* overexpressed their ODC levels as a self-protective mechanism against SAG, resulting in notable rises in their IC50
values than that of the wild-type strains (Singh et al., 2007). Moreover, the gene transfection followed by ODC expression was able to increase Sb(III) resistance in 2-fold compared to the wild-type parasites or empty vector transfected *L. guyanensis*. In fact, the parasites were more prone to the Sb effect with the inhibition of ODC and the opposite was experienced with the overexpression. For example, DMFO pre-treated *L. guyanensis* cells exhibited 648-fold susceptibility to Sb(III) for wild-type in comparison to the 1.5-fold observed in the ODC transfected clones (Fonseca et al., 2017). More remarkably, the canine-infected *L. infantum* clinical isolates showed increased RNA expression in Sb-resistant lines compared to the susceptible, especially in the absence of γ-GCS and TryS expression, indicating the functional relevance of ODC in elevating **T[SH]_2** levels in resistant lines (Gómez Pérez et al., 2016). There was a noticeable difference in ODC gene expression between the natural resistance and resistant mutants since only the naturally resistant *L. donovani* parasites could augment the expression, while the mutants remained unchanged compared to the reference Dd8 strain (Rai et al., 2013). Furthermore, the ODC expression was amplified in both genetic and protein levels in Sb-resistant Indian *L. donovani* and Peruvian *L. braziliensis*, and on the contrary, and a downregulation was observed in *L. donovani* isolates from Nepal (Adaui et al., 2011; Decuypere et al., 2005; Mukherjee et al., 2007), hence, permits queries about the exact biological role of this protein in Sb resistance.

Moreover, in *L. panamensis*, ODC failed to provoke Sb(III) resistance in laboratory selected stains, owing to the possible hindrance of expression due to the activations of alternative polyamine synthesis pathways or import of polyamines (Goyeneche-Patino et al., 2008). The intracellular amastigotes of clinical isolates showed strikingly decreased ODC expression in Sb(V)-resistant lines than that of the sensitive ones (Decuypere et al., 2005) that resulted due to the changes on ODC mediated thiol-biosynthesis in such a way that facilitates parasite-friendly intracellular environment and arrested activation of the Sb(V) in amastigotes.
(Decuypere et al., 2005). Supporting evidence was published on Sb-resistant \textit{L. guyanensis} mutants, whose ODC level had no relative difference compared to the parental strain (Dos Reis et al., 2017). The prevalence of contradictory evidence of expressions related to Sb resistance raises doubt on the diagnostic use of ODC. Accordingly, previous analysis based on Youden’s J statistics justified the foregoing results with the observation of only 50% specificity of ODC to detect the Sb(V) resistance in \textit{L. braziliensis} clinical isolates (Adaui et al., 2011).

\textbf{Trypanothione reductase (TR)}

TR and Thioredoxin peroxidase are among the several proteins involved in spermidine metabolism (Ilari et al., 2015), and it has become an attractive drug target since its unavailability in mammals (Krauth-Siegel and Inhoff, 2003; Vázquez et al., 2017). The protozoans have evolved a trypanothione/trypanothione reductase system instead of a glutathione/glutathione reductase system that is found in the mammalian cells (Fig. 1) (Baiocco et al., 2009). Trypanothione forms a complex with Sb(III) and it is efficiently transported via plasma membrane vesicles via an ATP-coupled efflux pump resulting in Sb resistance (Gómez Pérez et al., 2016; Mukhopadhyay et al., 1996). In a study, TR conferred oxidative protection to both amastigotes and promastigote stages against Sb(V) and Sb(III) and consequently intensified the treatment failure capacity of the non-responders (Zabala-Peñafiel et al., 2023). Hence, it is rational that Sb(III) inhibits TR in a reversible manner so as to secondarily avert the reduction of trypanothione leading to the accumulation of more disulphides, which would ultimately weaken the resistance (Fig. 2B) (Wyllie et al., 2004). Research findings corroborate the Sb(III) mediated inhibition of TR in \textit{Leishmania}, which eventually boosts the concentration of the disulphide forms of the intracellular trypanothione and glutathione which perturbs the cellular thiol redox potential (Wyllie et al., 2004). For instance, inhibition of TR leads to apoptotic death of \textit{Leishmania} parasites resulted in the instantaneous decline of thiol content.
(Ghosh et al., 2017), thus, the TR expression is of greater importance for protozoans, not only during drug pressure but also for the survival at any circumstance.

