Biomedicine & Pharmacotherapy 95 (2017) 577–582

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Review

Proteomic approaches for drug discovery against tegumentary leishmaniasis

Fernanda Negrão[a,b,*], Marcos Nogueira Eberlin[a], Selma Giorgio[b]

[a] Thomson Mass Spectrometry Laboratory, Institute of Chemistry, University of Campinas – UNICAMP, 13083-970 Campinas, SP, Brazil
[b] Department of Animal Biology, Institute of Biology, University of Campinas – UNICAMP, 13083-970 Campinas, SP, Brazil

ARTICLE INFO

Keywords:
Tegumentary leishmaniasis
Proteomics
Leishmania
Drug development

ABSTRACT

Tegumentary leishmaniases (TL) comprise various clinical forms, in which current therapeutic treatments lack in safety and efficacy. Recently the parasite is developing resistance mechanisms against anti leishmanial drugs startling the scientific community to recruit efforts to search for novel therapeutics. Proteomics hold promises for the treatment of leishmaniasis and investigation of parasite–host interaction since these set of methodological tools have provided a wealth of protein expression data on several *Leishmania* species. Firstly this review puts together the current treatment and challenges to fight tegumentary leishmaniasis. In addition, the 2 dimensional gel electrophoresis and mass spectrometry techniques in protein identification and characterization are described and discussed in the context of proteomics regarding *Leishmania* studies. In this review, we selected literature content on TL causative agents. Important proteomic findings related to differentiation proteome (promastigote and amastigote forms), *Leishmania*-macrophage interaction proteome and secreted and soluble proteins including molecules involved in parasite resistance and potential drug targets are examined and discussed. We also highlight open questions regarding drug research that can be addressed with proteomics approaches.

1. Introduction

The leishmaniasis are a group of diseases caused by protozoan parasites from > 20 different *Leishmania* species that are transmitted to humans by the bite of infected female sandflies. Globally, there are an estimated 1.5–2 million new cases and 70 000 deaths each year, and 350 million people are at risk of infection and disease [1]. The two main forms of the disease are tegumentary and visceral. Tegumentary leishmaniases (TL) comprise various clinical forms that depend on the *Leishmania* species as well as the host response [2].

TL are classified as localized cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis, disseminated leishmaniasis, leishmaniasis recidiva cutis, and mucosal leishmaniasis (ML) [2]. The CL is the most prevalent form and it is caused by all of the dermotropic *Leishmania* species being endemic in many countries [1]. It is specially found in Asia, the Middle East, Southern Europe and South America [1] but it is becoming increasingly reported in urban and peri-urban areas of the Old and New World [2]. TL is caused by *L. tropica* and *L. major* in the Old World and by *L. braziliensis*, *L. guyanensis*, *L. panamensis*, *L. peruviana*, *L. mexicana*, *L. amazonensis* and *L. venezuelensis* in the New World [3]. CL usually produces ulcers on the exposed parts of the body, such as the face, arms and legs [1]. Many lesions can cause serious disability and when ulcers heal, they leave permanent scars, which are the cause of serious social prejudice. The ML, common in Brazil, produces lesions that can lead to partial or total destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues [2]. Secondary infection plays a prominent role in the size and persistence of ulcers. This disabling form of leishmaniasis can lead to the sufferer being rejected by the community [1–3]. *L. braziliensis* is the primary species involved in New World mucosal leishmaniasis, although *L. panamensis*, *L. guyanensis*, and *L. amazonensis*. In the Old World, *L. major* and *L. infantum* also cause ML [2,32]. The *Leishmania* life cycle begins when parasites in their promastigote form are inoculated by a sandfly bite into the skin of a mammalian host. Macrophages phagocyte parasites, which turn into the amastigote form. Many will survive within the macrophages because of a variety of sophisticated defense mechanisms. *Leishmania* then multiply and spread to other macrophages [4,5].

The control of vectors and reservoirs in vector-borne diseases is difficult due to challenges of interventional programs, particularly in developing countries, where the prevalence is high [6]. The control of *Leishmania* also relies on the early diagnosis, vaccines and efficient treatment.

Although there have been early reviews regarding *Leishmania* biology and studies of proteins, recent proteomic approaches against TL deserve particular attention since they have led to a much deeper

* Corresponding author at: Thomson Mass Spectrometry Laboratory, Institute of Chemistry, University of Campinas – UNICAMP, 13083-970 Campinas, SP, Brazil.
E-mail address: negraosf@gmail.com (F. Negrão).

http://dx.doi.org/10.1016/j.biopha.2017.08.089
Received 19 July 2017; Received in revised form 14 August 2017; Accepted 23 August 2017

knowledge of its biochemistry [7–13]. The purpose of this review is to encompass important information regarding pre-proteomic and proteomic studies about TL causative agents. The highlight will be on how proteomic studies on *Leishmania* have contributed in the search of potent drug targets aimed to develop more effective and less toxic therapeutics against the disease.

2. Current treatment and challenges

The first-choice treatment for TL in most parts of the world is the pentavalent antimonials which were developed in 1945; amphotericin B and pentamidine are the second-line antileishmanial drugs, although they require long courses of parenteral administration [14]. The choice of treatment also depends on the causative *Leishmania* species [15]. Although spontaneous cure is the rule for CL the rate of recovery varies depending on the species of *Leishmania*, and may require months or years to complete healing. Most of the commonly used drugs are toxic and do not eliminate the parasite from infected individuals [14]. The major side effects of the first line treatment (antimonials) are arthralgy and myalgy but severe side effects related to cardiotoxocity or renal failure can occur mainly in older patients [16].

