



Research paper

Comparative proteomic profiling reveals distinct molecular signatures of drug resistance in clinical isolates of *Leishmania donovani*Dhiraj Gurjar^a, Baibaswata Saha^b, Kalpana Pai^b, Shyam Sundar^c, Alicia Ponte-Sucre^d, Neelam Bodhale^{a,e,*}, Bhaskar Saha^{f,**}^a National Centre for Cell Science, Pune 411007, India^b Department of Zoology, Savitribai Phule Pune University, Pune 411007, India^c Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India^d Laboratory of Molecular Physiology, Universidad Central de Venezuela, Caracas, Venezuela^e Dan L Duncan Comprehensive Cancer Center, Baylor College of Medicine, Houston, TX, USA^f JSPS Government Homoeopathic Medical College, Ramanthapur, Hyderabad 500013, India

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ABSTRACT

Leishmaniasis remains a major public health concern, with treatment options limited to amphotericin B (AmB), sodium stibogluconate (SSG), miltefosine, and paromomycin. Despite extensive clinical and field evaluations, the efficacy of these agents is increasingly undermined by toxicity, high cost, patient non-compliance, and the emergence of drug-resistance. Given this growing challenge, we conducted a comparative proteomic analysis of five *Leishmania donovani* clinical isolates: one drug-sensitive (BHU1066) and four drug-unresponsive to SSG (NS2), miltefosine (BHU1064), amphotericin B (BHU919), and paromomycin (BHU1020). Liquid chromatography-mass spectrometry identified proteins across these clinical isolates, with each drug-resistant isolate exhibiting a distinct profile of up-regulated proteins compared to the sensitive. Functional annotation revealed that genes involved in metabolic pathways, translation, RNA modification, chaperones, heat shock proteins, and signaling were prominently up-regulated in all drug-unresponsive clinical isolates. A subset of metabolic enzymes associated with glycolysis unique to *Leishmania* were consistently elevated, suggesting their potential role in mediating drug resistance. Approximately 20% of up-regulated proteins remain uncharacterized, highlighting novel candidates for future drug target discovery. Quantitative RT-PCR of selected differentially expressed genes confirmed significant drug-resistance-specific transcriptional modulation. These findings demonstrate that drug resistance in *L. donovani* is accompanied by extensive proteomic remodeling, emphasizing the need for comprehensive molecular studies to inform new therapeutic strategies against both drug-sensitive and drug-resistant leishmaniasis.

1. Introduction

The vector-borne zoonotic disease leishmaniasis, spread through sand-fly vectors and maintained in canines and rodents as the reservoirs for the disease-causing protozoan parasite of the genus *Leishmania*, affects 12 million people globally. According to WHO, 50–90 thousand new cases of visceral leishmaniasis (VL) and 0.6–1.0 million new cases of cutaneous leishmaniasis (CL) are annually recorded [1]. *Leishmania* has a digenetic, dimorphic life cycle, alternating between flagellated promastigotes in sand flies and non-flagellated amastigotes within

mammalian hosts. Following transmission through a sand-fly blood meal, parasites are phagocytosed by macrophages, where they survive and replicate by subverting host cellular signaling pathways to inhibit pro-inflammatory responses, suppress nitric oxide and reactive oxygen species (ROS) production, and skew cytokine profiles toward an anti-inflammatory state, often via increased IL-10 and TGF- β secretion [2–4]. These immunomodulatory mechanisms enable parasite persistence and systemic dissemination, particularly in VL [5]. Ultimately, disease outcome depends on the balance between parasite virulence and the host immune response with effective Th1 immunity and macrophage

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activation controlling infection, while immune dysfunction allows parasite spread and disease progression [6].

Despite extensive efforts, the absence of an effective vaccine against VL necessitates reliance on chemotherapeutic agents such as antimonials, amphotericin B, miltefosine, and paromomycin for disease management. However, the efficacy of these treatments is increasingly undermined by issues of patient non-compliance and the emergence of drug-resistant VL strains [7]. SSG, introduced in the 1940s, was initially highly effective against VL, but its efficacy declined by the 1980s–1990s due to widespread resistance, poor patient compliance, and significant toxicity, including cardiotoxicity and hepatotoxicity, leading to its replacement by safer alternatives [8–10]. Consequently, amphotericin B was introduced for VL treatment in the 1980s; despite its efficacy, the deoxycholate formulation is associated with nephrotoxicity, prolonged hospitalization, and cold-chain requirements [11,12]. The introduction of liposomal amphotericin B (L-AmB; AmBisome) in the early 2000s improved safety, reduced toxicity, shortened treatment regimens, and achieved cure rates >90% [13]. However, emerging resistance linked to altered sterol biosynthesis and membrane composition raises concerns about long-term efficacy, particularly in endemic, resource-limited settings [14]. Miltefosine, the first oral drug approved for VL in 2002, was initially favored due to its high efficacy and ease of administration and was incorporated into elimination programs in India, Nepal, and Bangladesh by 2010 [15]. Early studies reported cure rates of ~82% with low severe toxicity (3%), but subsequent reports documented declining efficacy and emerging resistance, likely related to its long half-life [16–19]. As a result, miltefosine use has been deprioritized due to reduced effectiveness, limited availability, and high cost [20]. Paromomycin, a broad-spectrum aminoglycoside, was approved for VL treatment in India in 2006 [21]. Rising resistance to monotherapies prompted the WHO in 2010 to recommend combination therapies [22]. These regimens shorten treatment, improve compliance, and reduce resistance risk. Although paromomycin is affordable and effective, its use is limited by 21-day intramuscular administration and potential nephro- and ototoxicity [23]. Despite their benefits, *Leishmania* can develop resistance even to combination therapies, especially under prolonged drug pressure or incomplete treatment [24]. Mechanisms include altered drug uptake, efflux pump overexpression, metabolic adaptations, and mutations in drug targets [25]. Combination regimens aim to delay resistance by targeting *leishmania* through multiple mechanisms. Common regimens include single-dose L-AmB with miltefosine or paromomycin, achieving ~94% cure rates, while Miltefosine with paromomycin reaches ~95%, whereas short-course amphotericin B deoxycholate with miltefosine is used where L-AmB is unavailable [26,27]. Since chemotherapy is the only curative option for VL, understanding and monitoring drug resistance in clinical isolates and laboratory models is critical.

To address this issue, we selected five clinical isolates of *L. donovani*; BHU1066 (drug-sensitive), NS2 (SSG resistant, lab adapted), BHU1064 (Miltefosine resistant), BHU919 (AmpB resistant) and BHU1020 (Paromomycin resistant). These clinical isolates were obtained from confirmed VL patients in endemic regions of Bihar, India, who were not responsive to specific drug treatments. At Banaras Hindu University (BHU, India), routine clinical characterization, including assessment of cross-resistance, and species confirmation had been completed prior to transfer, confirming that these isolates exhibit mono-drug resistance without cross-resistance. This study shows that drug resistance is accompanied by extensively modified gene expression.

