

Review

Experimental Strategies to Explore Drug Action and Resistance in Kinetoplastid Parasites [†]

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Abstract: Kinetoplastids are the causative agents of leishmaniasis, human African trypanosomiasis, and American trypanosomiasis. They are responsible for high mortality and morbidity in (sub)tropical regions. Adequate treatment options are limited and have several drawbacks, such as toxicity, need for parenteral administration, and occurrence of treatment failure and drug resistance. Therefore, there is an urgency for the development of new drugs. Phenotypic screening already allowed the identification of promising new chemical entities with anti-kinetoplastid activity potential, but knowledge on their mode-of-action (MoA) is lacking due to the generally applied whole-cell based approach. However, identification of the drug target is essential to steer further drug discovery and development. Multiple complementary techniques have indeed been used for MoA elucidation. In this review, the different ‘omics’ approaches employed to define the MoA or mode-of-resistance of current reference drugs and some new anti-kinetoplastid compounds are discussed.

Keywords: kinetoplastid; resistance; target; drug; omics; mechanism; resistance

1. Kinetoplastid Diseases

Leishmaniasis, Chagas disease, and sleeping sickness are caused by kinetoplastid protozoan parasites and are responsible for high morbidity and mortality rates, especially in developing countries [1–3]. These diseases are characterized by severe clinical manifestations such as hepatosplenomegaly, cardiomyopathy, and neuropathology, all of which may lead to fatality if left untreated [4–6]. Current therapies are known to be less than adequate due to the suboptimal administration routes and long treatment duration, the occurrence of severe adverse effects, and the growing incidence of treatment failures [3,5,7]. It is evident that treatment options should be improved and that new drugs will be needed to sustain adequate disease control.

Target-based and phenotypic screening are two standard approaches adopted by the pharmaceutical industry to identify novel active chemical entities. So far, the former has not been very successful given the lack of fully validated targets and the limited knowledge on their molecular biology [3,4,8]. The few targets that have been suggested for *Trypanosoma brucei* and *T. cruzi* have only been partially characterized [8–12] and ornithine decarboxylase is currently the only fully validated target involved in the mode-of-action (MoA) of eflornithine [8,13]. Identification of novel drug ‘leads’ is generally still achieved by phenotypic cell-based screening [14–17] but with the disadvantage of

lack of knowledge gained on the MoA. However, target elucidation remains pivotal for rational structure-based optimization of small molecules, which predicts adverse effects. Even though MoA studies are not essential for regulatory approval, they can strengthen successful drug development or redirect drug discovery [12,14,18–20]. Alternatively, knowledge on a drug's mode-of-resistance (MoR) can also provide useful information for anti-kinetoplastid drug research [21].

As described in several reviews [18,19,22], multiple strategies can be used to identify the MoA of compounds. The present review specifically focuses on the different 'omics' approaches which in recent years have emerged as valuable techniques to evaluate the MoA and MoR of anti-kinetoplastid compounds (Figure 1).