The TR protein augmentation was proportional to its activity with a more than doubled mean activity in non-responders versus responders, in the clinical isolates of *L. tropica* from ACL patients (Nateghi-Rostami et al., 2022). The high thiol levels observed in natural Sb-resistant *L. donovani* cells with concurrent amplification of TR and MRPA were not mediated by the γ-GCS (Mittal et al., 2007). Furthermore, a western blot analysis revealed 4-fold overexpression of TR in Sb(III) resistant natural canine isolates of *L. infantum* versus sensitive (Gómez Pérez et al., 2016). The TR expression seemed to be modulated during promastigote growth and however, it was upregulated in most of the parasite of Sb unresponsive isolates (9/10) along with some cured ones (3/11) (Adaui et al., 2011), and it pointed out a functionally irrelevant outcome.

Additionally, the TR expression was almost the same in between the Sb unresponsive and sensitive *L. donovani* clinical extracts and it was suggested that TR had no clear role in Sb resistance in *Leishmania* (Nateghi-Rostami et al., 2022). Studies conducted by Willie et al. did not show any correlation between the TR activity and the clinical Sb resistance in *L. donovani* isolates, which is why they suggested its negligible involvement in resistance phenotype (Wyllie et al., 2010). Likewise, TR showed a similar and consistently expressed pattern in both MA responsive and unresponsive *L. tropica*, whereas approximately 20% of samples from both types of TR was not affected by Sb (Oliaee et al., 2018). As summary, TR is an extremely important protein in Sb-resistant *Leishmania* with mostly elevated expressions despite possible outliers that could diminish the functional relevance.

**Thiol-dependent reductase 1 (TDR1)**

TDR1 is a tetramer with a functional domain containing omega class glutathione-S transferases.
that involves the reduction of Sb(V) to Sb(III), with the help of GSH as the reductant (Fig. 1) (Denton et al., 2004; Haldar et al., 2011). TDR1 was strikingly up regulated in the amastigotes than that of the promastigotes and it was attributed to the TDR1-mediated reduction of Sb(V) to Sb(III), whereas promastigotes were prominently sensitive to Sb(V) than the amastigotes (Denton et al., 2004; Shaked-Mishant et al., 2001). All the MA non-responders of a study showed a significantly elevated metabolic activity against H₂O₂ than the lower activity seen in responders, which was in line with the elevated trypanothione peroxidase levels in non-responders (Nateghi-Rostami et al., 2022). A 3-4 fold increased TDR expression was experienced in SSG-resistant parasites *L. donovani* and *L. tropica* than the sensitive line (Khanra et al., 2022).

Interestingly, the TRD1 expression was broadly variable in the *L. tropica* Sb responsive isolates with the majority of them having fold changes between 2.3 and 1124. In the meantime, the expression was not much prominent in the unresponsive lines having high expression only in several isolates (Oliaee et al., 2018). Moreover, the TDR1 expression was several-fold down-regulated in MA unresponsive clinical *L. tropica* (Oliaee et al., 2018).

**Tryparedoxin peroxidase (TryP)**

TryP is a principal enzyme that provides parasites with antioxidant defence through the detoxification of harmful peroxides (Flohé et al., 2003; Iyer et al., 2008). Since the Sb treatment is largely associated with the accumulation of deadly reactive oxygen species (ROS) in *Leishmania*, the enhanced expression of these proteins envisages the role of antioxidant defence and detoxification in the emergence of resistance (Fig. 2) (Mandal et al., 2007; Wyllie et al., 2004).

