The Role of Proteomics in the Study of Drug Resistance

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Abstract

The recent completion of the genomic sequencing of three species of *Leishmania*, *L. (L.) major*, *L. (L.) infantum*, and *L. (V.) braziliensis* has enormous relevance to the study of the leishmaniasis pathogenesis. However, since in *Leishmania* the control of gene expression relies on the stability or processing of the mature mRNA, as well as on the posttranslational modifications of proteins, the genomic sequences alone are insufficient to predict protein expression within the parasites. In this scenario, proteomic technologies provide feasible pathways to functional studies of this parasite. With the challenging increase of natural drug resistance by *Leishmania*, the combination of the available genomic resources of these parasites with powerful high-throughput proteomic analysis is urgently needed to shed light on resistance mechanisms and identify new drug targets against *Leishmania*. Diverse proteomic approaches have been used to describe and catalogue global protein profiles of *Leishmania* spp. reveal changes in protein expression during development, determine the subcellular localization of gene products, evaluate host-parasite interactions, and elucidate drug resistance mechanisms. The characterization of these proteins has advanced, although

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many fundamental questions remain unanswered. Here we discuss the recent proteomic discoveries that have contributed to the understanding of drug resistance mechanisms in *Leishmania* parasites.

10.1 Introduction

Since the publication of the genome data from *L. (L.) major* [1], *L. (L.) infantum*, and *L. (V.) braziliensis* [2], over 14 *Leishmania* spp. genomes have been sequenced to date (http://tritrypdb.org/tritrypdb/). Such achievements, in addition to the accumulation of genomic data from other *Leishmania* species, strains, and clinical isolates (http://www.genedb.org/, http://www.uniprot.org/, [3]), offer the prospects of new drug target identification and/or the exploration of particular metabolic pathways for drug development.

For example, analysis of genomic data from *L. (L.) infantum* and *L. (V.) braziliensis* has revealed the presence of a gene encoding cyclopropane fatty acyl phospholipid synthase [2]. Because this enzyme appears to be involved in the maintenance of the parasite's membrane and is not present in humans, it has been pointed as a putative chemotherapeutic target [2]. Such as this enzyme, many additional targets are probably encoded within the genome of *Leishmania* spp. The discovery of these targets is urgently needed given the increasing treatment failure observed with the mainstay chemotherapy, the pentavalent antimonials (Sb^V) [4], and the emergence of clinically resistant isolates [5–12].

Although all information concerning potential drug targets is contained in sequence databases, the promises of such target identifications are hampered by several factors. First, the limited functional annotation of the genomic sequence data determines that more than 50% of the predicted proteins have unknown functions [1, 2], which presents itself as an attractive challenge. Unfortunately, in 2017, 12 years after the first sequencing of a *Leishmania* genome, this scenario has not changed, and near 50% of the predicted proteins lacks functional annotation. Second, while the complexity of the cell cycle of these parasites would indicate that specific repertoires of genes are expressed in the promastigote and amastigote stages, global microarray genomic analyses have revealed that most *Leishmania* genes are constitutively transcribed [13–15], which is in agreement with the polycistronic organization of this parasite's genome [1]. Third, of the approximately 8000–9000 coding genes found in *Leishmania*, ~6200 are common to all trypanosomatids sequenced thus far, ~1000 are *Leishmania*-specific, and only ~200 genes (including some pseudogenes) are species-specific [1, 2, 16–18]. Hence, the modest differences between the genome sequences of *L. (L.) major*, *L. (L.) infantum*, *L. (V.) braziliensis*, and *L. (L.) mexicana* do not reflect the vast differences among the clinical phenotypes of leishmaniasis that are associated with each of these species. Consequently, the *Leishmania* genome sequences alone are insufficient to predict whole protein expression profiles throughout the life cycle of the parasite or under specific drug pressure or other experimental conditions. Interestingly, genome heterogeneity arisen from large-scale gene copy number variation, and extensive aneuploidy is

observed in natural *Leishmania* isolates in response to drug pressure [3, 19–22]. However, it is still unknown whether this variation in gene and chromosome copy number is followed by variation in protein abundance.

Gene regulation in *Leishmania*, as with other trypanosomatids, occurs principally at the posttranscriptional level [23, 24] mainly by mechanisms that involve RNA stabilization and 3′UTR signatures [25–27]. Seminal analyses of RNA transcripts using DNA microarrays in either broad gene expression studies [13, 14, 28–31] or studies limited to specific genes [32–34] have shown that less than 6 % of genes are modulated at the mRNA level during the different stages of the *Leishmania* life cycle. Gene expression studies specifically related to drug resistance in *Leishmania* have shown the same pattern [19, 20, 32, 35]. Changing this scenario, recently, RNA sequencing (RNA-seq) of *L. (L.) mexicana* transcriptome showed that over 3.000 genes (~40%) are differentially expressed between promastigotes and amastigotes [18].

Although mRNA quantification has resulted in the increase of knowledge of several cellular processes of *Leishmania*, the direct analysis of protein levels is advantageous because the relationship between transcript abundance and protein expression levels in this parasite has been shown to be poor [15, 36]. In addition, information concerning cellular localization, posttranslational modifications, or protein interactions cannot be obtained from mRNA data [37]. As aneuploidy and regulation at translational and posttranslational levels make the scenario of protein expression in this parasite more complex [36], high-resolution proteomic approaches have the potential of shedding light on protein patterns that define a clinical phenotype. This may include either a phenotype associated with a specific disease manifestation or one associated with the susceptibility or resistance to a specific drug.

Proteomic analyses, therefore, provide data that are of crucial significance for the description and comprehension of the biology of *Leishmania* parasites, which are not evident from the genome sequence or the mRNA transcripts. The proteome is defined as the set of proteins expressed by a cell or organism under specific conditions and at a given point in time. The field of proteomics intends to provide detailed descriptions and integration of protein data to better ascertain protein function in biological systems. By allowing the characterization of complex systems, proteomic approaches offer the opportunity to identify proteins involved with drug resistance in *Leishmania*, in addition to new drug targets for this parasite.

In general, most proteomic studies of *Leishmania* and other trypanosomatids involve protein fractionation from a protein mixture using SDS-PAGE and/or two-dimensional electrophoresis (2DE), followed by gel excision and enzymatic digestion of protein spots. Peptides are submitted to mass spectrometry (MS) methods that combine soft ionization sources [matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI)] [38–40] with various mass analyzers. Subsequent protein identification is accomplished by linking mass spectral data to genome sequence databases using bioinformatics tools [41, 42]. Gel-free shotgun liquid chromatography tandem mass spectrometry (LC/MS/MS) analyses have the potential to map more thoughtfully the *Leishmania* proteome

under different conditions [43–45]. High-resolution LC/MS/MS for quantitative analysis by isotopic labeling of proteins or even label-free approaches has in fact revealed new aspects of *Leishmania* stage-specific proteomes or specific parasite phenotypes [46–53].