2. Materials and methods

2.1. Parasite cultures and drug susceptibility assay

The lab-adapted *L. donovani* AG83 strain was maintained *in vitro* in RPMI-1640 medium supplemented with 10% FCS (GIBCO), 100 U/mL penicillin, 100 µg/mL streptomycin (GIBCO), and 2 mM glutamine. To

maintain virulence, parasites were injected intravenously into BALB/c mice and transformed *in situ* by harvesting infected spleens, aseptically crushing them, and incubating at room temperature (25 °C) for 7–15 days. The drug-nonresponsive clinical isolates (BHU919, BHU1020, and BHU1064), as well as the drug-sensitive clinical isolate BHU1066, were maintained similar to *L. donovani* AG83 strain. The SSG-resistant *L. donovani* NS2 was cultured in M-199 medium supplemented with 2% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1.36 mM glutamine. Drug-resistant clinical isolates were continuously cultured in the presence of sublethal drug concentrations that permitted viable growth while maintaining selective pressure, including amphotericin B (0.05 µg/mL), miltefosine (4.5 µM), SSG (12.5 µM), and paromomycin (6.25 µM).

For drug susceptibility assays, drug-resistant *L. donovani* clinical isolates in the late logarithmic phase were seeded at a density of 2.5×10^6 parasites per tube in 2 mL of complete RPMI-1640 medium. Parasites were exposed to increasing concentrations of miltefosine (1.5–24 µM), paromomycin (1.5–24 µM), amphotericin B (0.02–0.32 µg/mL), and sodium stibogluconate (SSG; 4–64 µM) and incubated for 72 h at 25 °C. Parasite viability was assessed by direct counting using a Neubauer hemocytometer.

2.2. Proteomic analysis of drug-resistant and drug-sensitive parasites

1.0×10^8 cells of stationary phase parasites were lysed in hot guanidine hydrochloride (GN) buffer (6 M Guanidium-HCL, 0.1 M Tris (pH 8.5), and protease inhibitor by Roche). For a 100 µL cell pellet, 400 µL of GN buffer was added, mixed well and kept at 90 °C for 10 min. For breaking nucleic acids and reducing the viscosity of slimy material, the lysate was sonicated and again kept at 90 °C for 5 min. The lysate was centrifuged at 23,940 ×g for 20 min at room temperature. The supernatant was collected carefully and stored in -80 °C deep freezer. The protein concentration was estimated by the Bradford method, keeping the lysis buffer for blank.

For sample preparation, 50 µL lysate was reduced with 5 mM TCEP, alkylated with 50 mM iodoacetamide, followed by digestion with trypsin (1:50 trypsin/lysate ratio) and incubated for 16 h at 37 °C. To remove the salts, the digests were cleaned using C18 silica cartridge and after that dried using speed vac. After drying pellet was resuspended in buffer A composed of 5% acetonitrile, 0.1% formic acid.

Mass spectrometric analysis of the peptide mixtures was performed by Valerian Chem Pvt. Ltd. (India) using an EASY-nLC 1000 system (Thermo Fisher Scientific) coupled with a 'nanoelectrospray ion source equipped Thermo Fisher-FUSION'. 25 cm PicoFit column (360 µm outer diameter, 75 µm inner diameter, 10 µm tip) filled with 1.9 µm of C18-resin (Dr. Maesch, Germany) was used to resolve 1 µg of the peptide mixture. Buffer A (0.1% formic acid in water) was used for loading the peptides and 0–40% gradient of buffer B (95% acetonitrile, 0.1% formic acid) was used for elution at flow rate of 300 nL/min for 60 min. For acquiring of MS data, a data-dependent top10 method was used which involves dynamic selection of the most abundant precursor ions from the survey scan.

The generated RAW files were analyzed using Proteome Discoverer (v2.2) against the Uniprot *L. donovani* (MHOM/IN/1983/AG83) proteome database (Proteome ID: UP000008980). To perform Sequest search, the settings were as follows: precursor- 10 ppm; fragment mass tolerances- 0.5 Da; enzyme specificity (the protease used to generate peptides): trypsin/P (cleavage site is C terminus of "K/R: must not be followed by "P"); maximum missed cleavage value: two; peptide spectrum match and protein false discovery rate: 0.01 FDR. For the database search, carbamidomethyl on cysteine was considered as a fixed modification, and oxidation of methionine and N-terminal acetylation were considered as variable modifications. Proteomic sample processing and LC-MS data acquisition were performed in duplicate to ensure reproducibility and statistical reliability, and were carried out by Valerian Chem Pvt. Ltd. (India).

Relative protein expression in this study was determined through comparative LC-MS-based proteomic analysis using abundance ratio values generated in Proteome Discoverer (v2.2). Protein expression in each drug-resistant clinical isolate was compared with the drug-sensitive reference isolate (BHU1066) using the abundance ratio (Resistant/Sensitive). In addition to this, protein detection patterns were also considered, including proteins exclusively detected in resistant isolates but not in the sensitive isolate. Proteins were categorized as differentially expressed based on their relative abundance ratios and/or consistent differences in detection between resistant and sensitive clinical isolates. The mass spectrometry proteomics data have been deposited to the MassIVE repository under accession number MSV000101040.

2.3. Bioinformatic studies; gene ontology and functional prediction

Functional annotation of mass spectrometry-identified proteins was performed using UniProt accession numbers. Gene Ontology (GO) terms related to biological processes, molecular functions, and cellular components, along with KEGG pathway information, were retrieved using DAVID, PANTHER, and TriTrypDB. Subcellular Localization was determined primarily using TriTrypDB; for proteins lacking this information, predictions were supplemented using DeepLoc 2.0.

2.4. Reverse transcriptase and quantitative real-time PCR

According to the manufacturer's instructions, RNA isolation from *Leishmania* promastigotes was performed using the NucleoSpin RNA Mini kit (Macherey-Nagel, Germany). For cDNA synthesis, 1 µg of RNA from each sample was incubated with 0.6 µg of random primers (6 nt; catalog number - 48190011) per 15 µL reaction, 0.1 M DTT, 500 µM dNTPs, 200 U M-MLV reverse transcriptase, and double-distilled water. Samples were incubated at 37 °C for 1 h, followed by 10 min incubation at 65 °C. cDNA from each sample was amplified with TaqDNA Polymerase (Life Technologies-BRL) in 25 µL reaction under the following conditions: 95 °C for 2 min, 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min for a total of 35 cycles. Equal cDNA input was confirmed for all samples by amplifying GAPDH. qRT-PCR was performed in triplicate in 0.1 µL strip tubes or plates (Axygen, Union City, CA) in a 10 µL reaction mixture containing 12–20 ng of cDNA, 2 ng of forward primer, 2 ng of reverse primer, and 2× SYBR Premix Ex Taq II (Takara). Real-time PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems) under the following conditions: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 34 s for 40 cycles. The mRNA expression levels of the target genes were normalized and expressed as relative fold change compared with AG83 *L. donovani* promastigotes. The primer sequences used for RT-PCR are provided in **Supplementary Table 3**.