In *Leishmania*, the Sb stress elevated the TryP expression in both cytosol and mitochondria, however, the cytosolic expression was more remarkable than the mitochondrial
counterpart (Das et al., 2018; Wyllie et al., 2008). The overexpression of TryP resulted in decreased Sb(III) sensitivity in *Leishmania*, while the overexpression of the enzymatically inactive form failed to bring about resistance, which corroborates the fact that TryP-mediated Sb resistance was independent of sequestration or membrane efflux of Sb(III) (Wyllie et al., 2008). A several-fold augmented TryP protein levels were reported in Sb-resistant *Leishmania* accompanied by activated H$_2$O$_2$ metabolism as well as increased tolerance to exogenous H$_2$O$_2$ than the respective sensitives or parental lines. (Andrade and Murta, 2014). Moreover, the elevation of TryP was also accompanied by a significant (> 2 time average) TR expression in the resistant extracts with an emphasis on the synergistic interactions of proteins involved in thiol-metabolism in gaining Sb resistance (Nateghi-Rostami et al., 2022). A three times TryP boost was observed in Sb-resistant *L. tarentolae* than observed in lysates of revertant, along with a positive correlation to the subsequent peroxidase activity (Wyllie et al., 2008). In addition, a comparative proteomic analysis has revealed a highly abundant TryP expression in Sb(III)-resistant *L. braziliensis* and *L. infantum chagasi* cells (Matrangolo et al., 2013). In contrast, the incorporation of TryP through transfection neither exhibited a significant gene expression nor tolerance of oxidative stress in *L. infantum* compared to the parental lines (Andrade and Murta, 2014).

**Heat shock proteins (HSP)**

The chemical inhibition of HSP was found to be a strategy to produce antileishmanial drugs (Das et al., 2020). Heat shock protein-83 (HSP83) and Heat shock protein-70 (HSP70) are involved in anti-leishmanial drug-mediated programmed cell death by interfering with mitochondrial membrane potential (Vergnes et al., 2007).

Matrangolo et al., demonstrated prominent expression profiles of HSP83, heat shock protein-60 (HSP60) and HSP70 in the resistant cell lines of both *L. braziliensis* and *L. infantum*.
*chagasi*, particularly the HSP70 equivalents having boosted in both cytoplasm and the mitochondria of the *L. infantum chagasi* lines. (Matrangolo *et al.*, 2013). HSP70 and HSP83 were reported to be overexpressed in the membrane-enriched fraction of the SAG-resistant *L. donovani* clinical isolates (Kumar *et al.*, 2010) and in the meantime, the increased expression pattern of HSP60 and HSP70 has been observed in relation to Sb-resistant mechanisms *L. (Viannia) panamensis* (Peláez *et al.*, 2012). The substantial abundance of HSP70 *in vitro* selected Sb-resistant *Leishmania* of both amastigotes and promastigotes, and the corresponding revertant cells in the absence of the drug pressure, could be suggestive of the stability and the functional significance of this protein in Sb-resistant *Leishmania* (Brochu *et al.*, 2004). Inversely, the Sb sensitivity was aligned with the downregulation of HSP83 in *L. donovani* (Kumar *et al.*, 2012). In addition, the CL caused by naturally Sb-resistant *L. tropica* was associated with HSP70 differential expression (Özbilgin *et al.*, 2021). The *L. infantum* promastigotes were superior to *L. tarentolae* in acquiring Sb(III) defence through robust HSP70 expression (Brochu *et al.*, 2004). Nonetheless, a study proposed HSP70 to have only a 75% success rate as an effective candidate prediction model in determining Sb resistance of CL clinical cases caused by *L. braziliensis* (Torres *et al.*, 2013).

However, a few of the findings possibly disprove the HSP mediated Sb resistance in *Leishmania* as well as imposing limitations on the suitability of this gene in predicting resistance. For instance, HSP83 was upregulated only in 40% of clinically resistant *L. donovani* lines compared to the LdAG83 reference strain (Kumar *et al.*, 2012) and also the transfection of the HSP70 gene was unable to circumvent the Sb effect and confer direct resistance towards Sb (Brochu *et al.*, 2004).