Proteomic studies of *Leishmania* spp. have focused on global proteome profiling [46, 49, 54–65], detailed descriptions of stage-specific protein expression [14, 36, 43, 47, 51, 66–76], posttranslational modifications (PTMs) [44, 52, 59, 73, 77–81], identification of proteins from subcellular proteomes and secretomes [45, 71, 82–91], and determining potential drug targets or proteins involved in drug resistance [50, 53, 56, 64, 92–100], among others.

Proteomic studies for identifying molecules potentially involved in the drug resistance of *Leishmania* spp. can be classified according to the approach carried out using either (1) axenic promastigotes and/or amastigotes that have been selected to drug resistance in vitro or (2) axenic promastigotes and/or amastigotes derived from clinical isolates that are considered naturally drug resistant. The compounds evaluated in these studies include drugs currently used for the treatment of leishmaniasis, such as Sb^V, amphotericin-B (AMB), and miltefosine (MIL), in addition to model drugs for the study of resistance, such as methotrexate (MTX) and arsenite, and drug under development, such as bicyclic nitro drugs [24, 50, 53, 56, 79, 92–99, 101–108] (Table 10.1). This chapter will discuss the applications of proteomic approaches to the study of *Leishmania* drug resistance, focusing on the identified molecules and on the inferred mechanisms of resistance to current medicines used for the treatment of leishmaniasis.

Table 10.1 Drugs analyzed in proteomic studies of *Leishmania* drug resistance

Drug	Drug status	Species analyzed	References
Antimonials	First-line drug	*L. (L.) infantum* *L. (L.) donovani* *L. (V.) panamensis* *L. (L.) braziliensis*	[79, 93–95, 102–106, 124]
Miltefosine	First-line drug	*L. (L.) donovani* *L. (L.) infantum*	[93, 96, 97, 99, 108]
Amphotericin-B	First-line drug	*L. (L.) infantum*	[107]
Methotrexate	Model drug	*L. (L.) major*	[56, 92]
α-Difluoromethylornithine	HAT drug	*L. (L.) donovani*	[50]
Arsenite	Model drug	*L. (L.) donovani*	[101]
Bicyclic nitro-drugs	Under development	*L. (L.) donovani*	[53]

HAT human African trypanosomiasis

10.2 Proteomic Approaches Used for Studying Drug Resistance in *Leishmania* spp.

10.2.1 Protein Expression Mapping by Two-Dimensional Electrophoresis

Proteomic studies of drug resistance in *Leishmania* spp. have traditionally used 2DE and 2D differential in-gel electrophoresis (2D-DIGE) for the comparative analysis of protein expression in drug-resistant and drug-sensitive parasites [56, 79, 92–99, 101–108]. This technique undoubtedly provided the basis for further developments in proteomics and, despite having certain limitations, is still used for protein expression mapping. The separation of complex cellular extracts by 2DE is achieved by coupling two independent electrophoretic separations, using isoelectric focusing in the first dimension and SDS-PAGE in the second [109, 110]. Soon after the first reports of 2DE appeared, this method was widely adopted by researchers around the world in several distinct applications [111–113].

The first works of what can be called the earliest *Leishmania* proteomics, even before the term "proteomics" was coined, came from the early 1980s. In these works, 2DE was used to (1) separate cell lysates of *L. (L.) tropica* for further detection of antigenic proteins using rabbit sera [114] and (2) for the comparative analysis of protein expression patterns from distinct *Leishmania* species that cause American tegumentary leishmaniasis with the aim of detecting species-specific markers [115]. However, issues concerning reproducibility, specifically involving the stability of the pH gradients, discouraged the widespread use of the method. Additionally, the absence of a protein identification system prevented the designation of interesting proteins. Identification was achieved by co-migration with purified proteins or through the use of antibodies. Using these methods, the regulation of tubulin expression during *Leishmania* differentiation was demonstrated [116]. Despite the drawbacks, valuable information was obtained, such as the demonstration that *Leishmania* resistance to MTX, an antiproliferative agent, is mediated by a mutation in the target enzyme, dihydrofolate reductase (DHFR), which alters the physicochemical properties of the protein [117].

The introduction of immobilized pH gradients in the first dimension [118] eliminated the reproducibility issues associated with pH stability. At the same time, N-terminal sequencing using traditional Edman chemistry applied to proteins separated by SDS-PAGE allowed the identification of peptides and proteins, as well as molecular mass determination [119]. However, it was the introduction of soft ionization techniques for peptides and proteins (MALDI and ESI) that allowed the acquisition of mass spectra of these molecules at the subnanomolar level and also changed the paradigm of protein identification [38–40]. Coupling 2DE and MS identification, Drummelsmith et al. observed up to a fourfold increase in the expression of several spots of trypanothione (TRYR) protein in transfected *L. (L.) major* promastigotes overexpressing the TRYR gene [56]. This assay validated the use of 2DE for drug resistance studies. Decades after 2D appeared, various studies started using fluorescent dyes, mainly the Cy dyes, which is the principle of 2D-DIGE, in

order to achieve a more reliable quantification of differences among the spots detected [47, 81].

As mentioned above, all proteomic studies on *Leishmania* drug resistance have used the combination of 2DE or 2D-DIGE and MS for protein identification. However, 2DE presents major limitations, including the inability to resolve low abundance proteins and hydrophobic proteins, which represent important sources of information in the case of drug resistance mechanisms. Also in quantitative studies, the protein co-migration is a significant issue when deciding which protein contributed the more for the change in intensity observed in one spot. Despite these limitations, 2DE is a well-characterized technique for protein separation, and it is distinguished by its visual array that allows the detection of posttranslational modified states [120, 121].

10.2.2 Other Proteomic Approaches for Studying Drug Resistance

Although the use of fluorescent dyes has turned 2D-DIGE into a quantitative tool with better sensitivity and reproducibility than 2D, the gel-based approaches are still very limited regarding linearity, dynamic range, and reliability for quantifying differences in protein abundance, being limited to the resolution of soluble and abundant proteins [118, 122]. These limitations have been surpassed by the development of mass spectrometry (MS)-based approaches. In MS-based proteomic approaches, proteins can be identified and quantified by means of detection and quantification of their peptides [123]. The main methods include labeling with stable isotopes or label-free approaches. Labeling methods introduce a mass tag into proteins or peptides, either metabolically, enzymatically, or by chemical means; labeling based on isobaric tags for relative and absolute quantification (iTRAQ) and stable isotope labeling by amino acids in cell culture (SILAC) has been used in several proteomic analysis of *Leishmania* parasites enabling quantification of stage-specific proteins, characterization of posttranslational modifications, and quantification of protein abundance in parasites selected for drug resistance [48, 50, 53, 72, 78, 107, 124]. On the other hand, label-free methods correlate the ion intensity signal of peptide mass spectra or the number of peptide spectral counts with the protein quantity [122, 125, 126]. Shotgun label-free methods for protein quantification in *Leishmania* have been little explored, but there is a nice example of the potential of this approach in the quantitative analysis of the proteome of *L. (L.) mexicana* reported by Paape et al. [43].

Using high-throughput proteomic technologies, pharmacoproteomics allows discovery and validation of novel drug targets and generates information about drug metabolism and transport as well as about drug efficacy, resistance, and toxicity [127, 128]. Successful examples of these applications can be found in cancer research [129, 130]. Pharmacoproteomics has started to be used successfully in the study of drugs under development for leishmaniasis treatment [53].