Current drugs against leishmaniasis lacks in safety and efficacy, which disrupts adhesion to the treatment. Recently, the parasite is developing resistance mechanisms against antileishmanial drugs alarming scientific community to recruit efforts to search for novel therapeutics. For example, free availability of anti-leishmanial drug in India increased the chances of misuse; thereby increasing the emergence of drug resistance [17].

As stated, unfortunately, drug therapy for TL has failed to significantly change since the beginning of the twentieth century, when it started. In addition, knowledge regarding the differences in the drug responses of the *Leishmania* species that are prevalent in different geographic areas and their clinical manifestation is slowly increasing [2]. Sensitivity of antimonials toward different *Leishmania* species varies differently [17]. It is observed that *L. brasilensis* is more sensitive to the treatment in comparison *L. Mexicana* [17]. There is also increasing awareness that drug treatment can be complicated by variation in the sensitivity of *Leishmania* species to drugs, variation in pharmacokinetics, and variation in drug-host immune response interaction [17–20].

Pentavalent antimonials (SbV) are the first choice treatment. The mechanism of action of antimonials is still poorly understood, but they seem to have a dual mode of action. One mode would be the perturbation of the redox-balance of the parasites and the other mode would be imposing extra oxidative and nitrosative stress upon the parasite through interaction with the host cell [21]. An interesting study comparing *L. panemensis* resistant to meglumine antimoniate and the wild type, tried to define the role of parasite sensitivity to SbV in treatment failure and to examine the mode of action of SbV [22]. The study showed the effects of SbV on the stabilization of cleaving DNA protein complexes associated with the topoisomerase, evaluated by same method described for *L. donovani* [23,24]. The median ED50 for the wild-type strain was considerably lower than the line selected for resistance. Treatment with both meglumine antimoniate and sodium stibogluconate stabilized DNA-protein complexes in the wild-type strain but not the resistant line. The ED50s of the SbVs for *Leishmania* strains from patients with relapses was comparable to those for the line selected for in vitro resistance, and DNA-protein complexes were not stabilized by exposure to meglumine antimoniate. The selective effect of the SbVs on the stabilization of DNA-protein complexes in *Leishmania* and the loss of this effect in naturally resistant or experimentally derived SbV-resistant *Leishmania* suggest that topoisomerase may be a target of antimonial drugs [22].

Amphotericin B is a polyene antibiotic that has been used as a second line treatment for leishmaniasis since the 1960s [17]. It has a selective activity against *Leishmania* due to the higher affinity of amphotericin B for ergosterol, the predominant sterol in these microbes, over cholesterol, the predominant sterol in the mammalian host cells [25]. An interest study compared amphotericin-resistant amastigotes and promastigotes of *L. mexicana* with control parasites to evaluate molecular differences, especially in the membrane [19]. Analyses of drug-resistant and control, wild type *L. mexicana* lines revealed dramatic differences in sterol composition, such as ergosta-5,7,24(241)-trienol contributed approximately 85% of the total sterol. In the amastigotes, the major sterols were a mixture of ergosta- and stigmasta-5,7,24(241)-trienols. In contrast, these sterols were undetectable in amphotericin-resistant parasites, which contained instead high levels of methylcholesta-sterols. The amphotericin B is therefore no longer able to enter the cytosol of resistant parasites [19].

Another treatment against leishmaniasis is pentamidine, which belongs to the diamidine class of drugs. The drug enters both promastigote and amastigote forms of the *Leishmania* cell via a carrier-mediated process which recognizes diamidines with high affinity [26]. Efforts to identify a physiological substrate for the transporter failed. Basic amino acids, polyamines and a wide variety of common metabolites also failed to inhibit pentamidine uptake. Resistance in this case was concluded not to associate with alterations at the level of a plasma membrane transporter, but with changes in the mitochondrial membrane potential [26]. The mitochondrion appears to be the target of pentamidine action and plays a crucial role in the mode of action for pentamidine in *Leishmania* parasites [26].

Because treatment is a growing problem, the development of new medicines that can replace or complement the presently available therapeutic alternatives is therefore necessary [27]. The decades of research that go into identifying the key proteins involved in *Leishmania* pathogenesis and intracellular survival are the groundwork for targets of new drug discovery.

3. Proteome findings on parasites causing TL

Previous studies and reviews also provide a framework for proteomics in the study of *Leishmania* [7,10,11,28,29]. In this review, however, we have selected important researches that focus on TL causative agents and discussed the challenges and perspectives for drug development regarding proteomic findings.

In the 1980s, the first proteome maps of *Leishmania* (*L. tropica*; *L. mexicana*; *L. braziliensis*) were published [30–32] before the term proteomics had been created [33]. Handman and colleagues evaluated protein isolates from four *L. tropica* isolates. Samples were biosynthetically labeled with S-methionine or surface radioiodinated, and the detergent lysates were analyzed by 2 dimensional gel electrophoresis (2DE). This study revealed two different protein patterns of four strains of *L. tropica* isolates [30].

Saravia and his team examined the relationship among different *Leishmania* strains and species (*L. mexicana* and *L. braziliensis*) using 2DE to identify subspecies. The study was successful in revealing an unexpected degree of disparity between this two species, at that time, when no further information was available [32].