2.5. Statistical analyses

The experiments were conducted in triplicate, using three distinct biological samples. The error bars in the figures represent the Mean ± Standard Error of the Mean (SEM). Statistical significance between the control and experimental groups was assessed using an unpaired *t*-test. Significance levels are indicated as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

3. Results

3.1. LC-MS analyses of proteome profiles of drug-sensitive and resistant parasites

Drug susceptibility assays were performed to confirm the resistant phenotypes of the clinical isolates used in this study. Increasing concentrations of amphotericin B, miltefosine, paromomycin, and sodium stibogluconate resulted in a dose-dependent reduction in parasite viability. IC₅₀ values were determined after 72 h of drug exposure and

indicated reduced drug sensitivity in the corresponding resistant isolates compared with the drug-sensitive isolate BHU1066. Specifically, the AmpB resistant isolate exhibited an IC₅₀ of 0.11 ± 0.053 µg/mL, while miltefosine, SSG, and paromomycin resistant isolates showed IC₅₀ values of 11.19 ± 3.47 µM, 21.75 ± 3.5 µM, and 13.12 ± 4.03 µM, respectively (**Supplementary Fig. 1**). These results confirm the stable drug-resistant phenotypes of the clinical isolates used for further studies.

The Pairwise comparison of the proteomes of all clinical isolates shows that each isolate has a distinct proteomic profile. Among them, NS2, BHU919, BHU1064, and BHU1020 exhibit a higher number of up-regulated genes compared to BHU1066. (**Fig. 1**). To understand the functional distribution of the differentially expressed genes within five clinical isolates, we segregated the genes based on different functional pathways and plotted them as the number of up-regulated and down-regulated genes (**Fig. 2**). The genes of all functional pathways, except lipid-cholesterol metabolism, were upregulated in NS2, BHU919, BHU1064, and BHU1020 compared with BHU1066. This quantitative

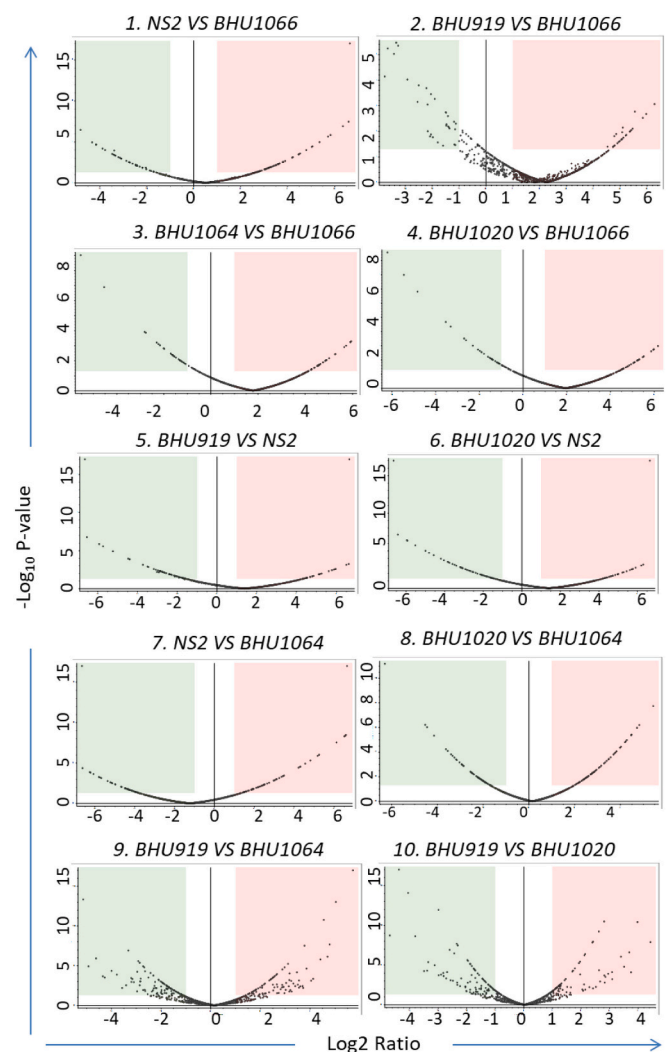


Fig. 1. Liquid chromatography-mass spectrometry analyses of proteome profiles of drug-sensitive and resistant parasites. Volcano plots showing differential protein expression across all pairwise comparisons of five *L. donovani* clinical isolates. Proteomic data were processed and quantified using Proteome Discoverer version 2.2. X-axis: log₂ fold change; Y-axis: log₁₀ P-value. Proteins exhibiting a ≥ 2-fold change in abundance with a P-value < 0.05 were considered significantly differentially expressed. Pink and green regions indicate significantly up- and down-regulated proteins, respectively, in each comparison. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

