Metabolomics (2022) 18:20
https://doi.org/10.1007/s11306-022-01880-0

REVIEW ARTICLE

Trypanosoma brucei: Metabolomics for analysis of cellular metabolism and drug discovery

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Received: 22 October 2021 / Accepted: 12 March 2022
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Abstract

Background *Trypanosoma brucei* is the causative agent of Human African Trypanosomiasis (also known as sleeping sickness), a disease causing serious neurological disorders and fatal if left untreated. Due to its lethal pathogenicity, a variety of treatments have been developed over the years, but which have some important limitations such as acute toxicity and parasite resistance. Metabolomics is an innovative tool used to better understand the parasite's cellular metabolism, and identify new potential targets, modes of action and resistance mechanisms. The metabolomic approach is mainly associated with robust analytical techniques, such as NMR and Mass Spectrometry. Applying these tools to the trypanosome parasite is, thus, useful for providing new insights into the sleeping sickness pathology and guidance towards innovative treatments.

Aim of review The present review aims to comprehensively describe the *T. brucei* biology and identify targets for new or commercialized antitrypanosomal drugs. Recent metabolomic applications to provide a deeper knowledge about the mechanisms of action of drugs or potential drugs against *T. brucei* are highlighted. Additionally, the advantages of metabolomics, alone or combined with other methods, are discussed.

Key scientific concepts of review Compared to other parasites, only few studies employing metabolomics have to date been reported on *Trypanosoma brucei*. Published metabolic studies, treatments and modes of action are discussed. The main interest is to evaluate the metabolomics contribution to the understanding of *T. brucei*'s metabolism.

Keywords Metabolomics · Anti-trypanosomal activity · *Trypanosoma brucei* · Mass spectrometry · Nuclear magnetic resonance

1 Introduction

African trypanosomiasis is a vector-borne disease caused by *Trypanosoma brucei* (*T.b.*), a parasitic protist transmitted by the bite of a tsetse fly. The *T.b.* species is divided into 3 subspecies: *T. b. gambiense*, accounting for an often chronic form of Human African Trypanosomiasis (HAT) in Central and Western Africa; *T.b. rhodesiense*, responsible for the acute HAT in Eastern and Southern Africa; and *T. b. brucei,* which infects domestic and wild animals. *T. b. gambiense* and *T.b. rhodesiense* account for 98% and 2% of reported human trypanosomiasis cases, respectively ("WHO | Human African trypanosomiasis," n.d.). In 2019, less than 1000 cases of HAT have been reported with approximately 2.5 million people screened per year ("WHO | Human African trypanosomiasis," n.d.).

T. brucei is an extracellular parasite that undergoes several developmental transformations both in the mammalian

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Published online: 19 March 2022

host's blood and in different compartments of the tsetse fly. Morphological and cellular differentiation events occur depending on the environment where the parasite is located (Matthews, 2005). The mammalian bloodstream form (BSF) is characterized by two stages named "long slender" and "short stumpy" (Rico et al., 2013). The slender form predominates when parasitemia increases. The long slender and the short stumpy forms are covered with a layer of Variant Surface Glycoproteins (VSG) that allows the parasite to evade the host immune system by antigenic variation and, eventually, survive. Progressively, the non-proliferating short stumpy form replaces the long slender trypanosomes resulting in the next phase of the life cycle (Nolan et al., 2000). This stumpy form is pre-adapted to survive in the insect vector. When the parasite is ingested by the tsetse fly during a blood meal, the intestinal conditions allow its morphological change into the procyclic form, followed by the epimastigote form and, finally, the infective metacyclic form in the vector's salivary glands (Fig. 1) (Hannaert, 2011; Matthews, 2005; Stijlemans et al., 2017). In the vector, high levels of proline are found in the hemolymph, digestive tract and salivary glands. Notably, the BSF uses glucose as its main source of ATP (Bakker et al., 1999; Matthews, 2005; Rico et al., 2013). Most of the glucose is converted into pyruvate through the glycolytic pathway, and into acetate, succinate and alanine to a lesser extent (Fig. 2). Noteworthy, most enzymes involved in glycolysis and some other core metabolic processes of trypanosomes and other Kinetoplastea protists, are sequestered in peroxisome-related organelles called glycosomes (Gualdrón-López et al., 2012). Contrarily, in the procyclic form, glucose is mainly converted into acetate and succinate, and to a lesser extent into alanine, pyruvate and lactate (Michels et al., 2021; Villafraz et al., 2021). Between blood meals