10.3 Proteomics of Drug Resistance in *Leishmania* spp.

10.3.1 Proteomics of Model Drugs for Understanding Resistance in *Leishmania*: Methotrexate and Arsenite Resistance

Model drugs such as MTX and arsenite have been widely used for the study of molecular mechanisms of drug resistance in *Leishmania* [131–136]. In fact, much of the current knowledge of resistance mechanisms and novel potential drug targets in this genus came from studies using resistant parasites obtained after in vitro selection with these drugs [137–140]. The resistance of *Leishmania* to these compounds includes events such as DNA amplification [131, 141, 142], decreased drug accumulation, and increased drug efflux [98, 143–145], among others. Methotrexate is an anticancer drug that inhibits DHFR which is responsible for the conversion of dihydrofolate to tetrahydrofolate. Derivatives of tetrahydrofolate are essential for the biosynthesis of purines and pyrimidines. Therefore, in the presence of MTX, DNA synthesis is prevented. Although this antifolate is toxic to *Leishmania*, it was found to be much more toxic to mammalian cells than for the parasites, thus preventing its use as a chemotherapeutic agent for leishmaniasis [146].

The first recorded proteomic study on drug resistance in *Leishmania* was conducted using *L. (L.) major* promastigotes that were induced in vitro to MTX resistance [56]. Comparison of MTX-resistant parasites to sensitive parasites using 2DE revealed the overexpression of the pteridine reductase PTR1, a known primary mediator of MTX resistance. It was demonstrated that the PTR1 overexpression was due to several gene amplification events in the resistant parasites [56]. As PTR1 is able to reduce dihydrofolate to tetrahydrofolate to a minimal extent, its overexpression could compensate for the inhibition of DHFR by MTX [56].

In a further study, Drummelsmith et al. [92] observed that *L. (L.) major* MTX-resistant promastigotes exhibited increased expression of proteins involved in stress response, such as chaperonins, heat-shock proteins, and enolase, as well as enzymes such as argininosuccinate synthetase (ARGG), which catalyzes the penultimate step in arginine biosynthesis. As in the case of PTR1, the overexpression of ARGG was also the result of gene amplification events, which was most likely a result of the structural proximity of the PTR1 and ARGG coding genes [92]. Other proteins with less easily predicted roles in drug resistance, such as methionine adenosyltransferase (MAT), were also identified in this study. This enzyme is overexpressed both in sensitive cells shocked with MTX and in mutants resistant to the drug, suggesting that it may play a significant role in the initial cellular responses to MTX in *L. (L.) major*. Unlike other proteins, the overexpression of MAT was not due to gene amplification events [92]. In addition, it was observed that increases in *S*-adenosylmethionine level, which is synthesized by MAT, correlated with the selection and emergence of MTX resistance in *L. major* [92].

Finally, a proteomic analysis of *L. (L.) donovani* induced to arsenite resistance was reported. However, as 2DE gels from wild-type and arsenite-resistant parasites were completely different, comparison of the differential protein expression between the two conditions was precluded [101].

10.3.2 Proteomics of Antimonial Resistance

Pentavalent antimonials in the forms of sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) are first-line drugs for the treatment of distinct forms of leishmaniasis [147]. Despite the fact that Sb^V have been used worldwide for almost 80 years, reports on the clinical resistance and descriptions of resistant parasites started to appear within the last 20 years [4–12]. This situation is particularly alarming in India where widespread failure to Sb^V treatment in previously untreated patients has been reported [see Chap. 4 in this volume; 8, 148, 149]. Recently, it was demonstrated that arsenic contamination of drinking water might have contributed to the development of antimonial resistance in *Leishmania* parasites circulating in the Bihar region [150]. The complexity of the resistance scenario is augmented and sometimes obscured by the variation in the clinical response to Sb^V due to species-specific sensitivity to these drugs [see Chap. 15 in this volume; 4, 151, 152].

The understanding of the mechanism of action of Sb^V drugs and resistance to them has come from laboratory parasites, in which resistance has been selected in vitro by the pressure of the drug. For antileishmanial activity, it is necessary that the Sb^V be reduced to the trivalent form Sb^{III}. Although debatable, reduction of the drug can apparently occur both in the macrophage and in the amastigote [153–156]. Reduction would be accomplished by either an enzymatic mechanism involving a thiol-dependent reductase [157] and/or an arsenate/antimonate reductase [158, 159] or by some nonenzymatic mechanism [160]. Regarding the internalization of the drug, it was demonstrated that AQP1, a transporter of trivalent metalloids [161], mediates the uptake of Sb^{III} in *Leishmania* [162]. The expression level of *aqp1* can correlate to the sensitivity to the drug [163, 164], and a major cluster of *L. (L.) donovani* isolates from the Indian subcontinent (ISC), which are resistant to Sb^V, presents a mutation in the *aqp1* gene that results in a nonfunctional protein and therefore reduced influx of Sb^{III} [3].

The activity of antimoniate seems to center around thiol redox metabolism [165], although early reports pointed to glycolytic and fatty acid β-oxidation pathways [166] or a programmed cell death (PCD) pathway involving DNA fragmentation non-mediated by caspase [167, 168]. On the other hand, increased levels of γ-glutamylcysteine synthetase and ornithine decarboxylase [137, 169], the enzymes involved in the synthesis of glutathione and polyamines, which are precursors of trypanothione, have been observed in parasites selected for resistance to Sb^{III} or arsenite [137, 145]. As a consequence, accumulation of trypanothione and glutathione contributes to the resistant phenotype [165, 170]. In addition, mechanisms for the increased efflux of Sb-thiol complexes [145] and/or decreased drug influx mediated by decreased/nonfunctional AQP1, besides drug sequestration involving a P-glycoprotein member of the ABC transporters (PgpA/MRPA), as well as other transporters (ABCC4, ABCC5, MRP1), might also influence the antimonial resistance [163, 171–177]. Despite some controversies, it seems that resistance to antimony is a multifactorial phenomenon involving various mechanisms such as decreased drug uptake, diminished metal reduction, increased glutathione and

trypanothione synthesis, and augmented drug efflux/sequestration [4, 139, 140, 178].

Proteomics of antimonial resistance have provided further evidence for some of the above mechanisms and have added new pieces to the resistance puzzle. Seminal studies comparing between Sb^{III}-sensitive and in vitro selected Sb^{III}-resistant axenic amastigotes of *L. (L.) infantum* using 2DE (pH range, 5–6) and ESI-MS/MS revealed some downregulated proteins in Sb^{III}-resistant parasites, including the LACK receptor, β-tubulin, proteasome pa26 subunit, pyruvate kinase, and the kinetoplastid membrane protein 11 (KMP-11) [94]. Although none of these proteins had previously been associated with antimony resistance, several of them have been further observed in other *Leishmania* species selected for antimony resistance or other drugs [79, 95, 96, 102, 124] (Table 10.2).