The identification of large molecules, such as proteins was achieved at 1980's, when mass spectrometry techniques were upgraded to analyze specifically large biomolecules. The identification of proteins was achieved by cleaving an intact protein into its peptides and analyzing these fragments by a new mass spectrometric (MS) technique developed by two independent groups in 1987 [34,35], which was MALDI-TOF (Matrix Assisted Laser Dessorption Ionization time of flight). This ionization technique is used to form intact species the TOF analyser is used to determine exact masses of proteins ions and then correlating such peptide masses against a database of known peptide and proteins expressed in the genome. In this way, it is possible to identify protein sequences to a high degree of accuracy without ever determining more than the masses of the peptide ions in question (Fig. 1a). Later on, in 1989, electrospray ionization (ESI) has emerged as a powerful

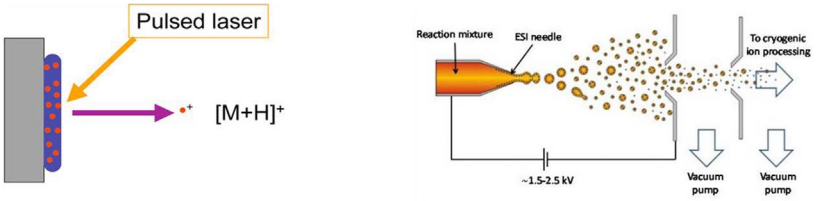

Fig. 1. Illustration of MALDI and ESI as ionization sources of biomolecules. (a) MALDI: a pulsed laser is directed over the matrix co-crystallized with biomolecules. The matrix is an organic molecule that transfers a proton to the analyte after laser bombardment. The ions are then directed to the mass analyser. (b) ESI: an electrospray produces charged droplets from a solution containing biomolecule ions that are transferred to the gas phase and then guided to the mass analyser.

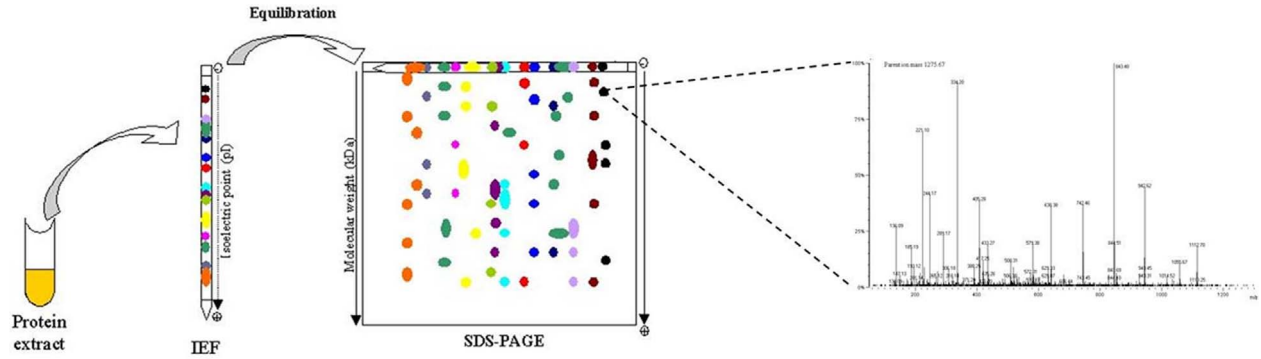

Fig. 2. Illustration of protein identification workflow: the protein extract is composed by a mixture of proteins. Isoelectric focusing (IEF) and sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) are preferred in 2-DE separation. In IEF/SDS-PAGE, the proteins applied in the first dimension will move along the gel and accumulate at their isoelectric point. After proteins are separated, each band is lysed by specific enzymes and proteins are cleaved into a peptide pool that is fingerprinted by MS.

technique for producing intact gaseous ions from large and complex species in solution. ESI improved the power of MS analysis applicable to the large and fragile polar molecules that play such vital roles in biological systems, such as proteins [36] (Fig. 1b).

Several studies have led to further improvement of proteomics to investigate the parasites' biology. To identify as many proteins as possible, new methodologies involving suitable lysis of *Leishmania* were developed, many containing urea and thiourea as reagents. A considerable number of studies about detailed 2DE proteome maps of *L. amazonensis*, *L. major*, *L. guyanensis* and *L. panamensis* were then published [37,38]. Fig. 2 shows an overview over the classic approach in proteomics: protein separation through two-dimensional electrophoresis (2-DE) and protein identification through MS analysis.

Proteomics is the study of ideally the entire set of proteins produced or modified by an organism at a specific time or condition [39]. There are two main ways to detect and quantify proteins: affinity reagent based methods, i.e. ELISA; Western blotting or immunohistochemistry staining; and MS based peptide identification and quantitation. Measuring proteins poses technical challenges, since biological samples are made-up of a large number of cells with different cell types [40]. Besides this challenge, *Leishmania* proteins are being largely identified by 2DE methods followed by MS analysis (Fig. 2).

Proteomics approaches are likely to unveil details at molecular level among hosts, parasites and vectors [7,41]. Proteomics hold promises for the treatment of infectious diseases such leishmaniasis; with this technology it would be possible to select proteins that can be used as biomarkers that characterize for example benign cutaneous lesions and cutaneous lesions which will expand to the mucosal region, and also as targets of drugs. Proteomics should also provide a better means to treat patients since smart drugs will be designed. The number of targeted proteins used for drug-design will therefore be increased. The knowledge of all parasite metabolic pathways and proteins of signaling systems and membranes will facilitate the development for new drugs with the help from bioinformatics approaches. The use of library of chemicals available on the database and combinatorial chemistry will be able to access possible chemicals as drugs against *Leishmania* targeted proteins.

Here, we present proteomic studies that have been published after the genome of *Leishmania major* was sequenced [42]. Since when, this achievement enabled the relatively straight-forward identification of protein-species excised from 2DE gels by mass spectrometry or from peptides separated by liquid chromatography. Many proteomic studies focusing on *Leishmania* infection have considered a variety of aspects related to parasite biology and host interactions, including parasite differentiation, drug resistance mechanisms, and the identification of immunogenic proteins for vaccine development [43–47].