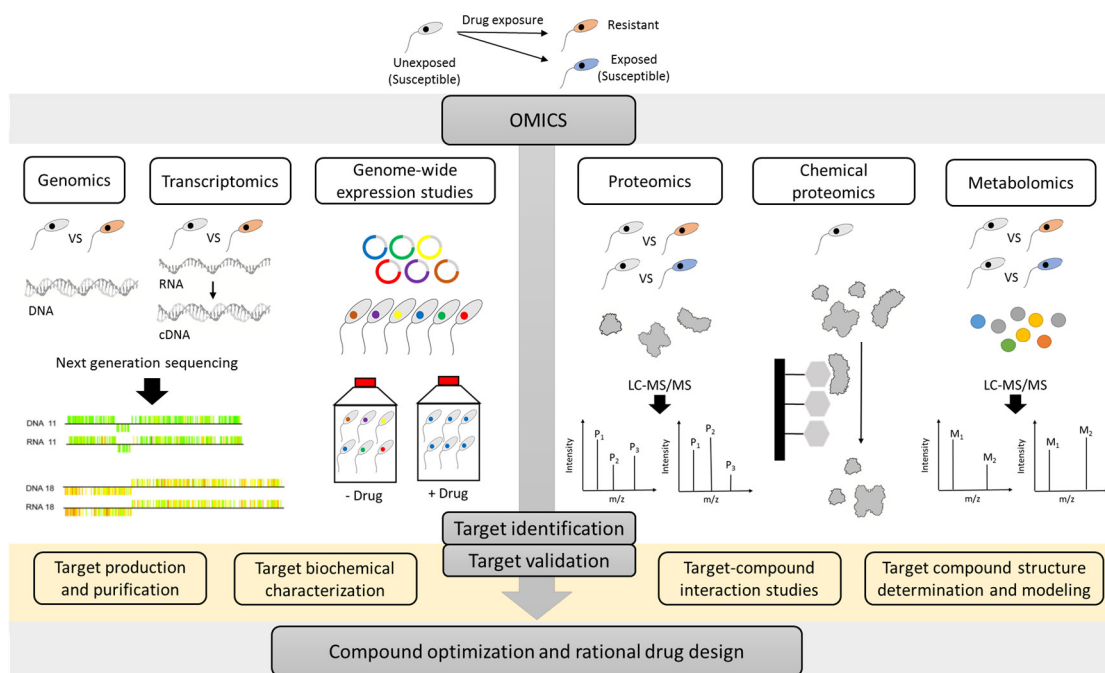


Figure 1. An overview of the multiple omics strategies that can be used to unravel the target of an anti-kinetoplastid compounds. Resistant parasites can be experimentally selected in the laboratory or obtained from the field. Comparative genomics, transcriptomics, proteomics/metabolomics approaches, genome-wide cosmid/RNAi libraries, and chemical proteomics can contribute complementary insights in the mode-of-action (MoA) or mode-of-resistance (MoR). Application of the various omics methodologies can enable the identification of drug targets, which may serve as starting point for additional hit finding, lead optimization and rational drug design. M: metabolite and P: protein. A graphical item in the figure about next generation sequencing was adopted from another publication [23].

2. Genomics

Forward genetics generally involve the identification of the gene(s) responsible for a resistant phenotype by performing a comparative study between a resistant line and its parent wild type [24]. For MoA/MoR studies of new chemical entities, an untargeted approach by next generation sequencing (NGS) is mostly used (Table 1) (reviewed for *Leishmania* by Leprohon et al. 2015 [25]). In general, whole genome sequencing (WGS) allows the detection of mutations such as single nucleotide polymorphisms (SNPs), as well as insertions and deletions (Indels), which can lead to resistance when occurring in drug targets or transporters [26–32]. Additionally, copy number variations (CNV) do occur in kinetoplastids and should therefore be incorporated in the analysis of altered drug tolerance [33–39]. CNVs arise as a result of the peculiar mechanism of genetic regulation in kinetoplastids where individual gene promoters are lacking and transcriptional regulation relies on RNA stability, resulting in the development of alternative tools to increase or decrease gene expression [40–43]. This could even lead to whole chromosome copy number variations (CCNV) which have been observed in

laboratory strains of *Leishmania* and *T. cruzi* [43–51]. These structural alterations are particularly observed in in vitro expanded parasites, suggesting that this phenomenon might be a short-term adaptation mechanism to external stress and to an in vitro culture environment [35,46,52]. On the other hand, *T. brucei* does not display karyotype alterations probably due to a different DNA replication and recombination mechanism [43,53]. The use of DNA microarrays, also known as comparative genomic hybridization assays (CGH), has allowed for the successful identification of karyotypic alterations in drug-resistant parasites [23,35,45,54,55]. Resistant strains are generally obtained upon in vitro selection on axenic parasites by stepwise increase of drug pressure. In some cases, mutagenesis prior to selection may generate more resistant mutants [26,56–60]. Selection on axenic stages has the advantage of being simple and relatively fast but lacks biological relevance in *T. cruzi* and *Leishmania* as these are intracellular pathogens in the vertebrate host. Accepting that this may not be a primary issue in MoA studies, it should be kept in mind that confirmation in the intracellular stage will ultimately be needed [61,62]. The downside of experimental resistance selection is that a lot of spontaneous mutations occur because of the high continuous drug pressure or the prior mutagenesis, further complicating the identification of the actual gene(s) responsible for the loss in drug susceptibility [50,63]. The use of multiple parallel resistant strains could help in finding the needle in the haystack by identifying commonly mutated gene(s) [27,32,46,55,56,64,65].

Table 1. Comparative whole genome sequencing (WGS) studies on laboratory-induced resistant strains to unravel the MoA or MoR of anti-kinetoplastid drugs. Experiments with *Leishmania* spp. and *T. cruzi* were performed on promastigotes and epimastigotes, respectively.

Organism	Drug/Compound	Main Relevant Gene(s)/Effect	Reference
<i>L. infantum</i>	AMB	MIL transporter	[66]
	AP	S-adenosylmethionine synthetase	[46]
	MIL *	MIL transporter	[28]
	MIL/PMM ^{CM}	MIL transporter/ Protein kinase CDPK1	[67]
	Sb	Multiple	[68]
	SNF	S-adenosylmethionine transporter	[56]
<i>L. donovani</i>	AMB	24-sterol methyltransferase	[69]
	MIL	Multiple	[69]
	MIL	Multiple	[61]
	MIL	MIL transporter	[70]
	NIO	Nitroreductase 2	[71]
	PMM	D-lactate dehydrogenase like protein Aminotransferase branched chain amino acids	[69]
	PMM	Multiple	[72]
	PZP	Cyclin-dependent kinase 12	[73]
	Sb	Multiple	[69]
<i>L. major</i>	MIL	Miltefosine transporter Pyridoxal kinase α -adaptin like protein	[27]
	Sb	Aquaglyceroporin 1	[55]
<i>L. braziliensis</i>	Sb	Multiple	[50]
<i>L. guyanensis</i>	Sb	Aquaglyceroporin 1	[65]

Table 1. Cont.