Regarding KMP-11, Western blot analysis confirmed that the levels of KMP-11 were lower in Sb^{III}-resistant parasites when compared to the parental wild-type parasites. However, overexpression of the protein did not alter the Sb^{III} susceptibility of parasites. In addition, Northern blot analysis revealed that the downregulation of KMP-11 was not due to a decrease in mRNA levels [94]. Decrease of KMP-11 abundance was also observed in the phosphoproteome analysis of *L. (V.) braziliensis* selected for antimonial resistance [79]. In *L. (L.) donovani*, it was shown that KMP11 could increase the lipid bilayer pressure [179, 180]. Thus, the marked decreased level of KMP-11, probably due to an increased turnover rate of this protein, could alter the interaction of transporters or putative efflux systems, enhancing activity for pumping Sb^{III} out of parasites [94]. On other hand, it has been proposed that the decrease of this protein could be part of a general mechanism of response to the stress caused by the drug pressure [79].

Argininosuccinate synthetase (ARGG) was the only protein identified as overexpressed in the *L. (L.) infantum* drug-resistant mutant. Increased abundance of ARGG was also observed in *L. braziliensis* resistant to antimonial [79] and in MTX-resistant *L. (L.) major* [92]. Both in Sb^{V}-resistant *L. (L.) donovani* and in MTX-resistant *L. (L.) major*, it was observed that the genomic region coding for this gene is amplified [20, 92], supporting the overexpression of ARGG [94]. However, the role that ARGG plays on resistance to Sb^{V} is unknown. The role in resistance of the other identified proteins remains to be established.

Regarding proteomic studies of field isolates, a proteomic analysis recently compared *L. (L.) donovani* parasites obtained from both a Sb^{V}-unresponsive and Sb^{V}-responsive patient to identify proteins involved in antimonial resistance [93]. First, the Sb^{V}-resistant and Sb^{V}-sensitive phenotypes of these isolates were corroborated by in vitro growth inhibition assays. Second, RT-PCR analysis showed that the expression levels of *aqp1*, *gsh1*, and *PgpA* (*mrpa*), which are genes associated with in vitro-induced resistance, were not differentially expressed between the sensitive and resistant clinical isolates. Third, it was shown that the parasites from the Sb^{V}-unresponsive patient were more resistant to Sb^{III}- and Sb^{V}-induced PCD. The PCD features analyzed were the mitochondrial membrane potential ($\Delta\psi m$), DNA fragmentation, and externalization of phosphatidylserine residues followed by membrane permeabilization [93]. Thus, based on previous evidence

suggesting that Sb^V kill *Leishmania* by a process involving several features of PCD [167, 168, 181], the soluble proteomes of these isolates naturally resistant and sensitive to Sb^V were analyzed by 2DE (pH range, 4–5) and MS/MS. Enolase, 14-3-3, ATP-dependent RNA helicase, dipeptidyl-peptidase III, 20 S proteasome α5 subunit, small kinetoplastid calpain-related protein SKCRP14.1, and many heat-shock proteins (HSP) were among the identified proteins. From this group, the 14-3-3 protein; the HSP83, whose abundances are increased in resistant parasites; and the SKCRP14.1, which is decreased in resistant parasites, were highlighted as having different roles in PCD. In fact, *Leishmania* HSP83 is an orthologue of the mammalian HSP90, which is involved in mitochondrial apoptotic pathways [182] (Table 10.2).

The genes coding for 14-3-3, HSP83, and SKCRP14.1 were cloned and the 14-3-3 and HSP83 constructs were transfected individually into the sensitive parasites, whereas the SKCRP14.1 construct was transfected into the resistant parasites [93]. While sensitive parasites transfected with the 14-3-3 construct did not show any increase in resistance to Sb^{III} or Sb^V when compared with sensitive control parasites, the sensitive parasites overexpressing HSP83 were more than twofold resistant to Sb^{III} compared with sensitive control parasites. In Sb^{III}-treated parasites, a more intense drug-mediated DNA fragmentation was observed in the control parasites when compared to HSP83-overexpressing parasites. It was also found that after treatment with Sb^{III}, the $\Delta\psi m$ was higher in HSP83-overexpressing parasites than in control cells [93]. Overexpression of SKCRP14.1 increased the sensitivity of resistant parasites to Sb^{III} and Sb^V, in addition to the sensitivity of transfectant parasites to Sb^{III}-induced DNA fragmentation. After treatment with Sb^{III}, no variations in the $\Delta\psi m$ were observed between SKCRP14.1 transfectants and control cells [93]. As will be described below, several of these effects were also observed in MIL-treated parasites [93], which highlights the phenomenon of cross-resistance but also reveals contrasting mechanisms involved in *Leishmania* drug resistance. Hence, although the network of molecules through which HSP83 and SKCRP14.1 interfere with drug-induced PCD pathways in *L. (L.) donovani* remains to be elucidated, this study demonstrated that these proteins modulate drug susceptibility in this parasite. It remains to be established if these phenomena are observed in other *L. (L.) donovani* clinical isolates and in other *Leishmania* species for which resistance has been reported. Interestingly, it recently was demonstrated that antimony-resistant *L. (L.) infantum* exhibited decreased abundance of SKCRP14.1, reinforcing the observation that antimonial resistance is associated with a decrease in cell death-related proteins [106]. In addition, increased abundance of HSP83, as well other HSPs and chaperones, has been further observed in different *Leishmania* species selected for antimonial resistance [79, 95, 103–106, 124], including *L. (L.) infantum*, *L. (V.) panamensis*, and *L. (V.) braziliensis* (Table 10.2). Remarkably, parasites selected for MIL, amphotericin-B, or alpha-difluoromethylornithine resistance also present a significant increase in various heat-shock proteins [50, 96, 107, 108], suggesting that these proteins are part of a general response to the stress caused by the drug pressure. The increased protection against drug-related stress and drug-related programmed cell death may contribute to the resistance phenotype as a whole

Table 10.2 *Leishmania* spp. proteins associated with drug resistance (proteins highlighted by the authors). Sb^{V}, pentavalent antimonial; Sb^{III}, trivalent antimonial

Protein	Species	Natural (N) or selected (S) resistance	Life stage	Increased (I) or decreased (D) abundance	Mechanism	References
Antimonials Sb^{V}-Sb^{III}						
MRPA (ABCC3) ABC-thiol transporter	*L. (L.) infantum*	S	Promastigotes	I	Increased drug sequestration	[106]
S-adenosylmethionine synthetase (SAMS)	*L. (V.) panamensis*	S and N	Promastigotes	I	Increased levels of intracellular T $[SH]_2$—Increased protection against ROS	[95, 104]
	L. (L.) infantum	S				
S-adenosylhomocysteine hydrolase (SAHH)	*L. (L.) donovani*	S	Promastigotes—Amastigotes	I	Increased levels of intracellular T $[SH]_2$—Increased protection against ROS	[104, 124]
	L. (V.) panamensis	S and N				
Rab7 homolog	*L. (V.) panamensis*	S and N	Promastigotes—Amastigotes	I	Transport of vesicles containing sequestered metal–thiol conjugates?	[95, 104]
	L. (L.) infantum	S				
Pteridine reductase (PTR1)	*L. (V.) braziliensis*	S	Promastigotes	I	Increased antioxidant response	[79, 95]
Tryparedoxin peroxidase	*L. (V.) braziliensis*	S	Promastigotes	I	Increased antioxidant response	[79, 95, 106]
Peroxiredoxin	*L. (L.) infantum*	S				