In this review, we selected literature content on TL causative agents, aiming to discuss on how proteomics can be a useful tool in the search of new drug targets.

3.1. Differentiation proteome (life cycle)

Proteomic studies have begun investigating mainly developmentally regulated proteins in *L. mexicana* (causative agent of CL) and *L. infantum*, *L. donovani* (causative agents of VL) using in vitro induced axenic amastigotes as models of authentic intracellular parasites [48,49]. The following studies reveal significant inter-species differences in amastigote-specific and complemented the intrinsic variations between *Leishmania* strains. It is essential to investigate expression analysis of individual *Leishmania* species strains to enable the elucidation of molecular mechanisms important for intracellular amastigote survival at specie-levels, aiming the development of promising therapeutics against the disease [39]. Since amastigotes are the intracellular form of the parasite, information obtained from these proteins contributes to enhance the resources to find therapeutic targets and it is also a promising field for the discovery of new virulence markers [11].

Nugent and Walker demonstrated the feasibility of protein profiling as a strategy for elucidating survival mechanisms and discovering novel developmentally regulated proteins in *Leishmania* parasites. Although the authors failed to suggest their findings as drug targets, their results are shown in the following paragraphs as a screening of important identified proteins. The researchers described robust methods for reproducible extraction and separation of *L. mexicana* proteins by gel electrophoresis technologies followed by mass spectrometry analysis [48]. A number of amastigote protein spots, heat-shock proteins including HSP60 and HSP70 were detected, while HSP83 (HSP90) was identified in all three developmental stages (procyclic promastigotes, metacyclic promastigotes and amastigotes) by immunoblotting.

Identification of a "uniquely" expressed HSP83 protein fragment in amastigotes may be consistent with its suggested role as a trigger for amastigote differentiation in *L. donovani* [50]. A large number of proteins associated with protein synthesis were also identified in amastigotes and many of these appeared as either "unique" spots (translation elongation factor eEF-1α, eukaryotic initiation factor eIF-5α, 40Sribosomal protein S2) or spots with highly upregulated expression. A protein of particular interest was the translation elongation factor eEF-1α and its isoforms. A high molecular weight isoform of this protein was expressed at high abundance and as a multi spot trail in all three developmental stages analyzed [48].

In another important study, Walker and co-workers employed gel electrophoresis technologies and selected differentially expressed proteins were identified by biological MS. A total of 75 protein spots were differentially expressed in amastigotes. Of these, 24 spots were "unique" to amastigotes (undetectable in the promastigote proteome even using sensitive silver staining), and the remaining 51 spots were up regulated compared to promastigotes. In addition, 29 protein spots were present exclusively in the promastigote proteome, and a further 16 displayed higher abundance in promastigotes than in amastigotes [48].

Recently Lynn et al. identified 189 *L. infantum* and 107 *L. mexicana* non-redundant proteins of which 20–40% showed differential expression levels between promastigote and amastigote lifecycle stages. Differentially expressed proteins mapped to several pathways including cell motility, metabolism, and infectivity as well as virulence factors such as eEF-1α, amastin and leishmanolysin (GP63). The authors suggested that differentially expressed proteins essential for pathogenesis, may ultimately identify novel potential therapeutic targets [13].

An important study by Moreira et al. investigated the differential phosphoprotein abundance associated with the drug-induced stress response and potassium antimonyl tartrate (SbIII)-resistance mechanisms. The study compared non-treated and SbIII-treated samples of *L. braziliensis*. They identified 48 different proteins distributed into different biological process categories. The category "protein folding/chaperones and stress response" is mainly implicated in response to SbIII treatment, such as heat shock proteins HSP70 and HSP83-1, while the categories "antioxidant/detoxification" (peroxidoxins and tryparedoxin peroxidases); "metabolic process" (metallo-peptidase, Clan MA(E), acidocalcisomal pyrophosphatase and protein nucleoside diphosphate kinase b), "RNA/DNA processing" (proliferative cell nuclear antigen (PCNA), Ran-binding protein 1, and nucleosome assembly protein) and "protein biosynthesis" (arginino succinate synthase, 40-S ribosomal protein S12 and lysyl-tRNA synthetase) are modulated in the case of antimony resistance. This study allowed the authors to profile the *L. braziliensis* phosphoproteome, identifying several potential candidates for biochemical or signaling networks associated with antimony resistance phenotype in this parasite [51].

3.2. Macrophage interaction proteome

Infected female sandflies transmit the disease by inoculating the promastigote form into the skin during their blood meal. In the vertebrate host, the parasites are phagocytosed by macrophages and dendritic cells in the dermis. After uptake and internalization of promastigotes into a phagosome, fusion with lysosomes proceeds as normal and the parasites survive in the phagolysosome. During this process, the promastigotes rapidly transform into amastigotes within 12–24 h and continue to grow and divide within the phagolysosomal compartment [52].

It is well described that phagocytosis of *Leishmania* leads to a burst of $O_2^{\bullet -}$ production through activation of NADPHoxidase [53]. Despite its susceptibility to exogenous ROS (reactive oxygen species) and NO• (nitric oxide) *Leishmania* can survive phagocytosis. The parasites have adapted to survive and replicate inside macrophages by deploying antioxidant systems and suppressing macrophage ROS production. It is known that parasites can counteract their endogenous ROS production through antioxidant systems or by actively decreasing ROS production. The parasite antioxidants are currently intensively explored as drug target molecules. Proteomics can however shed light to unknown interactions at molecular levels leading to novel antileishmanial drug targets.