Organism	Drug/Compound	Main Relevant Gene(s)/Effect	Reference
<i>L. mexicana</i>	AMB	Sterol 14 α -demethylase	[74]
	AMB	Sterol C24-methyltransferase	[75]
<i>L. panamanensis</i>	Sb	Multiple	[50]
	BOX	Multiple	[76]
<i>T. brucei</i>	FX	Nitroreductase	[63]
	MLP/PTM	Multiple	[64]
	NFX	Nitroreductase	[63]
	OX	Multiple	[30]
<i>T. cruzi</i>	BZN	Mutagenic	[39]
	GNF	Cytochrome B	[32]
	GNF	Proteasome inhibition	[31]
<i>T. congolense</i>	IC ^x	Multiple	[77]

^x Resistance selection was performed in vivo. * Resistance selection was performed in the intracellular amastigote stage. ^{CM} Chemical mutagenesis was used prior to resistance selection. AMB: Amphotericin B, AP: Allopurinol, BOX: Benzoxaborole, BZN: Benznidazole, IC: Isometamidium chloride, FX: Fexinidazole, GNF: GNF-series, MEL: Melarsoprol, MIL: Miltefosine, NFX: Nifurtimox, NH: Nitroheterocyclics, NIO: Nitro-imidazo-oxazine, OX: Oxaborole PMM: Paromomycin, PTM: Pentamidine, PZP: Pyrazolopyrimidine, Sb: Antimonial, SNF: Sinefungin.

3. Transcriptomics

Another technology that has frequently been used to elucidate the MoR in kinetoplastids is comparing the transcriptome of resistant and susceptible parasites (Table 2). Untargeted transcriptomics to compare treated versus untreated parasites was only performed once in *T. cruzi* [78]. However, the evaluation of mRNA transcript abundance in kinetoplastids has mostly been used in fundamental biology studies evaluating changes in expression between the various life cycle stages or to evaluate host-pathogen interactions [79–86]. The measurement of RNA levels can provide a broad range of information, but it should be kept in mind that gene expression in kinetoplastids relies on post-transcriptional regulation. Protein coding genes are transcribed as long polycistronic genes and are further processed into mature mRNAs by trans-splicing and polyadenylation [87,88]. Further post-transcriptional control relies on various mechanisms such as mRNA stability, translation, and degradation [40,89,90], often resulting in a weak correlation between mRNA and protein abundance. This discrepancy is considered one of the main disadvantages of transcriptomics in kinetoplastids [90]. As already mentioned, CNVs on chromosome or gene level are common events in kinetoplastids as one of their tactics to overcome the lack of transcriptional control. This modulation of RNA levels can often be ascribed to gene amplification or deletion events [33,35,91,92]. In some cases, elevated RNA levels could not be linked to CNVs and therefore probably relate to increase in stability [33,35,50,91,93,94]. Microarrays have been used to study the transcriptome but has a number of disadvantages, such as the high initial cost to construct specific chips for one of the kinetoplastids, as well as problems involving cross-hybridization and the inability to distinguish closely related genes. There is also concern about the lack in sensitivity and the possibility to miss low abundant genes [80,95–97]. More recently, high-throughput cDNA sequencing technology, also known as RNA-seq, has been developed [98]. This technique is fast, reliable, and allows the accurate quantification of transcript levels with high reproducibility and little technical variability [80,99]. In addition, RNA-seq provides a greater dynamic range compared to classical microarrays, thereby yielding more accurate information on the relative levels of transcription for genes that are expressed at very low or very high levels. Drawbacks are similar as those for WGS and include high cost, complex data analysis, and the need for adequate read depth [98,100,101]. To conclude, transcriptomics has increased the understanding of transcriptional

control in kinetoplasts and can be an informative tool in resistance studies by identifying differentially expressed genes that could play a role in the decreased susceptibility.

Table 2. Use of untargeted transcriptomics to unravel the MoR of drugs with anti-kinetoplastid effect by comparing resistant and susceptible parasites. Experiments with *Leishmania* spp. and *T. cruzi* were performed on promastigotes and epimastigotes, respectively.

Organism	Drug/Compound	Technique	Main Altered Expression	Reference
<i>L. infantum</i>	MTX	Whole genome microarray	Dihydrofolate reductase-thymidylate synthase ABCA ₂	[35]
	Sb	Whole genome microarray	Multidrug resistance protein A	[33]
	AMB	RNA-seq	24-sterol methyltransferase	[69]
	MIL	Whole genome microarray	Multiple	[102]
	MIL	RNA-seq	Multiple	[61]
	MIL	RNA-seq	Multiple	[69]
	NFV °	Whole genome microarray	ABC transporters	[23]
<i>L. donovani</i>	PMM	RNA-seq	D-lactate dehydrogenase like protein Aminotransferase branched chain amino acids	[69]
	Sb #	Whole genome microarray	Histones Mitogen-activated protein kinase 1	[103]
	Sb #	Genome microarray	ABC transporters	[97]
	Sb	RNA-seq	Multiple	[69]
<i>L. major</i>	MTX	Whole genome microarray	Dihydrofolate reductase-thymidylate synthase ABCA ₂	[35]
<i>L. braziliensis</i>	Sb	RNA-seq	Multiple	[50]
<i>L. mexicana</i>	AMB	RNA-seq	Sterol C24-methyltransferase	[75]
<i>L. panamensis</i>	Sb	RNA-seq	Multiple	[50]
<i>L. amazonensis</i>	Sb	Whole genome microarray	Multidrug resistance protein A Thiol metabolism	[94]
<i>T. brucei</i>	MLP/PTM	RNA-seq	Multiple	[64]
	SR	RNA-seq	Variant surface glycoprotein	[104]
<i>T. cruzi</i>	BZN #	RNA-seq	Adenine phosphoribosyltransferase	[105]
	BZN #	Genome microarray	ABCG ₁ transporter	[106]
	BZN x#	Genome microarray	Old yellow enzyme	[107]