**Other important genes with elevated expression in Sb resistance**

Only a few studies have been conducted on the histone modification and gene regulation in
trypanosomatids. There are two types of histones in *Leishmania*; core histones like H2A, H2B, H3, H4 and linker histone H1 (Martínez *et al*., 2002; Fasel *et al*., 1993; Soto *et al*., 1992, 1997).

Histone expression in *Leishmania* has been implicated as a coupled mechanism to DNA replication that affects the level of translation via post-transcriptional mechanisms (Abanades *et al*., 2009) and a few studies have discussed the histone expression in Sb-resistant in *Leishmania*. However, so far, the histone expression has not been characterised as a direct contributor to drug resistance, but it may have a function in the epigenetic programming of resistance genes in *Leishmania*. Apart from that, histone epigenetic markers were essential for the survival of *L. major* (Afrin *et al*., 2019; Anderson *et al*., 2013). Meantime, the studies aiming to explore the correlation between histone function in antimonial drug resistance are not well established yet.

Overexpression of H2A into *L. donovani* conferred decreased Sb susceptibility against not only SAG but developed resistance also against amphotericin-B and miltefosine (Singh *et al*., 2010). H1 was found to have elevated expression in nine out of ten Sb-resistant *L. donovani* isolates and H2A or H4 were upregulated in 50% of the SAG-resistant *L. donovani* clinical strains, however, it was found that a >2 fold down-regulation in all the sensitive lines compared to the LdAG83 sensitive reference line (Kumar *et al*., 2012). Moreover, H1 and H2A showed protein level upregulation in SAG-resistant *L. donovani*, kala-azar (Singh *et al*., 2010). Based on the present data, the histone modification could be a potential activator of the resistant genes but have to be further characterised to reveal its exact molecular relationship in Sb resistance.

The novel Sb-resistant markers, ARM56 and ARM58 were significantly elevated in resistant *Leishmania*, although with unknown function or molecular mechanisms in this regard. ARM proteins contain conserved domains with hydrophobic amino acids and form transmembrane structures, particularly, ARM56 and ARM58 are located as a subtelomeric cluster in chromosome 34 and their co-expression has been implicated in Sb resistance (Nevado...
Especially, the overexpression of ARM58 was found to minimise the Sb effect by reducing its accumulation in Leishmania cells by at least 70% (Schäfer et al., 2014). Most importantly, following in vitro selection for Sb(III), the ARM58 mRNA level exhibited 800% boost compared to the wild-type, and was also conferred protection on amastigotes as well as promastigotes against both Sb(III) and Sb(V) (Nühs et al., 2014). There was a clear correlation between the Sb resistance and ARM56/ARM 58 expression, whereas elevation of ARM 58 was identified as an exclusive feature in Sb-resistant field isolates, hence urges for extensive studies before validating this protein as an Sb-resistant marker (Rugani et al., 2019).

Another protein, pentamidine resistance protein (PRP1) belongs to the ABC transporter superfamily, and was initially considered to confer cross-resistance toward the SbCl₃ but not Sb(V) (Coelho et al., 2003), nonetheless, a recent finding illustrated a significantly elevated expression in SSG-resistant L. tropica and L. donovani resulting in more than 4-fold expression (Khanra et al., 2022).

Parasite surface antigen-2 (PSA-2) was consistently augmented in both the transcriptional and translational expression in SAG-resistant L. donovani clinical isolates resulting in more than 1.5-fold higher expression than the sensitive. Moreover the overexpression of this protein could transform the sensitive strains to resistance with a decline of their Sb susceptibility level in >12 fold (Bhandari et al., 2013). Arsenate reductase2 (ACR2) was a 3-4 fold expression in VL SSG-resistant L. donovani and L. tropica compared to the sensitive strain AG83 (Khanra et al., 2022).