The study of Menezes and colleagues represents the first trial to employ large-scale proteomic analysis to identify host cell protein expression in response to *Leishmania* infection. They hypothesized that macrophages from murine stain CBA, which are resistant to *L. major* express proteins associated with infection control compared to the same macrophages, which are susceptible to *L. amazonensis*. A total of 62 proteins were exclusively expressed in infected macrophages. From these proteins, a total of 15 proteins showed greater differences in expression (Phospholipase D1; RAS-related C3 botulinum substrate 2; glucuronidase, beta; PRD; coronin, actin binding protein 1B; PYD and CARD domain containing; Peripheral benzodiazepine receptor (TSPO); RAB1, member RAS oncogene family; Programmed cell death 5; Myoson light peptide 1; Hypoxia up-regulated 1; Cytochrome c oxidise; Alpha isoform of regulatory subunit A, protein phosphatase 2; SH3 domain protein 3; SERPINEI mRNA binding protein 1). Eleven of these 15 proteins exhibited reduced expression under *L. amazonensis* infection, and four proteins exhibited increased expression. One of the proteins with higher expression in *L. amazonensis* infection was phospholipase D1 (PLD1). PLD1 acts on phosphatidylcholine, releasing phosphatidic acid [54]. PLD1 has been associated with the recruitment of additional membrane for the formation of nascent phagosomes and the maintenance of phagosomes through fusion with endocytic vesicles [55]. This is typical of *L. amazonensis* infection, but not for *L. major* [10]. For the first time, proteins that are differentially modulated between these two species were identified through a qualitative approach. With significant levels of differential expression, 13 out of the 15 proteins were down-modulated in *L. amazonensis* or upmodulated in *L. major*-infected macrophages. That modulation might be responsible for distinct phenotypic macrophage responses was identified, leading to infection control under *L. major* and parasite survival under *L. amazonensis* [10]. The authors employed IPA-Ingenuity Systems® to build models of potential networks and connections among the differently expressed proteins. The networks that contain proteins modulated through *Leishmania* infection were involved in the cell signaling and cell death network, cellular movement and organization network, and the lipid metabolism and molecular transport network. This study shows that the comparison of different *leishmanial* species using proteomic approaches contributes to reveal details about specie-specific behavior of macrophage-parasite interactions at molecular level, which information are very promising to search new drug targets. Future studies are therefore required to obtain evidence of whether these differentially expressed proteins can be used as novel markers and targets for the control of *Leishmania* infection [10].

3.3. Secreted and soluble proteins

Despite the rich knowledge of differential host immune responses to cutaneous leishmaniasis, very little is known about how parasite-derived proteins contribute to the species-specific outcome of the disease. Many studies have established a widely used methodology for protein profiling that accelerates the search for novel *Leishmania* proteins/biomarkers. Proteins expressed and secreted by the parasite may contribute to parasite survival and disease progression, respectively. Further knowledge obtained from secreted and soluble proteins could therefore be an interesting field to search drug targets. Here, we discuss some studies [37,56] using 2-DE as a powerful tool to document protein expression profiles of TL causative agents, such as *L. amazonensis* and *L. major* [56].

Proteomic analysis of cellular fractions such as cytosol, organelles and/or nucleous, also called subproteomic studies, have been

characterized aiming to identify targets for intervention in the parasite multiplication. In this part of the review, we summarized available information about how protein content of the enriched microsomal fractions of *Leishmania* can be used to identify secreted proteins in the promastigote form of the parasite [56]. For example, Brobey and colleagues reported on proteomics studies in *Leishmania* using high-resolution 2-DE to compare the steady-state protein expression of two *Leishmania* species, *L. amazonensis* and *L. major* to document global differences on soluble proteins. The infectious promastigotes of both species were subjected simultaneously to 2-DE protein profiling. They looked for protein spots that were differentially expressed in either *L. amazonensis* or *L. major* by comparing spot intensity, aiming to examine the feasibility of exploring this procedure to search for species-specific *Leishmania* proteins. The study revealed that most *L. amazonensis* protein spots with an increased intensity relative to *L. major*. They also recognized spots that were readily detectable in the *L. amazonensis* gel, but barely visible in the corresponding locations in the *L. major* gel. For example, 4 spots were apparently detected in *L. amazonensis*, but not in *L. major*. Overall, 47 spots were confidently determined to be differentially expressed between *L. amazonensis* and *L. major*, although these spots were not identified in their study [37].

Oliveira and co-workers also aimed to identify potential secreted protein targets for further characterization. This time, they used 2DE gels followed by MS analysis to study the soluble protein content of *L. major* and *L. amazonensis*. Their study demonstrated that the overall protein profile of the microsomal extract differs between the two species. MALDI-TOF-MS peptide fingerprinting of 33 protein spots from *L. amazonensis* and 41 protein spots from *L. major* identified 14 proteins from each sample could be unambiguously assigned [56]. These proteins include the nucleotide diphosphate kinase, a calpain-like protease, atryparedoxin peroxidase and a small GTP-binding Rab1-protein, all of which have a potential functional involvement with secretion pathways and/or environmental responses of the parasite [56]. Proteins associated with energy metabolism pathway and with the 20S subunit of the proteasome are some of the proteins that were conserved between *L. amazonensis* and *L. major*. Two other proteins are also likely to be involved in drug resistance: the small GTP-binding Rab1-protein (*L. amazonensis*) and tryparedoxin peroxidase (*L. major*) and were both observed to be differentially expressed in a proteomic study with *Leishmania* methotrexate resistant strains [57]. The gene encoding the GTP-binding Rab1-protein may be involved in drug resistance in *Leishmania* [58]. The nucleoside diphosphate kinase was identified in both species. This enzyme plays a pivotal role in the nucleoside triphosphate and deoxynucleoside triphosphate regulation [59]. The proteins identified in this study show functional involvement of parasite responses which, in case of *Leishmania*, could be involved in the communication with the host cell during the invasion process [56]. These results are relevant as they can lead to better understanding of host/parasite interactions, clarifying how parasites are developing resistance against available drugs and open wings for the discovery of potential drug targets.