^x Resistance selection in vitro and in vivo. ^o Resistance selection on axenic amastigotes. [#] Use of clinical resistant isolates. AMB: Amphotericin B, BZN: Benznidazole, MIL: Miltefosine, MLP: Melarsoprol, MTX: Methotrexate, NFV: Nelfinavir, PMM: Paromomycin, PTM: Pentamidine, Sb: Antimonial, SR: Suramin.

4. Genome-Wide Expression Studies

The use of genome-wide overexpression or knockdown studies allows the identification of genes that may be involved in the MoA/MoR of drugs without the need of a time-consuming resistance selection process (Table 3). In general, genome wide knockdown studies will allow the identification of genes involved in drug uptake or activation, while overexpression studies identify the protein-binding target itself or genes that are involved in drug efflux or detoxification [108–110]. A cosmid-based gain-of-function screen has been developed in *Leishmania* and has recently been combined with NGS, also known as Cos-seq, allowing a full and unbiased view of the enriched genes [108]. Multidrug resistance associated protein A (MRPA), antimony (Sb) resistance marker

58 (ARM58), and heat shock proteins (HSPs) are a few examples of genes that have been identified when exposing cosmid libraries to Sb [108,111–113]. Additionally, this technique has confirmed N-myristoyltransferase as a drug target for pyrazolyl sulfonamide (PS) [114]. In some cases, cosmid libraries are transfected in resistant lines to identify the dominant negative mutations that underlie the resistant phenotype [26,115]. Yet another study combined the use of both susceptible and resistant strains to prepare and transfect the cosmid libraries [111]. All these studies have used the extracellular promastigote stage as it is known to be easier and faster. Only two studies opted to use intracellular amastigotes for the selection of cosmid bearing parasites [113,116], resulting in the identification of predominantly different genes compared to those identified in promastigotes [116]. These differences could be caused by a number of factors, not necessarily directly related to the drug, such as the host cell environment, parasite infectivity and intracellular multiplication [116]. For *T. brucei*, a tetracycline inducible overexpression library was developed and was able to confirm the drug target for difluoromethylornithine (DFMO), a N-myristoyltransferase inhibitor (N-MT), and even a novel benzoxaborole (BOX) [110,117]. Even though this strategy is promising, most of the genome wide expression screens in *T. brucei* are RNA interference (RNAi)-mediated knockdown studies. The use of these loss-of-function screens has been validated by using current anti-trypanosomal drugs all leading to the identification of known drug transporters or activators, such as amino acid transporter 6 (AAT6) for eflornithine (EFL), and nitro-reductase (NTR) for benznidazole (BZN) and nifurtimox (NFX) [109,118,119]. High-throughput detection of the phenotypes by NGS has been employed and is especially useful when more than a few targets are expected to play a role [120]. This technique is referred to as RNA interference target sequencing, also known as RIT-seq. Sadly, whole genome RNAi mediated knockdown studies are not available for *T. cruzi* and *Leishmania* spp. since they lack the genes encoding for the RNAi machinery, with the exception of the *L. viannia* subgenus [121]. For these species, the CRISPR/Cas9 technology could be an alternative tool to generate a loss-of-function library [122,123]. Some drawbacks of genome-wide expression studies are the inability to identify non-protein targets or drugs with complex multifactorial effects [110]. Knockdown of essential genes can also result in cell death reducing the chances of identifying such key genes in a RNAi screen [124]. At last, it should be taken into consideration that selection may favor parasites with a faster growth rate or increased infectivity [116,125]. To avoid false negative results, a good genome coverage is crucial and should be evaluated prior to selection [120].

Table 3. Genome-wide expression studies to unravel the MoR or MoA of anti-kinetoplastid drugs. Experiments with *Leishmania* spp. and *T. cruzi* were performed on promastigotes and epimastigotes, respectively.

Organism	Drug/Compound	Technique	Main Identified Genes	Reference
<i>L. infantum</i>	AMB/MIL/MTX/ PMM/PTM/Sb	Cos-seq	Multiple	[108]
	AMB/MIL/Sb *	Cos-seq	Multiple	[116]
	MIL	Cosmid library	Unannotated	[126]
	Sb §	Cos-seq	Heat shock proteins Antimony resistance marker	[113]
	SNF	Cos-seq	Adomet synthetase	[56]
<i>L. donovani</i>	BZF	Cos-seq	None	[127]
	MIL	^R Cosmid library	MIL Transporter	[26]
	PS	Cos-seq	N-Myristoyltransferase	[114]
<i>L. major</i>	MTX	Cosmid library	Dihydrofolate reductase-thymidylate synthase Pteridine reductase 1	[128]
	PTM	Cosmid library	P-glycoprotein	[129,130]
	TUB	Cosmid library	Toxic nucleoside resistance	[128]

Table 3. Cont.