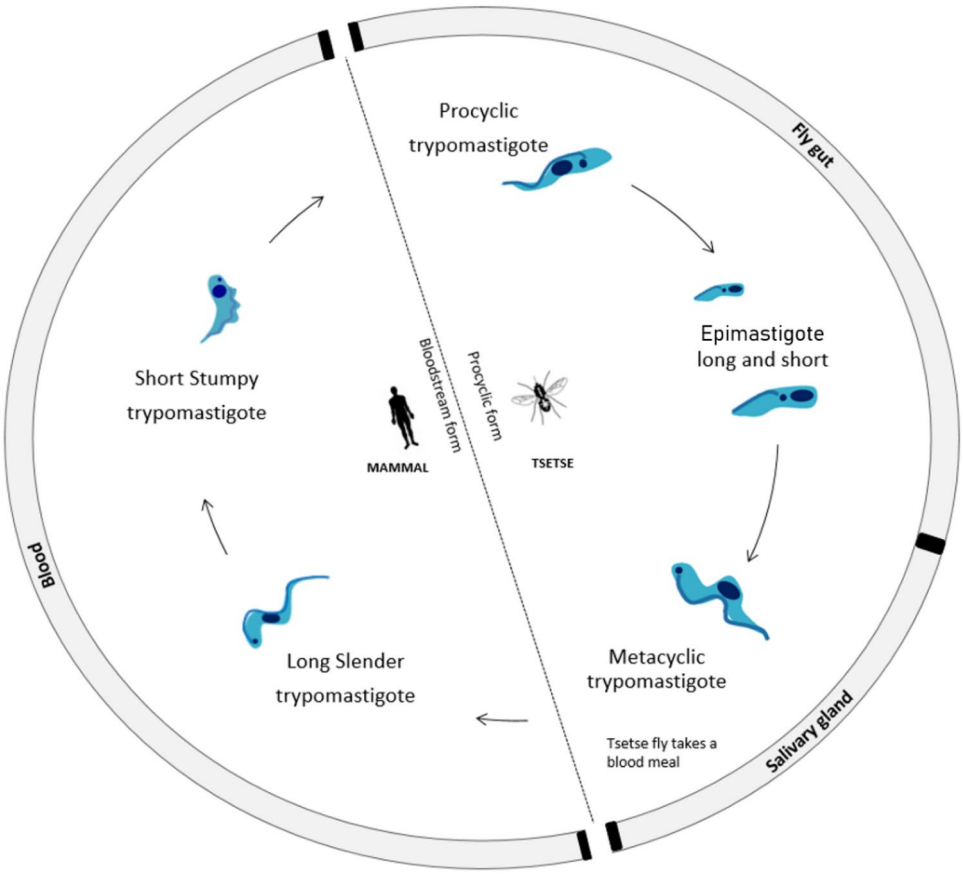


Fig. 1 Life cycle of African trypanosomes. Upon the bite by a tsetse fly infected with *Trypanosoma brucei*, metacyclic parasites (trypomastigotes) are transmitted to the mammalian host. These undergo then, in the bloodstream, differentiation into long slender trypomastigotes expressing a specific VSG layer at the surface that, by antigenic variation, enables them to escape the mammalian immune response. The long slender form trypomastigotes transform into short stumpy, non-dividing forms to preadapt for survival after transmission to the tsetse fly. After ingestion by tsetse, they are transformed, within the midgut, into procyclic forms without the VSG layer, which is replaced by a layer composed of the glutamate and proline rich surface proteins called EP and GPEET procyclins. They then migrate to the salivary gland where they metamorphose into the proliferative epimastigote forms and subsequently into the non-proliferative metacyclic forms with formation of a VSG layer, after which transmission to a new mammalian host is possible