In another study, Paape and co-workers developed a novel purification method for *L. mexicana* amastigotes to enable direct proteomic analysis using fluorescence parasite sorting in combination with gel free analysis. Their methodology improved proteome coverage and suggested proteins putatively secreted by the parasites. A set of 67 newly identified proteins of unknown function was presented. A novel proteomic data set of intracellular *L. mexicana* amastigotes have therefore extended the list of *Leishmania* proteins identified to date. Since amastigotes are the intracellular form of the parasite, these data is likely to provide a powerful resource to therapeutic targets, biomarkers discovery, metabolic pathways and vaccines [8,45].

More recently, Walker and co-workers using 2-DE gel electrophoresis, generated two distinct sub-proteomes (soluble in NP-40/urea and Triton X-114, respectively) of *L. panamensis* promastigotes lines resistant to antimony resistance, nine differentially expressed putative antimonial-resistance factors were detected and identified by MS analysis and divided in two major groups of molecules proteins involved in general stress responses and proteins with highly specific metabolic and transport functions, potentially contributing to the Sb-resistance mechanism. For example the mitochondrial ATPase subunit involved in multi-drug resistance; the elongation factor-2 and eukaryotic translation release factor responsible for maintaining the resistance phenotype and the small GTP-binding proteins of the Rab family involved in drug efflux [20].

Recently, Lima and co-workers employed a proteomic approach coupled to an in silico analysis and identified the most abundant and immunogenic proteins from *L. amazonensis*, *L. braziliensis* and *L. infantum* using gel electrophoresis technologies followed by MALDI-TOF/TOF MS analysis. They identified nine potential peptides specific of TL parasites, which were derived from 4 different proteins: elongation factor 2 (4 peptides), metallopeptidase, Clan MA(E)-Family M3 (1 peptide), Enolase (2 peptides) and peptidase m20/m25/m40 family-like protein, partial (2 peptides). These proteins and peptides may be potential candidates to improve the specificity and sensibility of TL diagnosis and drug targets aiming to control disease progression [9].

4. Perspectives of proteomic studies for drug discovery and drug resistance

In this review, we have emphasized how large-scale studies involving proteins contributed to the identification of protein functions, as well as networks related to the understanding of the responses of *Leishmania* parasites to a variety of occasions (differentiation life-cycle; macrophage-parasite interactions; secreted and soluble proteins). We also collected valuable information at the molecular level regarding the proteins involved in parasite resistance against available drugs [57], expressed proteins in infected macrophages [10], expressed proteins in the intracellular form of the parasite [8,45] and potential drug targets [7,41].

Leishmania parasites are adapted to survive inside macrophages and are developing resistance mechanisms against drugs that are currently available to fight leishmaniasis. Proteomics shed light to unknown interactions at molecular levels and reveal particularities of different species and its interactions with macrophages. Proteomic approaches can be helpful in the discovery novel protein targets for structural and functional studies.

Further proteomics are required to validate biomarkers and therapeutic targets. To that, studies involving biomarker's efficacy, safety, stability, quality control and clinical trials are necessary. More proteomic analysis on *Leishmania* isolates from different human LT cases, mainly *L. braziliensis*, which is less studied than other *Leishmania* species, are needed due to scarcity of amastigotes in the lesions and difficulty to cultivate promastigotes. More studies involving proteins are also necessary to identify markers of resistance in *Leishmania* isolates. Currently, we at the Department of Animal Biology of the Institute of Biology and the Thomson Laboratory of Mass Spectrometry at the Chemistry Institute of the University of Campinas – UNICAMP are investigating the proteins involved in disease progression caused by *Leishmania* through different MS techniques.

Acknowledgments

Funding for this project came from Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP – (2015/23767-0). F.N. acknowledges FAPESP (studentship 2016/11517-2) and University of Campinas.

References

[1] WHO, Leishmaniasis, WHO, 2016.
[2] H. Goto, J. Angelo, L. Lindoso, Cutaneous and mucocutaneous Leishmaniasis, Infect. Dis. Clin. NA 26 (2012) 293–307.