Organism	Drug/Compound	Technique	Main Identified Genes	Reference
<i>L. braziliensis</i> # <i>L. peruviana</i>	Sb	^{R/S} Cosmid library	Antimony resistance marker 58	[111]
<i>L. tarentolae</i>	MTX	Cosmid library	Biopterin and folic acid transporter	[131]
	Sb	Cosmid library	Heat shock proteins	[112]
	Sb	^R Cosmid library	Aquaglyceroporin 1	[115]
<i>T. brucei</i>	AMB/MIL/PMM/Sb	RIT-seq	Multiple	[132]
	BOX	RIT-seq	Aldehyde dehydrogenase 3	[133]
	BOX	Overexpression library	Cleavage and polyadenylation specificity factor 3	[117]
	CHA	RNAi library	Mitochondrial carrier protein family 14	[134]
	DFMO	Overexpression library	Ornithine decarboxylase	[110]
	dTUB	RNAi library	P ₂ nucleoside transporter Adenosine kinase	[135]
	EFL/MLP	RNAi library	Amino acid transporter 6/Adenosine transporter 1	[119]
	EFL/MLP/NFX/PTM/SR	RIT-seq	Multiple	[109]
	EFL/NFX	RNAi library	Amino acid transporter 6/Nitroreductase	[118]
	MTX/RX	RIT-seq	Folate transporter	[136]
N-MT	Overexpression library	N-myristoyltransferase	[110,117]	
PDE	RNAi library	cAMP response proteins	[60]	
TUB	RNAi library	Hexose transporter Hexokinase	[137]	

Use of clinical resistant isolates * Intracellular amastigote selection. § Both promastigote and intracellular amastigote selection. ^R Transfection was performed in resistant strains. ^{R/S} Transfection was performed in both resistant and susceptible strains. AMB: Amphotericin B, ARS: Arsenite, BZF: Benzothiope-phenol, BOX: Benzoxaborole, CHA: Choline analog, DFMO: Difluoromethylornithine, dTUB: 3'-deoxytubercidin, EFL: Eflornithine, MIL: Miltefosine, MLP: Melarsoprol, MTX: Methotrexate, NFX: Nifurtimox, N-MT: N-myristoyltransferase inhibitor, PDE: Phosphodiesterase inhibitor, PMM: Paromomycin, PS: Pyrazolyl sulfonamide, PTM: Pentamidine, RX: Raltitrexed, Sb: Antimonial, SNF: Sinefungin, SR: Suramin, TUB: Tubercidin.

5. Proteomics

In kinetoplastids, proteomics have been used to understand the differentiation processes in the various life cycle stages, to identify biomarkers, to support vaccine development, as well as to elucidate the MoA/MoR of drugs (Table 4) [85,138–141]. Several techniques can be used to compare the relative protein expression, but most employ labelling techniques such as 2D differential gel electrophoresis (2D-DIGE), stable isotope labeling of amino acids in cell culture (SILAC), or isobaric tags for relative and absolute quantitation (iTRAQ) [18,142]. Disadvantages of these labelling techniques are their high cost, the need of large amounts of protein fractions, the tedious sample preparation, and the high complexity of the acquired MS data [143,144]. An alternative is the use of label-free quantitative proteomics where relative protein abundance is determined by comparing peak intensities or number of tandem spectra between separately obtained LC-MS/MS runs [143,145,146]. 2D-DIGE is the mostly used technique in the study of MoR/MoA in kinetoplastids but is known to predominantly detect soluble and abundant proteins [139,146,147]. A way to evaluate a broader spectrum of the proteome is to additionally extract proteins from various specific cellular compartments or the use of differential extraction [138,139,148,149], such as the separate isolation of membrane-enriched and cytosolic proteins or the isolation of phosphoproteins, mitochondrial components, or the analysis of the secretome [68,150–156]. Most studies employing proteomics, especially in *Leishmania*, compared sensitive to resistant strains to elucidate the MoR of drugs. In contrast, most studies in *T. cruzi* and

T. brucei compared treated and untreated samples to evaluate the MoA, resulting in a large number of possibly involved proteins that need further mining and follow-up studies to confirm their actual involvement [79,85]. The extracellular parasite stages are mostly used in these proteomics studies, which is not ideal as the proteome may differ depending on the life cycle stage [157–160]. Some of the technological hurdles include the identification of proteins that are still unannotated [138,161] and the identification of proteins that could be differentially expressed purely due to stress response and not due to the resistant phenotype or drug exposure [112,150,162,163].

Table 4. Use of untargeted proteomics to unravel the MoA or MoR of anti-kinetoplastid drugs. Experiments with *Leishmania* spp. and *T. cruzi* were performed on promastigotes and epimastigotes, respectively. T/UT: comparison between treated and untreated parasites. R/S: comparison between resistant and susceptible parasites.