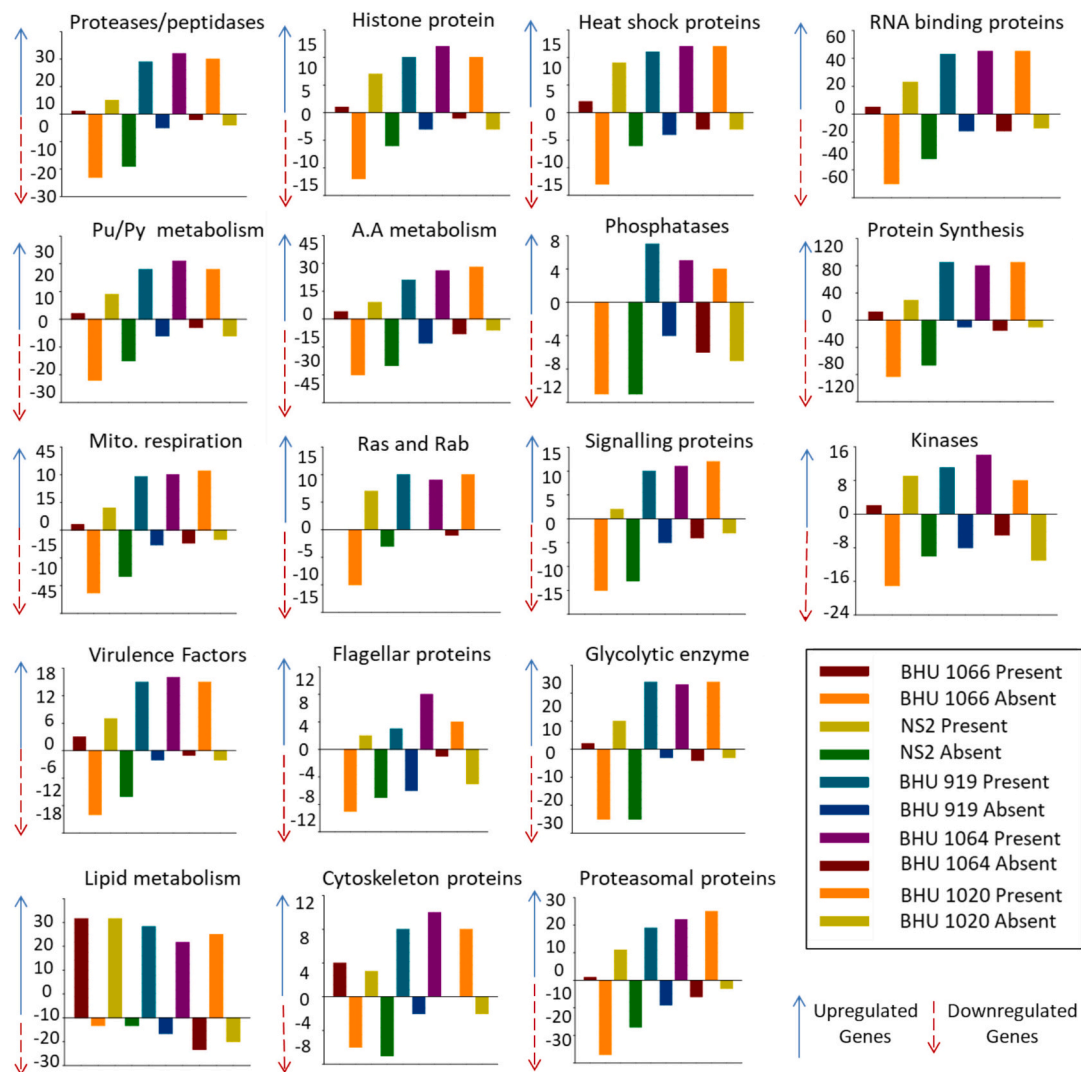


Fig. 2. Bar graphs showing the number of up- and down-regulated genes across major functional pathways in five *L. donovani* clinical isolates. Bar graphs showing the distribution of proteins across major functional pathways in five *L. donovani* clinical isolates. Data are shown for the drug-resistant isolates NS2, BHU919, BHU1064, and BHU1020 in comparison with the drug-sensitive clinical BHU1066. Bars represent proteins displaying differential abundance and detection between resistant and sensitive isolates across functional pathway.

up-regulation, as determined by LC-MS proteomic profiling, represents genuine increases in protein abundance and is indicative of functional changes in the affected pathways in drug-resistant parasites.

Among identified genes, to screen out the genes possessing role in drug resistance, we sorted the genes based on two criteria; 1. genes unique to specific drug-resistant clinical isolate (Fig. 3A-3D and Supplementary Table 1) and 2. Genes commonly up-regulated in all drug-resistant clinical isolate (Fig. 3E and Supplementary Table 2). The unique genes data suggest that there are certain sets of genes which are only up-regulated in specific drug-resistant clinical isolates and every clinical isolate is distinctly different from the others.

3.2. Genes up-regulated in all drug-resistant clinical isolates

To address multidrug and combination-therapy resistance in leishmaniasis, we analyzed genes commonly upregulated across drug-resistant clinical isolates to identify potential drug targets effective against both sensitive and resistant infections. Distinct functional category distributions were observed among isolates (Fig. 3A-D). Uncharacterized genes constituted the largest proportion of upregulated genes (32–50%), followed by metabolic enzymes (14–46%), highlighting their potential roles in resistance and parasite survival. Other recurring

categories included translation, signaling, RNA-associated proteins, proteasome-related proteins, and cytoskeletal components, with variable representation across isolates. Overall, these patterns reveal both shared and drug resistance-specific functional trends across these clinical isolates (Fig. 3A-D and Supplementary Table 1).

To refine these broad functional insights, we compared specific upregulated proteins in drug-resistant clinical isolates with the drug-sensitive BHU1066. This analysis revealed consistent upregulation of proteins involved in DNA modification, histone regulation, cytoskeletal organization, protein folding, stress response, proteolysis, and flagellar function (Supplementary Table 2).

Key glycolytic enzymes, including enolase, ATP-dependent 6-PFK, PGK, 6-PGI, and GPDH, are upregulated in drug-resistant clinical isolates. *Leishmania* glycolysis is distinctive because of glycosomal compartmentalization and phylogenetic divergence from the mammalian host, and glycolytic ATP synthesis has been associated with drug resistance in *L. major* [28]. Several TCA cycle enzymes, including aconitase, 2-OGDH [29], succinate-CoA ligase, and fumarate hydratase [30], were also upregulated. Aconitase, a 4Fe–4S iron-binding enzyme that catalyses citrate to isocitrate conversion, additionally contributes to iron homeostasis under low-iron conditions [31]. Furthermore, SCOT, a key enzyme in ketone body catabolism, was enriched in drug-resistant