[3] A. Pavli, H.C. Maltezou, Author's Personal Copy Leishmaniasis, an emerging infection in travelers, Int. J. Infect. Dis. 14 (2010) e1032–e1039.
[4] K. Leifso, G. Cohen-Freue, N. Dogra, A. Murray, W.R. McMaster, Genomic and proteomic expression analysis of Leishmania promastigote and amastigote life stages: the Leishmania genome is constitutively expressed, Mol. Biochem. Parasitol. 152 (2007) 35–46.
[5] R. Reithinger, et al., Review Cutaneous Leishmaniasis, infection.thelancet.com, 2007, http://dx.doi.org/10.1016/S1473-3099(07)70209-8.
[6] C. Cantacessi, F. Dantas-Torres, M.J. Nolan, D. Otranto, The past, present, and future of Leishmania genomics and transcriptomics, Trends Parasitol. 31 (2015) 100–108.
[7] M. Ehrmann, F. Kaschani, M. Kaiser, Chemical proteomics versus Leishmaniasis, Chem. Biol. 22 (2015) 309–310.
[8] D. Paape, M.E. Barrios-Llerena, T. Le Bihan, L. Mackay, T. Aebischer, Gel free analysis of the proteome of intracellular *Leishmania mexicana*, Mol. Biochem. Parasitol. 169 (2010) 108–114.
[9] B.S.S. Lima, L.C. Fialho, S.F. Pires, W.L. Tafuri, H.M. Andrade, Immunoproteomic and bioinformatic approaches to identify secreted *Leishmania amazonensis*, *L. braziliensis*, and *L. infantum* proteins with specific reactivity using canine serum, Vet. Parasitol. 223 (2016) 115–119.
[10] J.P.B. Menezes, et al., Proteomic analysis reveals differentially expressed proteins in macrophages infected with *Leishmania amazonensis* or *Leishmania major*, Microbes Infect. 15 (2013) 579–591.
[11] J.B. de Jesus, C. Mesquira-Rodrigues, P. Cuervo, Proteomics advances in the study of Leishmania parasites and leishmaniasis, Subcell Biochem . 74 (2014) 323–349.
[12] P. Cuervo, et al., Proteomic characterization of the released/secreted proteins of *Leishmania (Viannia) braziliensis* promastigotes, J. Proteomics 73 (2009) 79–92.
[13] M.A. Lynn, A.K. Marr, W.R. McMaster, Differential quantitative proteomic profiling of *Leishmania infantum* and *Leishmania mexicana* density gradient separated membranous fractions, J. Proteomics 82 (2013) 179–192.
[14] V.S. Amato, F.F. Tuon, H.A. Bacha, V.A. Neto, A.C. Nicodemo, Mucosal leishmaniasis: current scenario and prospects for treatment, Acta Trop. 105 (2008) 1–9.
[15] J. Arevalo, et al., Influence of *Leishmania (Viannia)* species on the response to antimonial treatment in patients with American tegumentary leishmaniasis, J. Infect. Dis. 195 (2007) 1846–1851.
[16] H. Goto, J.A. Lindoso, Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis, Expert Rev. Anti-Infect. Ther. 8 (2010) 419–433.
[17] S.L. Croft, S. Sundar, A.H. Fairlamb, Drug resistance in leishmaniasis, Clin. Microbiol. Rev. 19 (2006) 111–126.
[18] S. Decuypere, et al., Molecular mechanisms of drug resistance in natural leishmania populations vary with genetic background, PLoS Negl. Trop. Dis. 6 (2012) e1514.
[19] H.I. Al-Mohammed, M.L. Chance, P.A. Bates, Production and characterization of stable amphotericin-resistant amastigotes and promastigotes of *Leishmania mexicana*, Antimicrob. Agents Chemother. 49 (2005) 3274–3280.
[20] J. Walker, et al., Discovery of factors linked to antimony resistance in *Leishmania panamensis* through differential proteome analysis, Mol. Biochem. Parasitol. 183 (2012) 166–176.
[21] A. Stauch, et al., Treatment of visceral leishmaniasis: model-based analyses on the spread of antimony-resistant *L. donovani* in Bihar, India, PLoS Negl. Trop. Dis. 6 (2012) e1973.
[22] A. Lucumi, S. Robledo, V. Gama, N.G. Saravia, Sensitivity of *Leishmania viannia panamensis* to pentavalent antimony is correlated with the formation of cleavable DNA-protein complexes, Antimicrob. Agents Chemother. 42 (1998) 1990–1995.
[23] A.L. Bodley, T.A. Shapiro, Molecular and cytotoxic effects of camptothecin, a topoisomerase I inhibitor, on trypanosomes and Leishmania, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 3726–3730.
[24] A.K. Chakraborty, H.K. Majumder, Mode of action of pentavalent antimonials: specific inhibition of type I DNA topoisomerase of *Leishmania donovani*, Biochem. Biophys. Res. Commun. 152 (1988) 605–611.
[25] J.D. Berman, et al., Efficacy and safety of liposomal amphotericin B (AmBisome) for visceral leishmaniasis in endemic developing countries, Bull. World Heal. Organ. 76 (1998) 25–32.
[26] M. Basselin, H. Denise, G.H. Coombs, M.P. Barrett, Resistance to pentamidine in *Leishmania mexicana* involves exclusion of the drug from the mitochondrion, Antimicrob. Agents Chemother. 46 (2002) 3731–3738.
[27] T.S. Tiuman, A.O. Santos, T. Ueda-Nakamura, B.P.D. Filho, C.V. Nakamura, Recent advances in leishmaniasis treatment, Int. J. Infect. Dis. 15 (2011) e525–e532.
[28] P. Sampaio, T. Veras, J. Perrone, B. De Menezes, Using Proteomics to Understand How Leishmania Parasites Survive inside the Host and Establish Infection, Int. J. Mol. Sci. 17 (2016) 1270.
[29] S. Akpunarlieva, et al., Integration of proteomics and metabolomics to elucidate metabolic adaptation in Leishmania, J Prot. 155 (2017) 85–98.
[30] E. Handman, G.F. Mitchell, J.W. Goding, Identification and characterization of protein antigens of *Leishmania tropica* isolates, J. Immunol. 126 (1981) 508–512.
[31] E. Handman, R.E. Hocking, G.F. Mitchell, T.W. Spithill, Isolation and characterization of infective and non-infective clones of Leishmania tropica, Mol. Biochem. Parasitol. 7 (1983) 111–126.