Organism	Drug/Compound	Comparison	Extracted Proteome	Sample Preparation Prior to LC-MS/MS	Main Identified Protein(s)/Affected Pathways	Reference
<i>L. infantum</i>	AMB	R/S	Cytosolic and membrane enriched proteins	SILAC, SDS-PAGE	Glycolysis Tricarboxylic acid cycle	[150]
	MIL #	R/S	Whole parasite	2D-DIGE	Redox homeostasis Stress response	[164]
	MIL/Sb	R/S	Mitochondrial proteins	2D-DIGE	Pyruvate dehydrogenase Succinyl-CoA:3-ketoacid-coenzyme A transferase	[155]
	Sb	R/S	Cytosolic and membrane enriched proteins	SILAC, SDS-PAGE	Multidrug resistance protein A	[68]
	Sb °	R/S	Whole parasite	2D-DIGE	Argininosuccinate synthetase Kinetoplastid membrane protein 11	[165]
	Sb	R/S	Whole parasite	2D-DIGE	Antioxidant defense Stress response	[163]
<i>L. donovani</i>	AMB #	R/S	Secretome	SDS-PAGE	Carbohydrate metabolism Stress response	[154]
	ARS	R/S	Whole parasite	2D-DIGE	Multiple	[166]
	α-DFMO	R/S	Whole parasite	iTRAQ, SCX chromatography	Free radical detoxification Polyamine and trypanothione metabolic proteins	[167]
	MIL #	R/S	Whole parasite	2D-DIGE	Iron superoxide dismutase	[168]
	MIL	R/S	Whole parasite	2D-DIGE	Eukaryotic initiation factor 4A	[169]
	NIO	R/S	Whole parasite	SILAC, SDS-PAGE	Nitroreductase 2	[71]
	PMM	R/S	Whole parasite	SILAC, SDS-PAGE	Translation and vesicle-mediated trafficking	[170]
	Sb #	R/S	Whole parasite	2D-DIGE	Heat shock protein 83 Small kinetoplastid calpain-related protein	[171]
	Sb #	R/S	Whole parasite	iTRAQ, SCX chromatography	Glycolysis Stress response	[172]
	Sb #	R/S	Cytosolic and membrane enriched proteins	2D-DIGE	Heat shock proteins ABC transporter	[151]
	STQ	R/S + T	Whole parasite and cell fractionation	2D-DIGE	Multiple	[173]
<i>L. major</i>	MTX	R/S	Whole parasite	2D-DIGE	Pteridine reductase 1	[162]
	Sb #	R/S	Whole parasite	2D-DIGE	Multiple	[174]

Table 4. Cont.

Organism	Drug/Compound	Comparison	Extracted Proteome	Sample Preparation Prior to LC-MS/MS	Main Identified Protein(s)/Affected Pathways	Reference
<i>L. braziliensis</i>	Sb	R/S	Whole parasite	2D-DIGE	Antioxidant defense Stress response	[163]
	Sb	R/S T/UT	Phospho-proteins	2D-DIGE	Stress response Antioxidant defense	[153]
<i>L. panamensis</i>	Sb	R/S	Whole parasite and membrane enriched proteins	2D-DIGE	Stress response Metabolic and transport functions	[175]
<i>L. tropica</i>	Sb [#]	R/S	Whole parasite	2D-DIGE	Activated protein kinase c receptor Prostaglandin f2-alpha synthase	[176]
	Sb [#]	R/S	Mitochondrial proteins	SDS-PAGE	Multiple	[156]
<i>T. brucei</i>	CYM	R/S	Whole parasite	2D-DIGE	Putative nascent polypeptide associated complex subunit	[177]
	LPB	T/UT	Phospho-proteins	SILAC, IMAC	Vesicle transport Gene expression	[152]
	PP	T/UT	Whole parasite	IMAC	Flagellar pocket protein BILBO-1 Tb14-3-3-associated protein kinase	[178]
	SR	T/UT	Whole parasite	SILAC, SDS-PAGE	Multiple	[179]
	BZN [§]	R/S	Whole parasite	2D-DIGE	Transcription and protein destination	[180]
<i>T. cruzi</i>	NIZ	T/UT	Whole parasite	2D-DIGE	Activated protein kinase C receptor Asparagine synthetase	[181]
	NIZ	T/UT	Whole parasite	2D-DIGE	Redox metabolism Energy production	[182]
	PIP	T/UT	Whole parasite	2D-DIGE	Tryparedoxin peroxidase Methionine sulfoxide reductase	[183]
	VDM	T/UT	Whole parasite	SDS-PAGE	Multiple	[78]

[°] Experiment used axenic amastigotes. [#] Clinical resistant samples were used instead of laboratory induced resistance. [§] Both in vitro and in vivo selected resistant strains were used. IMAC: FeCl₃-charged metal affinity chromatography. AMB: Amphotericin B, ARS: Arsenite, BZN: Benznidazole, CYM: Cymelarsan, α -DFMO: DL- α -Difluoromethylornithine, LPB: Lapatinib, MIL: Miltefosine, MTX: Methotrexate, NIO: Nitro-imidazo-oxazine, NIZ: Naphtoimidazole, PIP: Piplartine, PMM: Paromomycin, PP: Pyrrolopyrimidine. Sb: Antimonial, SR: Suramin, STQ: Sitamiquine, VDM: Vanadium analogue.