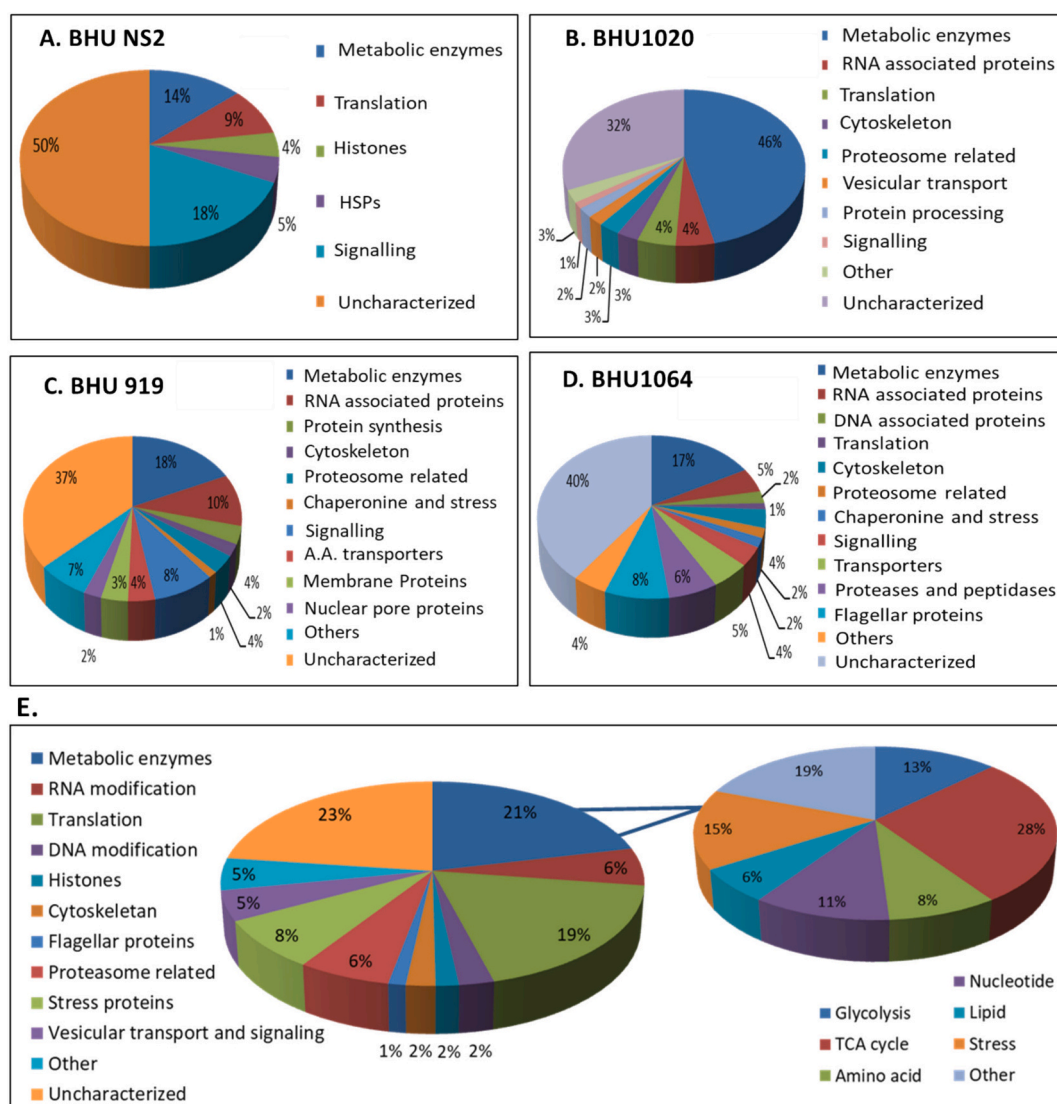


Fig. 3. Pie charts showing the functional categorization of genes associated with drug resistance in *L. donovani*. Proteins were identified through comparative proteomic analysis and categorized based on differential detection and relative abundance patterns between drug-resistant clinical isolates and a drug-sensitive BHU1066 isolate. Proteins were filtered using two criteria: (A-D) proteins detected exclusively or showing higher relative abundance in individual drug-resistant clinical isolates, and (E) proteins commonly detected with higher relative abundance across all resistant clinical isolates compared with the sensitive clinical isolate. Functional categorization was performed based on annotated biological roles.

Leishmania parasites [29,32].

Gluconeogenic enzymes F-1,6-BP [33] and phosphoenolpyruvate carboxykinase, both essential for *Leishmania* virulence, were upregulated and have been previously linked to drug resistance. Cytosolic malate dehydrogenase, a component of malate-aspartate shuttle, was likewise upregulated and has been earlier reported in paromomycin resistant *L. donovani* [34]. NAD-specific glutamate dehydrogenase, alanine aminotransferases and glycine cleavage system H protein are important enzymes of amino acid metabolism which suggest enhanced utilization of amino acids by drug-resistant *Leishmania*. Among nucleotide metabolism, aspartate carbamoyl transferase, orotidine-5-phosphate decarboxylase/orotate phosphoribosyltransferase, inosine-5'-monophosphate dehydrogenase, inosine-uridine preferring nucleoside hydrolase and nonspecific nucleoside hydrolase are up-regulated. No studies previously have shown high expression of these genes in drug-resistant *Leishmania* parasites. 3, 2-trans-enoyl-CoA isomerase and enoyl-CoA hydratase/isomerase family proteins involved in lipid catabolism through β -oxidation were upregulated and have been previously reported to be abundant in infective metacyclic promastigotes,

suggesting their role in *Leishmania* virulence [35]. Inositol phosphosphingolipid phospholipase C-like proteins, essential for sphingomyelin degradation and *Leishmania* virulence [36], and diphosphomevalonate decarboxylase, involved in ergosterol biosynthesis and protection against antimony stress, were upregulated [37]. Adenosyl homocysteine and S-adenosylmethionine synthetase, key enzymes of sulphur amino acid metabolism involved in glutathione and trypanothione precursor synthesis, were up-regulated in our study and have also been reported in antimony resistant *Leishmania* [38].

Ascorbate-dependent peroxidase is crucial for the redox defense system of *Leishmania* and essential for parasite differentiation and survival inside host, was also upregulated in our study [39]. Cystathionine- γ -lyase, an enzyme required for cysteine biosynthesis via reverse trans-sulphuration, was upregulated. Cysteine serves as a precursor for coenzyme A, iron-sulphur clusters, glutathione, and trypanothione, all of which play pivotal roles in redox regulation and cellular signaling. [40,41]. Tryparedoxin, which plays crucial roles in metabolic rewiring during oxidative and nitrosative stress, was upregulated [42]. Other metabolic enzymes up-regulated in drug-resistant *L. donovani* are

carboxylase, aldehyde dehydrogenase, mitochondrial short chain dehydrogenase, sucrose-phosphate synthase-like protein, isopentenyl-diphosphate delta-isomerase, N-acyl-L-amino acid amidohydrolase, amidohydrolase, GDP-mannose pyrophosphorylase, Ubiquinol-cytochrome-c reductase and probable vacuolar ATP synthase catalytic subunit A, which are important for energy production, redox balance, and metabolic regulation.

Proteins related to RNA modification, including RNA-binding proteins, KH domain-containing proteins, RNA helicases, Scd6-like Sm domain-containing proteins, Tudor domain-containing proteins, Nop10p, nuclear ribonucleoprotein Sm D2, polyadenylate-binding protein, and DEAD-box RNA helicase, were upregulated. Translation-related proteins, such as 40S and 60S ribosomal proteins, tRNA synthetases, elongation factors, and other translation factors, were also upregulated in all drug-resistant clinical isolates, with mitochondrial RNA-binding protein 1, 40S ribosomal proteins S18 and S19, 60S ribosomal protein L13 [43] [44], and elongation factor Tu [45] previously reported as upregulated in resistant strains. Proteins involved in DNA modification, including DNA polymerase, replication protein A subunit, ATP-dependent helicase, homeobox-associated leucine zipper, and RuvB-like DNA helicases, were similarly upregulated. Collectively, this upregulation may contribute to drug resistance in *L. donovani* by enhancing RNA stability, protein synthesis, and DNA repair mechanisms. Histone H4, histone H3, histone H2B variant, and H1 histone-like protein were more abundant in drug-resistant *L. donovani*, with H4, H3 and H2B variant are previously reported as up-regulated in drug-resistant *Leishmania* [43].

The cytoskeletal proteins, including actin, calmodulin, kinesin-like protein, C-terminal motor kinesin along with flagellar proteins such as intra-flagellar transport protein 81, flagellar Member 7 and flagellar radial spoke protein-like, were up-regulated in all drug-resistant clinical isolates, suggesting a role in maintaining cellular structure, motility, and parasite infectivity under drug pressure.

The proteasome-ubiquitin complex and various proteases, which regulate protein turnover and degrade misfolded proteins, were highly expressed in these drug-resistant clinical isolates. Proteasome related proteins, including the proteasome endopeptidase complex, proteasome regulatory non-ATPase subunits, ubiquitin-activating enzyme E1, ubiquitin-activating enzyme-like protein, ubiquitin ligase, WD domain G-beta repeat proteins, as well as proteases such as p45, metallopeptidases clan MG family M24, prolyl oligopeptidase, cysteine peptidase C, cysteine protease C, aminopeptidase are up-regulated. The upregulation of these proteins may contribute to drug resistance by enhancing protein quality control, stress adaptation, and parasite survival, and also reinforces their role as virulence factors and potential therapeutic targets [6]. Cysteine protease/peptidase C are cathepsin B like proteases are virulence factors involved in secretome and cell death regulation of *Leishmania* [46]. p45, a member of methionine aminopeptidase family, has demonstrated prophylactic efficacy upon immunization with recombinant *L. donovani* p45 [47]. Inhibition of prolyl peptidase has been shown to prevent parasite invasion in murine macrophages suggesting involvement of prolyl peptidase in invasion macrophage invasion process [48]. *Leishmania* aminopeptidases of also contributes to parasite virulence and represent potential therapeutic targets *Leishmaniasis* [49] [50].