Conclusions

Antimony resistance in Leishmania is mainly achieved by the expression modification of Sb transporter genes, Sb-reducing enzymes, and thiol-synthesising enzymes. Out of the genes studied in the present review, MRPA, γ-GCS, ODC, TR, TDR1, TryP, and HSP illustrated a
general likelihood of upregulation while AQP1 and MAPK showed a tendency to downregulate in the Sb-resistant Leishmania and vice versa, therefore, the resistance may have been orchestrated by the functional relevance of genes. Likewise, the relative gene expression in Sb resistance can exhibit similarities among different resistant isolates, but there are chances for deviations to be made from the mostly accepted phenomena. The reason for the presence of inconsistencies of protein functions may be due to molecular adaptations like polymorphism and post-translational modifications. Altogether, the gene expression-based confirmation of Sb resistance in Leishmania by examining the upregulation or downregulation of a particular gene compared to a control, will be useful in scientific studies to investigate the underlying biology, however, its application in patient diagnosis of clinical resistance may not be reliable due to possible false positive or false negative results and in some cases and the inadequacy of research work. Nonetheless, it is possible to minimise the potential misinterpretations provided that multiple resistance gene expressions are included in the analysis and if their relative expressions are highly significant. The current review circumscribed the Sb resistance-related gene expression, to facilitate future research that will fulfil the unmet need for the detection of biomarkers for Sb-resistant leishmaniasis, which remains as an obvious need to achieve effective disease control and elimination.

Financial support. Indo-Sri Lanka fellowship awarded to RKM (INSA/DST-ISRF/2022/45); Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number U01AI136033 and Indian Council of Medical Research under award number 63/8/2013-BMS. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health and Indian Council of Medical Research.
Authorship. All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

Author contributions. Conceptualisation: [Rajamanthrilage Kasun Madusanka, Angamuthu Selvapandiyan]; Methodology: [Rajamanthrilage Kasun Madusanka, Angamuthu Selvapandiyan]; Formal analysis and investigation: [Rajamanthrilage Kasun Madusanka, Angamuthu Selvapandiyan, Hermali Silva, Nadira Karunaweera]; Writing - original draft preparation: [Rajamanthrilage Kasun Madusanka]; Writing – review and editing: [Rajamanthrilage Kasun Madusanka, Angamuthu Selvapandiyan, Hermali Silva, Nadira Karunaweera]; Funding acquisition: [Nadira Karunaweera, Angamuthu Selvapandiyan]; Supervision: [Angamuthu Selvapandiyan, Nadira D. Karunaweera, Hermali Silva]; All authors read and approved the final manuscript.

Competing interests. Rajamanthrilage Kasun Madusanka, Nadira D. Karunaweera, Hermali Silva and Angamuthu Selvapandiyan declare that they have no conflict of interest.

Ethical approval. Not applicable.
References


Anderson, B. A., Wong, I. L. K., Baugh, L., Ramasamy, G., Myler, P. J., and Beverley, S.


Dos Santos Ferreira, C., Silveira Martins, P., Demicheli, C., Brochu, C., Ouellette, M.,


potassium antimonyl tartrate (SbIII) resistant *Leishmania tarentolae*. *Molecular and Biochemical Parasitology, 108*, 131–135.


Mandal, G., Wyllie, S., Singh, N., Sundar, S., Fairlamb, A. H., and Chatterjee, M. (2007). Increased levels of thiols protect antimony unresponsive *Leishmania donovani* field


**PAHO/WHO Leishmaniasis Fact Sheet.** (2017).

https://doi.org/10.1017/S0031182023001129 Published online by Cambridge University Press


Rai, S., Bhaskar, Goel, S. K., Nath Dwivedi, U., Sundar, S., and Goyal, N. (2013). Role of


https://doi.org/10.1017/S0031182023001129 Published online by Cambridge University Press


Elevated levels of tryparedoxin peroxidase in antimony unresponsive *Leishmania donovani* field isolates. *Molecular and Biochemical Parasitology*, 173, 162–164.