6. Chemical Proteomics

Another way to analyze the proteome is to use affinity-based techniques where protein targets can be identified by the direct interaction with the compound/drug of interest [18,19,142,184–186]. This technology shows added value in the identification of the possible drug target(s) in kinetoplastids, especially for *T. brucei* (Table 5). Affinity techniques usually entail the need to chemically modify the small molecules to enable immobilization on a matrix, a process that can be lengthy and tedious and is not always fail-proof as immobilization of the drug may impede or alter target binding [18,142]. Non-specifically bound proteins are often detected but this can be avoided by the inclusion of appropriate experimental controls, e.g., using an inactive analogue bound to the matrix [30,142,187–189] or the use of free compounds in a competition experiment [30,73,187,190–192]. In some cases, SILAC has been employed to analyze these differential eluates to increase sensitivity and specificity, and to reduce technical variation [30,73,193]. An alternative to the standard affinity chromatography for the elucidation of drug targets is the addition of chemo- or photoreactive groups on small molecules to enable covalent binding to the target protein [191,192,194–196]. An added advantage of this technique is the possibility to incubate the probes in live cells instead of protein lysates [192,195,197]. When comparing differential proteomics (discussed above) with affinity-based techniques, the latter

provides a more concise list of putative drug targets showing direct interaction with the small molecule. However, compounds with a low affinity to their target may be missed and the need of chemical modification is a hurdle and cannot always be applied [18]. A potential alternative is the use of label-free proteomics, such as drug affinity response target stability (DARTS), thermal protein profiling (TPP), or stability of proteins from rate of oxidation (SPROX), which are based on the idea that small molecules stabilize the target protein and therefore protect from respectively proteolysis, heat-denaturation or oxidation [185,198–202]. The use of TPP has recently confirmed N-myristoyltransferase as a drug target in *Leishmania* [114].

Table 5. Use of chemical proteomics to unravel the MoA of anti-kinetoplastid drugs. Experiments with *Leishmania* spp. and *T. cruzi* were performed on promastigotes and epimastigotes, respectively.

Organism	Drug/Compound	Technique	Main Identified Target(s)	Reference
<i>L. donovani</i>	PMM	Affinity chromatography	Paraflagellar rod proteins Prohibitin	[170]
	PZP	SILAC and Affinity chromatography	Cdc-2-related kinases Cyclins	[73]
<i>L. major</i>	dAP	Affinity chromatography	Mitogen-activated protein kinases Cdc2-related kinases	[190]
<i>L. mexicana</i>	PVB	Affinity chromatography	Cyclin-dependent kinase Casein kinase 1	[189]
	ARM	Photo-affinity labelling	X	[191,203]
	AZN	Activity-based probes	Cathepsin B-like protease	[195]
	B-THP-T	Photo-affinity labelling	F ₀ F ₁ -ATP synthase	[197]
	CYM	Affinity chromatography	Glycerol-3-phosphate dehydrogenase	[204]
<i>T. brucei</i>	dAP	Affinity chromatography	Mitogen-activated protein kinases Cdc2-related kinases	[190]
	MPH	Affinity chromatography	Adenosine kinase	[188]
	NQ	Affinity chromatography	Polypharmacology	[205]
	OX	SILAC and Affinity chromatography	Polypharmacology	[30]
	VS	Activity-based probes	Cysteine peptidase precursor Cathepsin B-like protease	[192]
<i>T. cruzi</i>	BZN	Affinity chromatography	Aldoketo reductase Protein transport protein Sec23A	[206]
	PVB	Affinity chromatography	Cyclin-dependent kinase Casein kinase 1	[189]

X: no identification step performed. ARM: Artemisinin, AZN: Azadipeptide nitrile, B-THP-T: Bis-tetrahydropyran 1,4-triazole, BZN: Benzimidazole, CYM: Cymelarsan, dAP: 2,4-Diaminopyrimidine, MPH: 4-[5-(4-phenoxyphenyl)-2H-pyrazol-3-yl]morpholine, NQ: Naphtoquinone derivatives, OX: Oxaborole, PMM: Paromomycin, PVB: Purvalanol B, PZP: Pyrazolopyrimidine, VS: Vinyl sulfone.