Multiple HSPs and chaperonins were upregulated in drug-resistant parasites, suggesting enhanced tolerance to drug-induced stress. These stress proteins prevent protein misfolding and aggregation [51]. Increased chaperones levels have been reported in *Leishmania* strains resistant to antimonials, AmpB, methotrexate [52], as well as in infective promastigotes forms of *L. amazonensis* [53]. Overexpression of HSP83 in drug-sensitive *L. donovani* reduced programmed cell death and increases antimony resistance, suggesting its role in drug resistance mechanism [54].

Intracellular trafficking is crucial for any living cell for nutrient intake, targeting and secretion [55]. Small GTPases of the Rab family are

essential regulators of intracellular trafficking pathways that enable parasites like *Leishmania* to acquire nutrients from their host cells [56]. Rab1 of *L. donovani* plays role in conventional secretory pathway [57]. In *Leishmania* hemoglobin is endocytosed via receptor-mediated endocytosis [58] and then internalized into Rab5-positive early endosome [59] followed by targeting to lysosomal compartment in a Rab-7 dependent manner [60]. The isoforms of Rab5, Rab5a and Rab5b differentially regulate fluid-phase and receptor mediated endocytosis in *Leishmania* [61]. Rab GDP dissociation inhibitor (GDI) is another regulator of intracellular trafficking of *Leishmania* and studies show that overexpression of *Leishmania* GDI enhances lysosomal targeting of internalized hemoglobin and the secretion of gp63 in the parasites [62]. The probable vesicular-fusion protein nsf and vesicular-fusing ATPase have role in SNARE complex disassembly which is crucial for regulation of SNARE complex mediated membrane fusion [63]. Probable 14–3–3 protein, activated protein kinase c receptor (Fragment) and probable CAS/CSE/importin domain protein are the proteins involved in cell signaling up-regulated in drug-resistant *Leishmania*. 14–3–3 is the protein involved in endocytosis and negative regulation of mitotic nuclear division and previously reported overexpressed in drug-resistant *Leishmania* [54].

3.3. Selection of unique genes and qPCR validation of unique genes

Next, to validate the proteomic findings, quantitative RT-PCR was performed on genes corresponding to proteins that were uniquely present in these clinical isolates. We shortlisted 30 genes unique to each resistant clinical isolate that showed a ≥ 1.5 -fold change in expression and had known or predicted roles in drug resistance. Functional annotation, including Accession ID, predicted name, cellular component, biological process, and molecular function, was carried out using various bioinformatics tools (**Supplementary Table 4**). Due to the unavailability of specific antibodies, qPCR was used to evaluate transcript levels of selected genes. Expression was assessed in the drug-sensitive isolate BHU1066 and three drug-resistant isolates (BHU1064, BHU1020, and BHU919), using the reference strain AG83 for fold-change normalization (**Fig. 4**). This analysis enabled correlation of protein abundance with transcript levels, and qRT-PCR validation confirmed drug resistance-specific transcriptional changes in *L. donovani*.

In BHU1064, qPCR analysis of 18 uniquely expressed genes showed consistently higher expression than in other clinical isolates. Elevated transcripts of redox and oxidative stress-related genes, including trypanothione reductase, thioredoxin domain-containing protein, and SOD, indicate enhanced ROS detoxification. Genes involved in metabolic pathways, such as pteridine transporter and reductase, inositol-3-phosphate synthase, and UDP-glucose 4'-epimerase, were also upregulated, supporting survival under drug-induced stress. In addition, increased expression of structural and motility-associated proteins (GP63, flagellar radial spoke, and intraflagellar transport proteins), signaling regulators (protein kinase and phosphatase 2C), and the universal minicircle sequence binding protein suggests coordinated adaptations in virulence, signaling, and mitochondrial maintenance associated with drug resistance.

Among the seven uniquely upregulated proteins identified in BHU1020, qPCR confirmed elevated transcript levels for proteins involved in carbohydrate metabolism and redox balance, including D-xylulose reductase and 6-phosphogluconate dehydrogenase which play key roles in the pentose phosphate pathway and oxidoreductase activity. Increased expression of metabolic and cytoskeletal proteins such as 6-phosphogluconate dehydrogenase, dynein heavy chain, and flagellar member 8 suggests adaptations in energy metabolism and motility. Additionally, proteins related to post-transcriptional regulation and mitochondrial RNA processing showed enhanced expression, indicating elevated metabolic and regulatory functions. Interestingly, dynein heavy chain, critical for microtubule-based movement, was not showing