(continued)

Table 10.2 (continued)

Protein	Species	Natural (N) or selected (S) resistance	Life stage	Increased (I) or decreased (D) abundance	Mechanism	References
Heat shock proteins/chaperones						
HSP83, HSP70, HSP60 Protein disulfide isomerase	*L. (L.) donovani*	N and S	Promastigotes—Amastigotes	I	Increased general response to stress/protection against drug-related programmed cell death	[79, 93, 95, 103–106, 124]
	L. (V.) panamensis	S				
	L. (V.) braziliensis	S				
	L. (L.) infantum	S				
Small kinetoplastid calpain-related protein (SKCRP14.1)	*L. (L.) donovani*	N	Promastigotes—Amastigotes	D	Decreased cell death-related proteins	[93, 106]
	L. (L.) infantum	S				
Proliferative cell nuclear antigen (PCNA)	*L. (L.) donovani*	N	Promastigotes	I	Increased DNA repair?	[79, 95, 102]
	L. (V.) braziliensis	S				
Glycolytic enzymes						
Triose phosphate isomerase Fructose-1,6-bisphosphate aldolase Glyceraldehyde 3-phosphate dehydrogenase Hexokinase Glycosomal malate dehydrogenase	*L. (L.) donovani*	N and S	Promastigotes	I	Increased glycolysis—Increased parasite fitness? General stress response mechanism	[93, 95, 102, 103, 106, 124]
	L. (L.) infantum	S				
	L. (V.) panamensis	S				
	L. (V.) braziliensis	S				

Aldehyde dehydrogenase Enolase						
TCA-related enzymes						
Pyruvate dehydrogenase Dihydrolipoamide dehydrogenase Isocitrate dehydrogenase	*L. (L.) infantum*	S	Promastigotes	I or D	General stress response mechanism Increased parasite fitness? (only for I)	[95, 99, 106]
Transcription, translation-related proteins						
Translation initiation factor 1-alpha Elongation factor 1-alpha Elongation factor 2 Elongation factor 5-alpha 60s ribosomal L23a Various ribosomal proteins	*L. (L.) infantum*	S	Promastigotes	I	General stress response mechanism Increased parasite fitness?	[79, 95, 102–104, 106, 124]
	L. (L.) donovani	N and S				
	L. (V.) panamensis	S				
	L. (V.) braziliensis	S				
Oligopeptidase B (OPB)	*L. (V.) panamensis*	S	Promastigotes	I	Increased virulence—Increased parasite fitness?	[104]
Cysteine leucine rich protein	*L. (L.) donovani*	N	Promastigotes	I	Increased virulence—Increased parasite fitness?	[102]
Succinyl-CoA:3-ketoacid-coenzyme A transferase	*L. (L.) infantum*	S	Promastigotes	I	Compensatory production of acetyl-CoA?	[99]
Histone 4	*L. (L.) infantum*	S	Promastigotes	I	General stress response mechanism Increased parasite fitness?	[106]
Cyclophilin-A	*L. (V.) braziliensis*	S	Promastigotes	D	???	[95]
	L. (L.) infantum	S				

(continued)

Table 10.2 (continued)

Protein	Species	Natural (N) or selected (S) resistance	Life stage	Increased (I) or decreased (D) abundance	Mechanism	References
Activated protein kinase c receptor (LACK)	*L. (L.) infantum*	S	Promastigotes	I	Increased signal transduction?	[94]
GP63, Leishmanolysin	*L. (L.) infantum*	S	Promastigotes	D	???	[106]
Amastin, surface protein	*L. (L.) infantum*	S	Promastigotes	D	???	[106]
Argininosuccinate synthetase (ARGG)	*L. (L.) infantum*	S	Amastigotes Promastigotes	I	???	[79, 94]
	L. (V.) braziliensis	S				
Kinetoplastid membrane protein (KMP-11)	*L. (L.) infantum*	S	Amastigotes	D	General stress response mechanism?	[79, 94]
	L. (V.) braziliensis	S	Promastigotes			
Ca^{2+} homeostasis-related proteins						
Hypothetical protein containing an EF-hand calcium binding domain	*L. (L.) infantum*	S	Promastigotes	I	Ca^{2+} homeostasis—Increased protection against ROS	[99]
Calcium binding protein				D		
Tetratricopeptide repeat (TPR) domain protein	*L. (V.) braziliensis*	S	Promastigotes	I	General stress response mechanism	[79]
Calcireticulin	*L. (V.) braziliensis*	S	Promastigotes	I	General stress response mechanism	[79]

Beta-tubulin	*L. (L.) donovani*	N and S	Promastigotes	I	Signal transduction?—General stress response mechanism?	[79, 94, 95, 102, 124]
	L. braziliensis	S				
	L. (L.) infantum	S				
Nucleoside diphosphate kinase b (NDKb)	*L. braziliensis*	S	Promastigotes	I	???	[79]
Succinyl-CoA ligase	*L. braziliensis*	S	Promastigotes	D	???	[79, 95]
RNA helicase	*L. (L.) donovani*	N	Amastigotes-Promastigotes	D	???	[79, 95]
	L. braziliensis	S				
	L. (L.) infantum	S				
Miltefosine (HePC)						
Transcription, translation-related proteins						
Eukaryotic initiation factor 4A (eIF4A)	*L. (L.) donovani*	S	Promastigotes	I	General stress response mechanism	[96, 97]
Elongation factor 1-beta	*L. (L.) infantum*	N			Increased parasite fitness?	
Heat shock proteins						
HSP83, HSP60, stress-induced protein sti1	*L. (L.) infantum*	N	Promastigotes	I	Increased general response to stress/protection against drug-related programmed cell death	[96, 108*]
HSPA9B	*L. (L.) donovani*	S		D		
Peroxiredoxin	*L. (L.) infantum*	N	Promastigotes	I	Increased antioxidant response	[96]
Proliferative cell nuclear antigen (PCNA)	*L. (L.) infantum*	N	Promastigotes	I	Increased DNA repair?	[96]

(continued)

Table 10.2 (continued)

Protein	Species	Natural (N) or selected (S) resistance	Life stage	Increased (I) or decreased (D) abundance	Mechanism	References
Mitochondrial ATPase β-subunit	*L. (L.) infantum*	N	Promastigotes	I	Increased DNA repair?	[96]
TCA-related enzymes						
Pyruvate dehydrogenase Dihydrolipoamide dehydrogenase Isocitrate dehydrogenase	*L. (L.) infantum*	S	Promastigotes	D	General stress response mechanism Increased parasite fitness?	[99]
Ca^{2+} homeostasis-related proteins						
Hypothetical protein containing an EF-hand calcium binding domain	*L. (L.) infantum*	S	Promastigotes	I	Ca^{2+} homeostasis—Increased protection against ROS	[99]
Calcium binding protein				D		
Succinyl-CoA:3-ketoacid-coenzyme A transferase	*L. (L.) infantum*	S	Promastigotes	I	Compensatory production of acetyl-CoA?	[99]
Activated protein kinase c receptor (LACK)	*L. (L.) infantum*	N	Promastigotes	I	Increased signal transduction?	[96]
S-adenosylmethionine synthetase (SAMS)	*L. (L.) infantum*	N	Promastigotes	D	Increased levels of intracellular T $[SH]_2$—Increased protection against ROS	[96]
Amphotericin-B (AmB)						
S-adenosylmethionine synthetase (SAMS)	*L. (L.) infantum*	S	Promastigotes	I	Increased levels of intracellular T $[SH]_2$—Increased protection against ROS	[107]