Figure 1. The Sb metabolism and related gene expression in wild-type *Leishmania amastigote*. TR- trypanothione reductase; TDR1- thiol-dependent reductase 1; γ-GCS- gamma-glutamylcysteine synthetase; MRPA- multidrug resistant protein A; AQP1- quaglyceroporin; ODC- ornithine decarboxylase; TryP- tryparedoxin peroxidase; MAPK- mitogen activated protein kinase; GSH- glutathione; T(SH)2- trypanothione; TS2- trypanothione disulphide; ROS- reactive oxygen species
Fig. 2. The comparison of Sb metabolism and related gene expression in Sb resistant versus sensitive amastigotes. The vertical red and blue arrows indicate the gene upregulation and downregulation respectively. The purple vertical arrows exhibit the increase/decrease of each component. TR- trypanothione reductase; TDR1- thiol-dependent reductase 1; γ-GCS- gamma-glutamylcysteine synthetase; MRPA- multidrug resistant protein A; AQP1- quaglyceroporin; ODC- ornithine decarboxylase; TryP- tryparedoxin peroxidase; MAPK- mitogen activated protein kinase; T(SH)2- trypanothione; TS2- trypanothione disulphide; ROS-reactive oxygen species. The (i1), (i2), (i3), (a1), (a2), and (a3) are mechanisms that affect the intracellular Sb concentration (A) (i1); decreased Sb influx; (i2)- decreased Sb(V) to Sb(III) conversion; (i3)- decreased T(SH)2 synthesis; (ii)- decreased Sb-thiol conjugate formation; (iii)- increased Sb(III) accumulation; (iv)- decreased cell apoptosis. (B) (a1)- decreased Sb influx; (a2)- increased Sb(V) to Sb(III) conversion; (a3)- increased T(SH)2 synthesis; (b)- increased Sb-thiol conjugate formation; (c)- decreased Sb(III) accumulation; (d)- increased cell apoptosis.
Table 1. The list of Sb resistance related genes in *Leishmania*.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Abbreviation</th>
<th>Functions/Relevance</th>
<th>Expected expression in Sb resistant <em>Leishmania</em> (Upregulation/Down regulation)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multidrug resistant protein A</td>
<td>MRPA</td>
<td>Sb detoxification and intracellular sequestration</td>
<td>Upregulation</td>
<td>(Barrera <em>et al</em>., 2017; El Fadili <em>et al</em>., 2005; Fekrisoofiabadi <em>et al</em>., 2019; Moreira <em>et al</em>., 2013; Mukherjee <em>et al</em>., 2007)</td>
</tr>
<tr>
<td>Aquaglyceroporin 1</td>
<td>AQP1</td>
<td>Uptake of Sb(III)</td>
<td>Downregulation</td>
<td>(Khanra <em>et al</em>., 2022; G. Mandal <em>et al</em>., 2015; S. Mandal <em>et al</em>., 2010; Marquis <em>et al</em>., 2005; Monte-Neto <em>et al</em>., 2015; Sharma <em>et al</em>., 2015)</td>
</tr>
<tr>
<td>Gamma-glutamylcysteine synthetase</td>
<td>γ-GCS</td>
<td>Thiol biosynthesis</td>
<td>Upregulation</td>
<td>(Carter <em>et al</em>., 2006; Fonseca <em>et al</em>., 2017; González-Chávez <em>et al</em>., 2019; Grondin <em>et al</em>., 1997; Mukherjee <em>et al</em>., 2007)</td>
</tr>
<tr>
<td>Mitogen activated protein kinase 1</td>
<td>MAPK1</td>
<td>Signal transduction cellular stress response, proliferation, infectivity, differentiation, and apoptosis</td>
<td>Downregulation</td>
<td>(Garg and Goyal, 2015; Leonard <em>et al</em>., 2004; Mann <em>et al</em>., 2006)</td>
</tr>
</tbody>
</table>