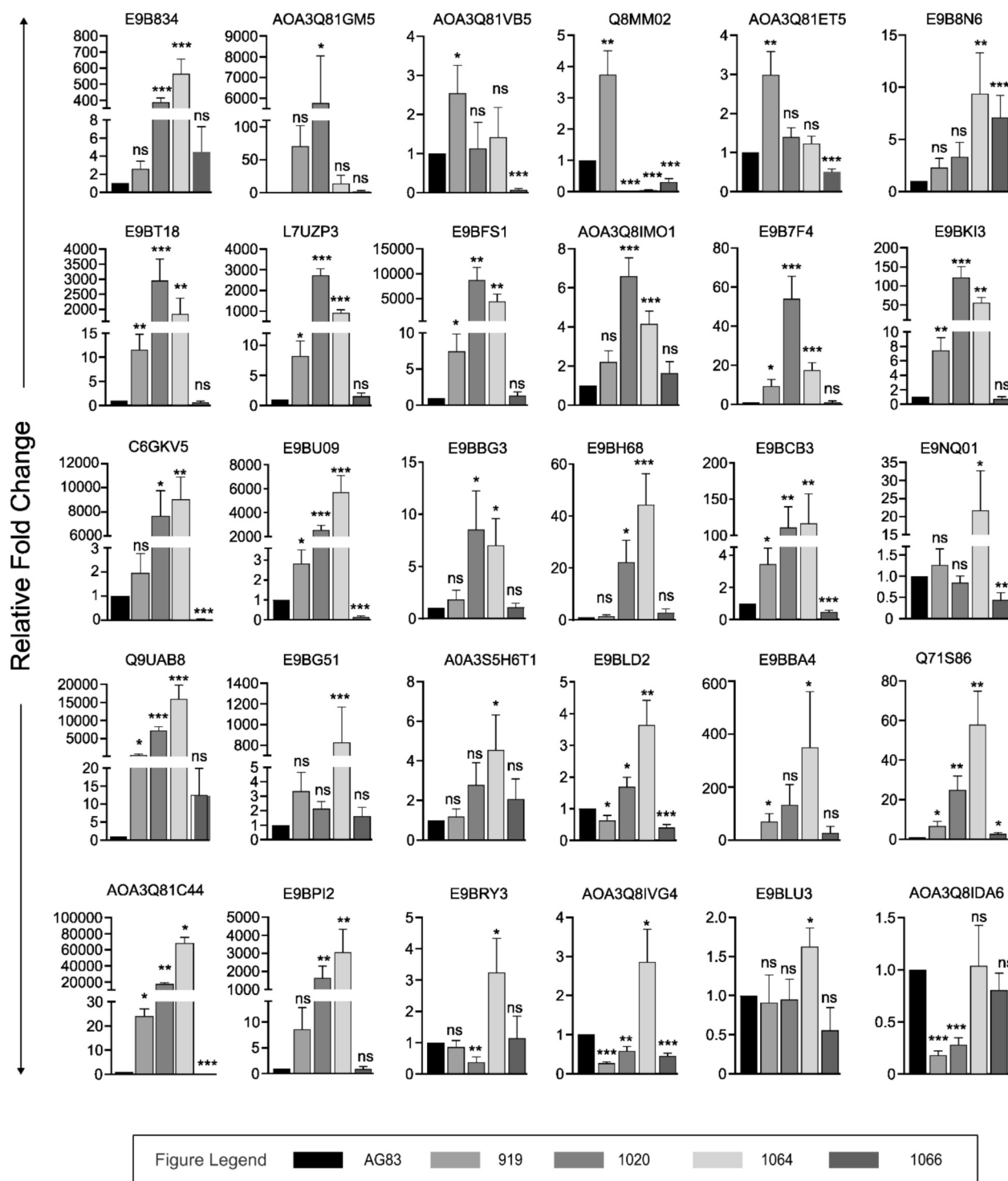


Fig. 4. Bar graphs showing the relative expression levels of drug resistance-specific unique genes across five *L. donovani* clinical isolates. Gene expression levels were normalized to β -tubulin, and relative fold changes were calculated using the drug-sensitive reference isolate AG83 (value = 1). Data represent mean $\pm$ SEM from three independent experiments ($N = 3$). Statistical significance was determined using a *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

high expression at the transcript level despite its detection in proteomics, likely reflecting post-transcriptional control or greater protein stability. Similarly, the leucine zipper transcription factor-like protein 1 (E9B8N6), uniquely expressed in the drug-sensitive 1066, localises to the cytoplasm and may be involved in cilium assembly and cargo trafficking to the periciliary membrane, potentially contributing to flagellar function.

In BHU919, proteomics initially identified five upregulated proteins,

with qPCR validating three: acyl-CoA dehydrogenase, prostaglandin F2- α synthase, and Pumilio protein 6. These proteins indicate BHU919-specific adaptations in lipid metabolism and post-transcriptional regulation, possibly affecting membrane composition and RNA stability. Conversely, axonemal dynein light chain and phosphorylated CTD-interacting factor 1 were downregulated at the mRNA level, suggesting differential regulation between transcript and protein levels.

The NS2 isolate was used only for proteome profiling because it

exhibits higher growth and proliferation, larger cell size, heavy glycosylation, and high survival, and it requires low-nutrient conditions (2% M199 with glutamine), unlike the other isolates cultured in 10% RPMI with glutamine. Consequently, NS2 could not be included in qPCR validation. Some of the proteins uniquely expressed in NS2 are related to chromatin and gene regulation such as Histone H4, NLI interacting factor-like phosphatase, and a coiled-coil domain-containing protein, likely influence DNA structure and gene expression through nucleosome formation, dephosphorylation, and post-translational modifications. Proteins involved in protein folding and translation, including Heat shock protein 70 and 60S ribosomal protein L23a, support proper protein synthesis and cellular homeostasis. Transport and membrane-related proteins, such as Nucleoporin NUP110, a conserved hypothetical protein, and Concanavalin A-like lectin, facilitate nucleocytoplasmic transport, membrane channel formation, and carbohydrate recognition. Lastly, the serine/threonine protein kinase modulates cellular signaling through phosphorylation, playing a key role in regulatory pathways [64].

4. Discussion

The present study provides a comprehensive proteomic insight into the molecular landscape of drug resistance in clinical isolates of *L. donovani*, offering valuable contributions toward understanding the mechanisms underlying resistance to frontline anti-leishmanial therapies. Our findings reveal that each drug-resistant clinical isolate possesses a unique proteomic fingerprint, yet shares overlapping adaptations, particularly in metabolic reprogramming, stress response, and gene expression regulation [Fig. 5].

Previously, our group demonstrated that all these clinical isolates exhibit comparable *in vitro* growth kinetics, except for NS2, but differ in splenic parasite load. Despite comparable *in vitro* growth, the drug-sensitive BHU1066 causes significantly lower infection than drug-resistant clinical isolates, supporting the association between drug

resistance and increased virulence. The rationale for the present study stems from our earlier findings demonstrating distinct splenic cytokine profiles and differential expression of T cell associated transcription factors among these clinical isolates. Importantly, those studies established that drug resistance in *L. donovani* operates independently of host immunosuppression [65]. Building on this foundation, the current study aims to further elucidate the mechanisms linking drug resistance to specific antileishmanial drugs. As a prerequisite for these investigations, we first confirmed the drug resistance status of the clinical isolates used. These isolates exhibited stable and significantly reduced sensitivity to amphotericin B, miltefosine, paromomycin, and sodium stibogluconate relative to the drug-sensitive clinical isolate BHU1066. The well-defined resistance phenotypes of these isolates provided a reliable framework for subsequent analyses.

The emergence of drug-resistant *L. donovani* severely compromises the efficacy of frontline antileishmanial therapies and represents a critical barrier to VL control. Resistance mechanisms include genetic mutations, genomic plasticity, and biochemical adaptations [25]. For instance, miltefosine resistance is linked to mutations in the transporter gene (*LDMT*), reducing drug uptake. Antimonial resistance involves enhanced oxidative stress tolerance and drug efflux [66]; and amphotericin B resistance stems from altered sterol composition and increased ABC transporter expression [67,68]. Genomic alterations such as gene amplification, SNPs, and chromosomal rearrangements further aid rapid adaptation under drug pressure [69].

In this study, comparative proteomic analysis of drug-sensitive and drug-resistant *L. donovani* isolates identified numerous differentially expressed proteins. We analyzed the data using two approaches: isolate-specific uniquely abundant proteins and proteins commonly upregulated across resistant isolates. While unique proteins may reflect drug-specific adaptations, commonly upregulated proteins are likely more informative, as resistant strains can evolve toward multidrug resistance. Consequently, future antileishmanial therapies should be effective against sensitive, mono-drug-resistant, and multidrug-resistant strains.