Glycolytic enzymes						
Fructose-1,6-bisphosphate Aldolase Glyceraldehyde 3-phosphate dehydrogenase Hexokinase Enolase	*L. (L.) infantum*	S	Promastigotes	I	General stress response mechanism Increased parasite fitness?	[107]
TCA-related enzymes						
Aconitase Dihydrolipoamide dehydrogenase Malate dehydrogenase Succinate dehydrogenase flavoprotein	*L. (L.) infantum*	S	Promastigotes	I	General stress response mechanism Increased parasite fitness?	[107]
Trypanothione reductase	*L. (L.) infantum*	S	Promastigotes	I	Increased antioxidant response	[107]
Tryparedoxin peroxidases	*L. (L.) infantum*	S	Promastigotes	I	Increased antioxidant response	[107]
Iron superoxide dismutase	*L. (L.) infantum*	S	Promastigotes	I	Increased antioxidant response	[107]
Heat shock proteins/chaperones						
HSP83, HSP70, HSP60 T-complex protein 1 (several subunits)	*L. (L.) infantum*	S	Promastigotes	I	Increased general response to stress/protection against drug-related programmed cell death	[107]
Various peptidases	*L. (L.) infantum*	S	Promastigotes	I	Increased parasite fitness—virulence?	[107]
Transcription, translation-related proteins						
Various protein synthesis-related proteins Various ribosomal proteins	*L. (L.) infantum*	S	Promastigotes	I	Increased parasite fitness?	[107]

(continued)

Table 10.2 (continued)

Protein	Species	Natural (N) or selected (S) resistance	Life stage	Increased (I) or decreased (D) abundance	Mechanism	References
H1A-2 P-type H^+-ATPase	*L. (L.) infantum*	S	Promastigotes	D	Protection against drug-related programmed cell death	[107]
Methotrexate (MTX)						
Pteridine reductase (PTR1)	*L. (L.) major*	S	Promastigotes	I	Increased antioxidant response	[56, 92]
Beta-tubulin	*L. (L.) major*	S	Promastigotes	I	Signal transduction?—General stress response mechanism	[56]
Methionine adenosyltransferase (MAT)	*L. (L.) major*	S	Promastigotes	I	Increased levels of intracellular T $[SH]_2$—Increased protection against ROS	[92]
S-adenosylmethionine synthetase (SAMS)	*L. (L.) major*	S	Promastigotes	I	Increased levels of intracellular T $[SH]_2$—Increased protection against ROS	[92]
Argininosuccinate synthetase (ARGG)	*L. (L.) major*	S	Promastigotes	I	???	[92]
α-difluoromethylornithine (DFMO)						
S-adenosylmethionine synthetase (SAMS)	*L. (L.) donovani*	S	Promastigotes	I	Increased levels of intracellular T $[SH]_2$—Increased protection against ROS	[50]
Cystathione β-lyase like protein	*L. (L.) donovani*	S	Promastigotes	I	Increased levels of intracellular T $[SH]_2$—Increased protection against ROS	[50]
Dihydrofolate reductase (DHFR-TS)	*L. (L.) donovani*	S	Promastigotes	I	Increased levels of intracellular T $[SH]_2$—Increased protection against ROS	[50]

Nucleoside diphosphate kinase b (NDKb)	*L. (L.) donovani*	S	Promastigotes	I	???	[50]
Heat shock proteins/chaperones						
HSP83, HSP70, HSP60	*L. (L.) donovani*	S	Promastigotes	I	???	[50]
Transcription, translation-related proteins						
Various protein synthesis-related proteins Various ribosomal proteins	*L. (L.) donovani*	S	Promastigotes	I	Increased parasite fitness?	[50]
TCA-related enzymes and fatty acid metabolism						
Isocitrate dehydrogenase 3-ketoacyl-CoA thiolase Oxoglutarate ehydrogenase	*L. (L.) donovani*	S	Promastigotes	I D	General stress response mechanism Increased parasite fitness?	[50]
Tryparedoxin	*L. (L.) donovani*	S	Promastigotes	I	Increased antioxidant response	[50]
Histone 2 Histone 3 Histone 4	*L. (L.) donovani*	S	Promastigotes	I	General stress response mechanism Increased parasite fitness?	[50]
Glycolytic enzymes						
Fructose-1,6-bisphosphate Aldolase Glucose-6-phosphate 1-dehydrogenase, Phosphoenolcarboxykinase	*L. (L.) donovani*	S	Promastigotes	D	General stress response mechanism Increased parasite fitness?	[50]
Various peptidases	*L. (L.) donovani*	S	Promastigotes	I	Increased parasite fitness?	[50]

(continued)

Table 10.2 (continued)

Protein	Species	Natural (N) or selected (S) resistance	Life stage	Increased (I) or decreased (D) abundance	Mechanism	References
Cyclophilins	*L. (L.) donovani*	S	Promastigotes	I	General stress response mechanism Increased parasite fitness?	[50]
Bicyclic nitro-drugs						
Hypothetical NADH:FMN dependent oxidoreductase	*L. (L.) donovani*	S	Promastigotes	D	Reduced drug bio-activation	[53]

?: suggested mechanism with different level of evidences
???: unknown mechanism

[79, 104], resulting in parasites able/adapted to cope with the oxidative stress caused by drug, probably by maintaining functional proteostasis mediated by HSPs.

Cell membrane proteins are very interesting for understanding drug transport in resistant parasites. In this context, Kumar et al. reported a set of overexpressed proteins in membrane- and cytosolic-enriched fractions of an *L. (L.) donovani* clinical isolate resistant to Sb^V, when compared to a sensitive isolate [102]. The six proteins identified in the membrane-enriched fraction were two ABC transporters, a fragment of HSP83, a cysteine-leucine-rich protein (CLrP), a GPI transamidase, and a 60S ribosomal protein (L23a). Remarkably, these authors further demonstrated that CLrP is a glycosylated protein with dual localization, in the membrane and nucleolus, whereas the 60s ribosomal L23a protein (60sRL23a) is localized in the cytosol [183, 184]. It was also shown that antimonial-resistant clinical isolates of *L. (L.) donovani* present higher mRNA and protein levels of CLrP and 60sRL23a as compared to antimonial-sensitive parasites [183, 184]. Overexpression of CLrP or 60sRL23a in a sensitive isolate of *L. (L.) donovani* significantly decreased its responsiveness to Sb^V and Sb^{III}, in the case of CLrP, and also to MIL and paromomycin, in the case of 60sRL23a. Such reduction on drug sensitivity was followed by increased parasite infectivity to murine macrophages or increased proliferation rate, for CLrP- or 60sRL23a-overexpressing mutants, respectively [183, 184]. Such studies reveal that resistant parasites exhibit a higher fitness than sensitive parasites, showing increased infectivity capability to host cells and increased proliferation rate.