7. Metabolomics

Metabolomics enables the identification and quantification of many metabolites within a biological system as downstream product of gene, protein, and environmental interactions. As such, the metabolome can reflect the organism's or cell's physiological state and is closest to the phenotype [92,207]. Not only does this technique allow for the discovery or fine tuning of molecular mechanisms, it also allows for the identification of pathways involved in a drug's

MoA since drugs that inhibit enzymes cause alterations in the level of their substrates and products [18,62,208]. The experimental set-up is quite straightforward, where cells are grown in culture with and without drug and cell extracts are analyzed using mass spectrometry or nuclear magnetic resonance [62,161,209,210]. The drug exposure (duration and intensity) should be well chosen to allow the distinction between drug-specific effects and non-specific metabolic changes due to stress or secondary responses. Separation of the cellular extracts can be performed by liquid/gas chromatography or capillary electrophoresis, which are mostly complementary, to reduce complexity of the sample [62,161,209,211–214]. Metabolomics can provide an unbiased method to discover MoA pathways by comparing treated and untreated cell lines (Table 6) (reviewed by Creek et al. and Vincent et al. [62,215]). Alternatively, the comparison between susceptible and resistant strains can allow investigation of the MoR [66,70,72,74,211,216–221]. The primary challenge of the technique lays in the data deconvolution and in the identification of metabolites, as the kinetoplastid metabolomes have not yet fully been elucidated [215]. Even so, new pathways can be discovered, and compounds can still be classified based on their metabolic fingerprint [222–224].

Table 6. Application of untargeted metabolomics to unravel the MoA of anti-kinetoplastid drugs. All references in this table compared the metabolome between treated and untreated samples. Experiments performed with *Leishmania* were performed on promastigotes unless mentioned otherwise.

Organism	Drug/Compound	Main Affected Pathway(s)	Reference
<i>L. infantum</i>	AN	Pentose Phosphate pathway Preiss-handler salvage pathway	[225]
	MIL	Internal lipid metabolism	[217]
	Sb	Oxidative stress	[216]
	Sb	Redox metabolism Amino acid metabolism	[211]
	SRT	Polyamine and trypanothione biosynthesis Tricarboxylic acid cycle Phospholipid and sphingolipid metabolism	[226]
<i>L. donovani</i>	MIL °	Sphingolipids and sterol metabolism	[212]
	MIL	Polyamine metabolism from arginine to trypanothione (ROS)	[213]
<i>L. major</i>	FU/FdU	Deoxynucleotide metabolism	[227]
	MIL	Sphingolipids and sterol metabolism	[212]
<i>L. mexicana</i>	AN	Pentose Phosphate pathway Preiss-handler salvage pathway	[225]
	BS °	Deoxynucleotide metabolism Oxidative stress	[228]
	FU/FdU	Deoxynucleotide metabolism	[227]
<i>L. amazonensis</i>	LPV	Sterol metabolism	[229]
<i>T. brucei</i>	BOX	Methionine metabolism	[223]
	BOX	S-adenosyl methionine Methylation	[76]
	EFL	Polyamine pathway	[230]

Table 6. Cont.

Organism	Drug/Compound	Main Affected Pathway(s)	Reference
<i>T. brucei</i>	FdU	Thymidylate synthase	[221]
	FOA/FU	RNA modification	[221]
	SNF	S-adenosyl methionine 5'-methylthioadenosine	[223]
	NFX	Carbohydrate and nucleotide metabolism	[230]
<i>T. cruzi</i>	BZN	Thiol metabolism	[231]

^o Use of axenic amastigotes. AN: 6-aminonicotinamide, BOX: Benzoxaborole, BS: Bisabolane sesquiterpene, BZN: Benznidazole, EFL: Eflornithine, FdU: 5-fluoro-2'-deoxyuridine, FOA: 5-fluoroorotic acid, FU: 5-fluorouracil, LPV: Lopinavir, MIL: Miltefosine, NFX: Nifurtimox, Sb: Antimonial, SNF: Sinefungin, SRT: Sertraline.

8. Conclusions

Phenotypic screening has allowed the identification of new or repurposed chemical entities that display promising anti-kinetoplastid activity [14–17]. This cell-based strategy, however, does not give adequate insights into the MoA, which is useful for further compound optimization and overall success in drug development [14,18,19]. ‘Omics’ approaches offer tools to identify drug targets in an untargeted manner in an either direct (affinity) or indirect approach, resulting in the generation of a (long) list of potential target candidates. Although this may seem to be a disadvantage, combining results of complementary techniques permits fine-tuning of acquired data [18,30,56,114,232]. Target validation by either chemical or genetic means can further confirm a potential role in the MoA [19,120,233]. Importantly, it should be noted that such an undertaking often represents a first step in drug target validation and that additional experiments (such as enzymatic, biophysical and structural studies of the target-compound complex) plays a significant role in paving the road toward future optimization of the molecule by rational drug design. One major hurdle using ‘omics’ techniques is the functional annotation of genes, proteins and metabolites, since they are not always available for kinetoplastids [138,161,215]. Elucidation of the MoA is mostly based on the comparison between treated and untreated parasites or between resistant and susceptible strains. The latter will predominantly identify genes/proteins that are related to the MoR without necessarily providing information about the MoA. On the other hand, when comparing treated to untreated parasites, it is important to distinguish stress-related factors to the real drug effects by using adequate controls or an adapted drug concentration and exposure duration. Lastly, the kinetoplastids have multiple life cycle stages whereby different results can be obtained depending on the used parasite stage or form [116,192,234]. This should be kept in mind and additional confirmation in the host-relevant stage may be needed. In conclusion, a wide variety of complementary ‘omics’ tools are available for studies inquiring into drug actions and resistance mechanisms. While not always straightforward, their combination may enable novel target identification and trigger rational drug design.

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