[32] N.G. Saravia, M.A. Gemmell, S.L. Nance, N.L. Anderson, Two-dimensional electrophoresis used to differentiate the causal agents of American tegumentary leishmaniasis, Clin. Chem. 30 (1984) 2048–2052.
[33] D. Paape, T. Aebischer, Contribution of proteomics of *Leishmania* spp. to the understanding of differentiation, drug resistance mechanisms, vaccine and drug development, J. Proteomics 74 (2011) 1614–1624.
[34] M. Karas, D. Bachmann, U. Bahr, F. Hillenkamp, Matrix-assisted ultraviolet laser desorption of non-volatile compounds, Int. J. Mass Spectrom. Ion Process. 78 (1987) 53–68.
[35] K. Tanaka, et al., Protein and polymer analyses up to m/z 100 000 by laser ionization time of flight mass spectrometry, Rapid Commun. Mass Spectrom. 2 (1988) 151–153.
[36] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Electrospray ionization for mass spectrometry of large biomolecules, Science 246 (1989) 64–71.
[37] R.K.B. Brobey, F.C. Mei, X. Cheng, L. Soong, Comparative two-dimensional gel electrophoresis maps for promastigotes of *Leishmania amazonensis* and *Leishmania major*, Braz. J. Infect. Dis. 10 (2006) 1–6.
[38] R. Góngora, et al., Mapping the proteome of *Leishmania Viannia* parasites using two-dimensional polyacrylamide gel electrophoresis and associated technologies, Biomedica 23 (2003) 153–160.
[39] J. Walker, et al., Identification of developmentally-regulated proteins in *Leishmania panamensis* by proteome profiling of promastigotes and axenic amastigotes, Mol. Biochem. Parasitol. 147 (2006) 64–73.
[40] H.A. Ebhardt, A. Root, C. Sander, R. Aebersold, Applications of targeted proteomics in systems biology and translational medicine, Proteomics 15 (2015) 3193–3208.
[41] M.H. Wright, et al., Global analysis of protein N-myristoylation and exploration of N-myristoyltransferase as a drug target in the neglected human pathogen *Leishmania donovani*, Chem. Biol. 22 (2015) 342–354.
[42] A.C. Ivens, et al., The genome of the kinetoplastid parasite, *Leishmania major*, Science 309 (2005) 436–442.
[43] M.A. Dea-Ayuela, L. Ordoñez-Gutierrez, F. Bolás-Fernández, Changes in the proteome and infectivity of Leishmania infantum induced by in vitro exposure to a nitric oxide donor, Int. J. Med. Microbiol. 299 (2009) 221–232.
[44] S.K. Gupta, et al., Proteomic approach for identification and characterization of novel immunostimulatory proteins from soluble antigens of *Leishmania donovani* promastigotes, Proteomics 7 (2007) 816–823.
[45] D. Paape, et al., Transgenic, fluorescent *Leishmania mexicana* allow direct analysis of the proteome of intracellular amastigotes, Mol. Cell. Proteomics 7 (2008) 1688–1701.
[46] C. Henriques, G.C. Atella, V.L. Bonilha, W. de Souza, Biochemical analysis of proteins and lipids found in parasitophorous vacuoles containing *Leishmania amazonensis*, Parasitol. Res. 89 (2003) 123–133.
[47] K. Hassani, E. Antoniak, A. Jardim, M. Olivier, Temperature-induced protein secretion by *Leishmania mexicana* modulates macrophage signalling and function, PLoS ONE 6 (2011).
[48] P.G. Nugent, S.A. Karsani, R. Wait, J. Tempero, D.F. Smith, Proteomic analysis of *Leishmania mexicana* differentiation, Mol. Biochem. Parasitol. 136 (2004) 51–62.
[49] Y. El Fakhry, M. Ouellette, B. Papadopoulou, A proteomic approach to identify developmentally regulated proteins in Leishmania infantum, Proteomics 2 (2002) 1007.
[50] M. Wiesgigl, J. Clos, Heat shock protein 90 homeostasis controls stage differentiation in *Leishmania donovani*, Mol. Biol. Cell 12 (2001) 3307–3316.
[51] Dde.S. Moreira, et al., Phosphoproteomic analysis of wild-type and antimony-resistant *Leishmania braziliensis* lines by 2D-DIGE technology, Proteomics 15 (2015) 2999–3019.
[52] R.K. Singh, H.P. Pandey, S. Sundar, Visceral leishmaniasis (kala-azar): challenges ahead, Indian J. Med. Res. 123 (2006) 331–344.
[53] R. Minakami, H. Sumimotoa, Phagocytosis-coupled activation of the superoxide-producing phagocyte oxidase, a member of the NADPH oxidase (Nox) family, Int. J. Hematol. 84 (2006) 193–198.
[54] L. Wang, et al., Involvement of phospholipases D1 and D2 in sphingosine 1-phosphate-induced ERK (extracellular-signal-regulated kinase) activation and interleukin-8 secretion in human bronchial epithelial cells, Biochem. J. 367 (2002) 751–760.
[55] M. Corrotte, et al., Dynamics and function of phospholipase D and phosphatidic acid during phagocytosis, Traffic 7 (2006) 365–377.
[56] A.H.C. de Oliveira, et al., Subproteomic analysis of soluble proteins of the microsomal fraction from two Leishmania species, Comp. Biochem. Physiol. D: Genomics Proteomics 1 (2006) 300–308.
[57] J. Drummelsmith, I. Girard, N. Trudel, M. Ouellette, Differential protein expression analysis of leishmania major reveals novel roles for methionine adenosyltransferase and S-adenosylmethionine in methotrexate resistance, J. Biol. Chem. 279 (2004) 33273–33280.
[58] J.F.M. Marchini, A.K. Cruz, S.M. Beverley, L.R.O. Tosi, The H region HTBF gene mediates terbinafine resistance in *Leishmania major*, Mol. Biochem. Parasitol. 131 (2003) 77–81.
[59] L. Lascu, A. Giartosio, S. Ransac, M. Erent, Quaternary structure of nucleoside diphosphate kinases, J. Bioenerg. Biomembr. 32 (2000) 227–236.