Metabolic isotopic labeling of *L. (L.) infantum* resistant to Sb^{III} followed by comparative proteomic analysis of membrane and cytosolic fractions allowed the observation of increased levels of the ABC transporter MRPA (ABCC3) for the first time in a proteomic study [106]. The increased abundance of MRPA, a well-known protein involved in antimonial sequestration, was accompanied by alterations in the abundance of other transporters such as folate/biopterin transporters that presented diminished abundance in resistant parasites. Interestingly, folate/biopterin transporters have been previously pointed out as potential chemotherapeutic targets in *Leishmania* [185, 186].

It is pertinent to mention that in the proteomic studies that aimed to analyze *Leishmania* membrane proteins associated with drug resistance, the identification of a higher number of membrane proteins, more representative of this fraction, has been hampered possibly by the inherent limitation of 2DE for resolving hydrophobic proteins. Furthermore, the methods hitherto used for sample preparation do not favor the representativeness of such proteins. Such limitations can be overcome using better solubilizing agents and gel-free shotgun proteomic approaches, as demonstrated for other cells and tissues [187–189].

Seminal proteomic studies of antimonial resistance in *L. (L.) donovani* isolates identified β-tubulin, enolase, fructose-1,6-bisphosphate aldolase, the proteasome subunit α5, a carboxypeptidase, a fragment of HSP70, and the proliferative cell nuclear antigen (PCNA) [102]. Interestingly, further expression analyses, by Western blot and qPCR, confirmed that promastigote and amastigotes of resistant parasites exhibit ≥threefold and ~fivefold increased levels of PCNA, respectively,

compared to the antimonial-sensitive parasites [190]. Overexpression of PCNA in antimonial-sensitive isolate resulted in significant increase of Sb^V IC_{50}, and parasites overexpressing PCNA exhibited less DNA fragmentation compared to wild-type sensitive parasites upon treatment. In addition, parasites overexpressing PCNA modulated negatively nitric oxide (NO) production in infected macrophages [190].

Because many of the soluble proteins detected in drug resistance studies are among the abundant proteins commonly identified in proteomic studies of *Leishmania* and other trypanosomatids [59, 68, 71, 191], it is difficult to elucidate a clear role in resistance for them. However, as mentioned above, further proteomic analyses of *Leishmania* resistance mechanisms either to Sb^V or to other drugs have corroborated that HSPs, glycolytic enzymes, TCA-related enzymes, transcription-/translation-related proteins, peptidases, as well as DNA repair-related proteins, among other noncanonical resistance proteins, exhibit altered abundance in resistant parasites (Table 10.2) [50, 79, 95, 96, 103–108, 124]. Such findings reinforce the idea that resistant parasites exhibit a better general fitness than sensitive parasites, mediated by the (1) remodeling of their glycolytic metabolism, (2) increasing of virulence factor abundance, (3) and more efficient protein homeostasis and DNA repair, which together result in an increased proliferation and infectivity capability to host cells. Some of these phenotypic traits have been corroborated in a mutant *Leishmania* line that is deficient in glucose transport [100]. A detailed description of the association between fitness and drug resistance in *Leishmania* can be found in Chap. 15, this same volume. Proteomic studies of antimonial resistance have also shown that enzymes that are precursors of trypanothione, such as *S*-adenosylmethionine synthetase (SAMS) and *S*-adenosylhomocysteine hydrolase (SAHH), present increased abundance in *L. (V.) panamensis*, *L. (L.) infantum*, and *L. (L.) donovani* resistant parasites [95, 104, 124]. In addition, proteins involved in redox homeostasis, such as tryparedoxin, peroxiredoxin, and pteridine reductase, are also more abundant in resistant parasites [79, 95, 106]. Together, these findings corroborate the hypothesis that antimonial resistance is closely associated with nitrosative and oxidative stress resistance and remodels the parasite thiol redox metabolism.

10.3.3 Proteomics of Miltefosine Resistance

MIL [hexadecylphosphocholine (HePC]), an alkyl phospholipid compound, is the only oral drug currently available for the treatment of leishmaniasis. Originally intended for breast cancer treatment, MIL proved to be effective against *Leishmania* both in vitro and in animal models [192, 193]. This drug was registered and approved for visceral leishmaniasis (VL) treatment in India in 2002, followed by Germany in 2004. In Colombia in 2005, MIL was approved for the treatment of cutaneous leishmaniasis (CL), where it reached cure rates of over 91% [194, 195]. In 2005, the governments of India, Nepal, and Bangladesh adopted MIL as the first-line treatment for VL elimination [196, 197]. The oral administration of MIL avoids the need of patient hospitalization in VL cases and reduces the inconvenience of

injectable drugs, augmenting treatment adherence [195, 198]. Despite the recent approval of MIL for disease treatment, clinical failures during treatment of VL and CL caused by different *Leishmania* species have already been reported [199–201]. MIL is registered for the oral treatment of canine leishmaniasis in several European countries since 2007 (Milteforan®) and was authorized recently (2016) for the treatment of dogs with VL in Brazil, despite studies showing that the improvement in the clinical symptoms was not followed by parasitological clearance [202]. In fact, that study did not recommend the use of MIL for dog treatment, especially in endemic areas of Brazil where dogs have a crucial role in the maintenance and transmission of the parasite [202]. In addition, failure treatment has been reported in naturally infected dogs treated with MIL [203].

Although MIL exhibits in vitro activity against various *Leishmania* species [204], the mechanism of action of this compound is not well understood. However, based on evidence obtained in tumor cell lines, it is known that MIL acts by triggering apoptotic pathways [205]. Evidences of PCD induced by MIL have also been reported for *L. (L.) donovani* promastigotes [206]. This drug appears to affect the integrity of cellular membranes by interfering with lipid metabolism, resulting in the decrease of phosphatidylcholine synthesis [207, 208]. In addition, intracellular drug accumulation seems to be required for the drug's activity. Accumulation involves, among other steps, the translocation of the drug across the cellular membrane, which is accomplished with a recently identified complex of proteins including a P-type ATPase termed *L. (L.) donovani* MIL transporter (LdMT) and its β-subunit, LdRos3 [209]. Interestingly, the expression levels of these proteins are diminished in *L. (V.) braziliensis*, which would help to explain the low sensitivity of this species to the drug [210]. In addition, a common feature of MIL-resistant parasites consists of a decrease in drug accumulation mainly due to either the decreased uptake or increased efflux of the drug [211].