This is an Open Access article, distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives licence (http://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is unaltered and is properly cited. The written permission of Cambridge University Press must be obtained for commercial re-use or in order to create a derivative work.

https://doi.org/10.1017/S0031182023001129 Published online by Cambridge University Press
<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Abbreviation</th>
<th>Function</th>
<th>Regulation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ornithine decarboxylase</td>
<td>ODC</td>
<td>Spermidine biosynthesis</td>
<td>Upregulation</td>
<td>(Fonseca <em>et al.</em>, 2017; Gómez Pérez <em>et al.</em>, 2016; Haimeur <em>et al.</em>, 1999; Mukherjee <em>et al.</em>, 2007)</td>
</tr>
<tr>
<td>Trypanothione reductase</td>
<td>TR</td>
<td>Reduction of trypanothione</td>
<td>Upregulation</td>
<td>(Ghosh <em>et al.</em>, 2017; Krauth-Siegel and Inhoff, 2003; Nateghi-Rostami <em>et al.</em>, 2022; Zabala-Peñafiel <em>et al.</em>, 2023)</td>
</tr>
<tr>
<td>Thiol-dependent reductase 1</td>
<td>TDR1</td>
<td>Reduction of Sb(V) to Sb(III)</td>
<td>Upregulation</td>
<td>(Denton <em>et al.</em>, 2004; Nateghi-Rostami <em>et al.</em>, 2022)</td>
</tr>
<tr>
<td>Heat shock protein-60</td>
<td>HSP-60</td>
<td>Unknown</td>
<td>Unknown</td>
<td>(Matrangolo <em>et al.</em>, 2013; Peláez <em>et al.</em>, 2012)</td>
</tr>
<tr>
<td>Heat shock protein-83</td>
<td>HSP-83</td>
<td>Unknown</td>
<td>Unknown</td>
<td>(Kumar <em>et al.</em>, 2012; Matrangolo <em>et al.</em>, 2013)</td>
</tr>
<tr>
<td>Histone 1</td>
<td>H1</td>
<td>Linker protein of nucleosome</td>
<td>Unknown</td>
<td>(Martínez <em>et al.</em>, 2002; Fasel <em>et al.</em>, 1993; Singh <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>Histone 2A</td>
<td>H2A</td>
<td>Core histone of nucleosome</td>
<td>Unknown</td>
<td>(Abanades <em>et al.</em>, 2009; Singh <em>et al.</em>, 2010; Soto <em>et al.</em>, 1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------</td>
<td>------------------------------------</td>
<td>----------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>Histone 4</td>
<td>H4</td>
<td>Core histone of nucleosome</td>
<td>Unknown</td>
<td>(Kumar et al., 2012; Soto et al., 1997)</td>
</tr>
<tr>
<td>antimony resistance marker 56</td>
<td>ARM56</td>
<td>Unknown</td>
<td>Unknown</td>
<td>(Rugani et al., 2019)</td>
</tr>
<tr>
<td>antimony resistance marker 58</td>
<td>ARM58</td>
<td>Unknown</td>
<td>Unknown</td>
<td>(Nühs et al., 2014; Rugani et al., 2019; Schäfer et al., 2014)</td>
</tr>
<tr>
<td>Pentamidine resistance protein 1</td>
<td>PRP1</td>
<td>Unknown</td>
<td>Unknown</td>
<td>(Coelho et al., 2003; Khanra et al., 2022)</td>
</tr>
<tr>
<td>Parasite surface antigen-2</td>
<td>PSA-2</td>
<td>Immunogenic antigen</td>
<td>Unknown</td>
<td>(Bhandari et al., 2013)</td>
</tr>
<tr>
<td>Arsenate reductase2</td>
<td>ACR2</td>
<td>Reduction of Sb(V) to Sb(III)</td>
<td>Upregulation</td>
<td>(Khanra et al., 2022; Zhou et al., 2004)</td>
</tr>
</tbody>
</table>