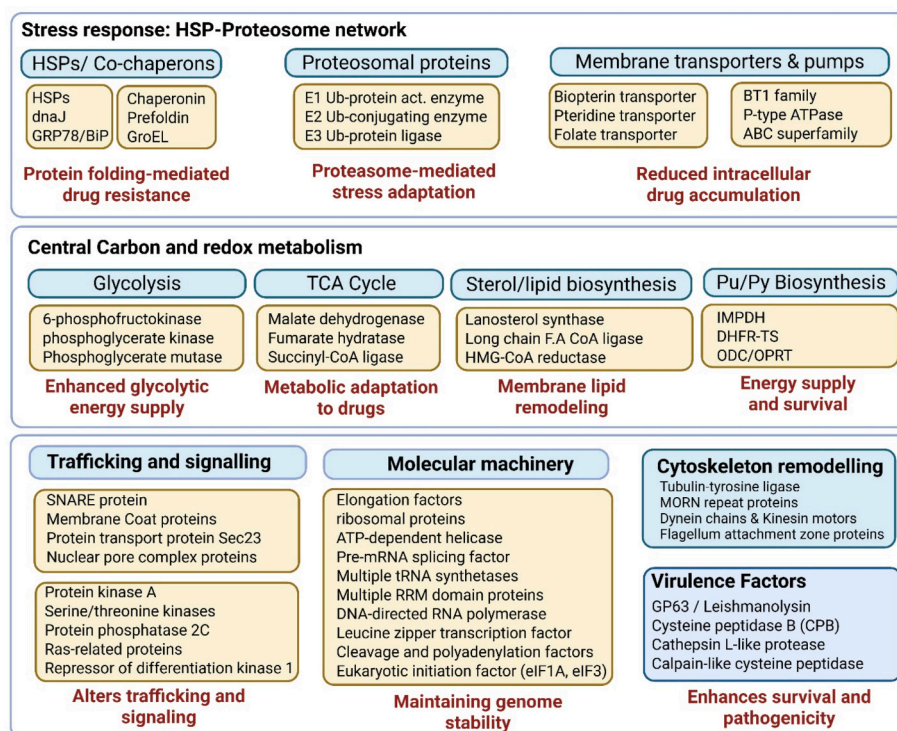


Fig. 5. Integrated overview of cellular pathways contributing to *Leishmania* drug resistance. The diagram summarizes how stress response, central carbon and redox metabolism, trafficking and signalling, molecular machinery, cytoskeleton remodeling and virulence factors cooperatively promote protein homeostasis, metabolic adaptation, altered drug transport and enhanced survival of drug-resistant parasites.

Although many upregulated proteins participate in essential cellular processes, their consistent elevation in resistant isolates reflects an adaptive response to drug pressure and a direct role in drug resistance. This study reveals both unique and shared resistance associated proteins, suggesting that despite drug specific selective pressures, *Leishmania* employs a core set of adaptations, particularly those involving metabolism, stress response, and translation [Fig. 5]. These convergences point to potential universal resistance markers and broad-spectrum therapeutic targets. All resistant *L. donovani* isolates showed global upregulation of proteins involved in glycolysis, the TCA cycle, amino acid metabolism, and nucleotide biosynthesis [Fig. 5]. Key enzymes, including enolase, phosphoglycerate kinase, and ATP-dependent 6-phosphofructokinase, were consistently elevated, enhancing energy production and metabolic adaptability in drug-resistant phenotypes [70,71]. The glycosomal localization of these glycolytic enzymes, distinct from their mammalian counterparts, further emphasize their potential as selective drug targets [72].

Furthermore, Proteins involved in RNA modification and translation were consistently enriched in resistant *L. donovani* isolates, suggesting that enhanced protein synthesis supports rapid adaptation under drug-induced stress. Heat shock proteins and chaperonins, including HSP83, were also markedly upregulated, reflecting improved protein homeostasis that helps parasites survive chemotherapeutic challenges. In addition, proteasomal components and proteases, including cysteine protease C and prolyl oligopeptidase, were upregulated in resistant *L. donovani*, supporting protein turnover and contributing to both drug resistance and virulence. Approximately 20% of upregulated proteins remain uncharacterized, representing potentially novel, parasite-specific resistance factors and drug targets that warrant further investigation. The involvement of endocytosis and intracellular trafficking proteins, including Rab family GTPases and vesicular-fusion proteins, further suggests that modulation of host-parasite interactions and nutrient acquisition pathways may also contribute to the resistant phenotype. In our study, we identified 30 clinical isolate-specific proteins with known or predicted roles in drug resistance for further functional analysis. The results confirmed concordant transcriptional changes for the majority of targets, lending confidence to the proteomic findings.

Finally, our findings reinforce previous reports that drug resistance in *Leishmania* is multifactorial, involving metabolic adaptation, altered stress responses, and post-transcriptional regulation [25].

While this study presents a comprehensive proteomic landscape of drug-resistant *L. donovani* isolates, it also highlights a key challenge in translating large-scale omics data into effective antileishmanial therapies. Despite extensive datasets, few molecular findings progress to validated drug targets, contributing to high attrition in drug development. The commonly upregulated and uncharacterized proteins identified here provide a valuable resource for future prioritization and functional studies. Although experimental validation was beyond the scope of this study, one could envision integrating this proteomic information with *in silico* tools such as druggability prediction, essentiality mapping, and structural modeling - alongside molecular, biochemical, and cell biological approaches to prioritize and functionally characterize promising candidates. These could then be evaluated through genetic manipulation, enzymatic assays, subcellular localization studies, recombinant expression, or even incorporated into structure-based virtual screening pipelines using available compound libraries. By presenting a high-resolution proteomic compendium alongside a structured framework for future functional exploration, our study aims to contribute strategically to more efficient and collaborative antileishmanial drug discovery efforts. These insights emphasize the complex and layered nature of drug resistance and highlight the importance of integrating proteomic data with functional studies to uncover novel targets for intervention.

A limitation of this study is that while drug susceptibility, growth, and virulence were previously evaluated for these clinical isolates,

comprehensive phenotypic and biochemical comparisons among the five isolates were not performed. Such comparisons are important to fully elucidate how drug-specific traits contribute to resistance mechanisms and virulence beyond proteomic signatures. Future work could integrate these analyses through standardized phenotypic assays, biochemical profiling, and multi-omics integration to reveal actionable differences for targeted therapies. Also, validation using laboratory-generated resistant parasites was not conducted, which could enhance study reliability by providing controlled models to confirm clinical isolate findings and dissect causal resistance pathways. Finally, while qRT-PCR confirmed transcriptional changes for select proteins, broader translational validation of key upregulated proteins was not performed due to the unavailability of commercial antibodies against these *Leishmania*-specific candidates, limiting direct confirmation of proteomic abundance changes at the protein level. Future considerations include testing larger panels of clinical isolates from diverse patients and regions to confirm reproducibility of these drug phenotypes across broader *Leishmania* populations.

5. Conclusion

In conclusion, this study demonstrates that drug resistance in *L. donovani* clinical isolates is accompanied by extensive and distinct proteomic alterations. Each resistant isolate exhibits a unique set of upregulated proteins compared to the drug-sensitive reference. Comparative proteomic profiling identified 1825 proteins across five isolates, with consistent enrichment of proteins involved in metabolism, translation, RNA modification, chaperones, heat shock proteins, and signaling. Approximately 20% of the upregulated proteins remain uncharacterized, representing potential novel therapeutic targets. We employed two approaches to understand resistance: one identifying proteins uniquely upregulated in each isolate, revealing drug resistance-specific signatures, and another focusing on proteins commonly upregulated across all resistant isolates, uncovering a conserved subset essential for survival under drug pressure. These findings highlight both the diversity and convergence of resistance mechanisms, supporting integrated strategies targeting conserved parasite pathways and exploring host-directed approaches. Overall, the results emphasize the complexity of drug resistance in *L. donovani* and the need for continued molecular research to guide effective therapies and surveillance.

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CRediT authorship contribution statement

Dhiraj Gurjar: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Baibaswata Saha:** Methodology. **Kalpana Pai:** Writing – review & editing, Conceptualization. **Shyam Sundar:** Writing – review & editing. **Alicia Ponte-Sucre:** Writing – review & editing. **Neelam Bodhale:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Bhaskar Saha:** Writing – review & editing, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.parint.2026.103267>.

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