As described above, in *L. (L.) donovani* field isolates, HSP83 and SKCRP14.1 were implicated in the modulation of parasite sensitivity to Sb^V through a mechanism involving features of PCD [93]. In the same study, it was observed that Sb^V-resistant parasites were also cross-resistant to both MIL and AMB when compared with the Sb^V-sensitive parasites. It was also shown that the parasites from the Sb^V-unresponsive patient were more resistant to MIL-induced PCD. Besides being resistant to antimonial, the HSP83-overexpressing parasites were also resistant to MIL and were less sensitive to drug-mediated DNA fragmentation when compared to control parasites. In addition, 10 μM MIL first induced a more rapid hyperpolarization of the mitochondria in HSP83 transfectants when compared to the control cells, followed by a depolarization that took place more slowly in HSP83-overexpressing parasites than in control ones [93]. However, the effect of MIL treatment on SKCRP14.1-overexpressing parasites was the opposite of that observed with antimonial treatment. Resistant parasites transfected with SKCRP14.1 became more resistant to MIL compared with the transfectant control. In addition, SKCRP14.1 overexpression was significantly protected against MIL-induced mitochondrial depolarization and led to resistance against MIL-mediated DNA fragmentation when compared with the control [93]. These results reveal the contrasting

roles of the proteins in the resistance mechanisms of *Leishmania* and highlight the importance of setting the individual action scenarios for each drug.

Recently, a study was conducted using a MIL-resistant *L. (L.) donovani* isolate, which was selected in vitro by sequential exposure to the drug [97]. In this study, the total cell extracts of sensitive and resistant promastigotes were analyzed by 2DE (pH range, 4–7), and two differentially expressed spots were identified by LC/MS/MS. The identified spots corresponded to the probable eukaryotic initiation factor 4A (eIF4A), a protein belonging to the DEAD-box subfamily of ATP-dependent helicases. This protein participates in the regulation of translation initiation, and it has been reported that its overexpression confers lithium resistance in *Saccharomyces cerevisiae*, probably by restoring protein synthesis [212].

In a recent study, Carnielli et al. used 2D-DIGE/MS to study the differences in protein abundances between MIL-sensitive and MIL-resistant *L. (L.) infantum* isolates from VL patients with different MIL treatment outcomes [96]. Among 46 spots exhibiting different intensity, 22 proteins were identified. Proteins with increased abundance in MIL-resistant isolates were associated with (1) redox homeostasis, such as peroxiredoxin and *S*-adenosylmethionine synthetase (SAMS); (2) stress response, including several HSPs; (3) DNA repair, such as PCNA and mitochondrial ATPase β-subunit; and (4) glycolytic and TCA-related enzymes, among others. A very similar group of proteins was observed in proteomic studies of *L. (L.) infantum* in vitro selected for amphotericin-B resistance [107] and in *L. (L.) donovani* selected for resistance against DL-α-difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase, the first enzyme of the polyamine biosynthetic pathway [50] (Table 10.2). These results corroborate the multifactorial character of drug resistance phenomenon in *Leishmania* and also show that irrespective of the chemotherapy used to select the resistant lines, resistant parasites respond in similar ways to the drug pressure (either in vivo or in vitro) exhibiting increased resistance to oxidative and nitrosative stress, remodeling their glycolytic metabolism and increasing their virulence.

10.4 Proteomic Challenges in the Study of Drug Resistance

Proteomic studies of drug resistance in *Leishmania* have increased over the last decade. The reports reviewed here illustrate the value of proteomic approaches for the identification of proteins and mechanisms involved in resistance phenomenon. Those studies show that proteomic screens are useful in defining new roles for already well-characterized proteins in addition to assigning roles for proteins of unknown function. A summary of the proteins identified from proteomic studies using either resistant parasites selected in vitro or parasites from clinical isolates that are considered naturally drug resistant (proteins highlighted by the authors) is presented in Table 10.2. As can be seen in this table, many proteins identified in these studies have been implicated in *Leishmania* drug resistance using other approaches, but many other proteins are new or even unexpected in the scenario of drug resistance.

Several proteins classically described as being involved in various resistance mechanisms are membrane proteins, and this fraction has not been widely explored in drug resistance studies in *Leishmania*. This fact points to the first challenge: the deep analysis of distinct subcellular fractions of the parasite. Besides the contribution of protein annotation, subcellular proteomic analysis offers the possibility of inferring protein function and elucidating biochemical pathways in drug resistance, which can be exploited for purposes of drug development. In addition, it should be taken into account that the approach used for the proteomic studies revisited here has been 2DE, with the already mentioned limitations, applied to whole cell extracts and analyzing only some pH ranges. As a result, a large part of the *Leishmania* "resistance" proteome remains to be analyzed, which points to the second challenge: the need for a comprehensive proteomic study using better solubilizing detergents for sample preparation and gel-free methods [213] that ensures greater coverage of the proteome. Such an approach will require more powerful and specific bioinformatics tools to cope with the analysis of the enormous quantity of data that would be produced. In fact, data analysis represents a considerable bottleneck in the proteomic studies of parasites, mainly because ~50% of the coding genes do not have a functional annotation, which is why it represents the third challenge.

In very nice example of the exploitation of pharmacoproteomics for the study of drug targets and mechanisms of action, Wyllie et al. [53] studied by proteomic and genomic approaches the effects of bicyclic nitro-compounds on *L. (L.) donovani*. Nitro drugs are being used as part of a combination therapy for human African trypanosomiasis (HAT) [214], and bicyclic nitro-compounds are potential candidates for the treatment of VL (www.dndi.org). Comparing susceptible and drug-resistant parasites, authors identified the hypothetical NADH/FMN-dependent oxidoreductase as the activating nitroreductase (NTR2) and demonstrated that its overexpression rendered parasite hypersensitive to bicyclic nitro-compounds. In addition, it was demonstrated that knockout of NTR2 rendered parasites completely resistant to the compounds [53]. This study shows the potential of pharmacoproteomics to study drug mechanisms and resistance in trypanosomatids.

A common trait of proteomic studies in *Leishmania*, as well as in other organisms, is the recurrent identification of a group of proteins that correspond to the most abundant ones [215]. This precludes the identification of the less abundant proteins and obscures the studied phenomenon. Thus, a dedicated analysis of *Leishmania* most abundant proteins with the subsequent construction of an interactive database containing raw mass data and mass spectra data of these proteins would allow a better exploitation of the proteomic studies, saving time and optimizing resources [216]. In addition, the wide use of transfection models and the potential exploitation of a putative RNA interference (RNAi) pathway, at least in *L.* (*Viannia*) parasites [2, 217], would reinforce and complement the proteomic analysis of changes associated with drug resistance. Finally, as far as we know, proteomic studies of the resistance to other drugs used for leishmaniasis treatment, such as pentamidine, paromomycin, and azoles, have not been reported.

10.5 Concluding Remarks

As the cellular proteome is a dynamic scenario, it should be considered that inter- and intraspecific *Leishmania* genetic variation, in addition to host immune responses and host genetic background, might influence the resistant or sensitive phenotype of the parasites [218]. Thus, despite being rich and detailed, proteomic profiles represent specific patterns that need to be contextualized into a "biological system" level where the complexity must be governed by well-defined mechanisms. The continued advances in proteomic technology development, together with genome data and bioinformatics analysis, could reveal effective therapeutic strategies for species-specific treatments in the future, individualizing the epidemiological settings and valorizing the patients [219]. A large endeavor joining expertise, technologies, facilities, and knowledge would be desirable for obtaining and (re-) interpreting proteomic data of drug resistance in *Leishmania*.

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