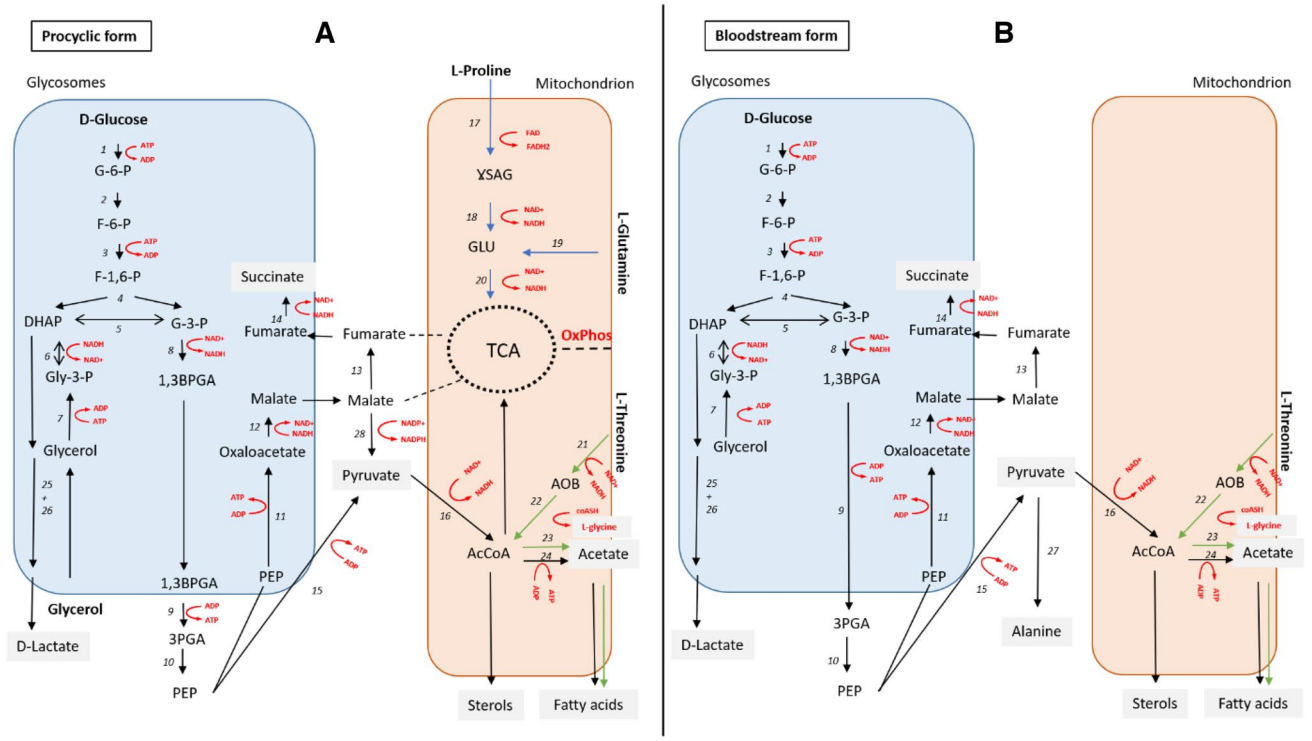


Fig. 2 **A** Schematic representation of the energy metabolism of D-glucose, L-proline, L-glutamine and L-threonine in the procyclic form. **B** Schematic representation of the energy metabolism of D-glucose and L-threonine in the bloodstream form. In blue L-proline and glutamine metabolism, in green threonine metabolism and in black glucose metabolism. This scheme implemented the most recently reported findings (Villafraz et al., 2021). G-6-P glucose 6-phosphate, F-6-P fructose 6-phosphate, F-1,6-P fructose 1,6-bisphosphate, G-3-P glyceraldehyde 3-phosphate, DHAP dihydroxyacetone phosphate, BPGA bisphosphoglycerate, Gly-3-P glycerol 3-phosphate, PGA phosphoglycerate, PEP phosphoenolpyruvate, ɣSAG glutamate γ-semialdehyde, GLU glutamate, TCA tricarboxylic acid cycle, AcCoA acetyl-CoA, AOB amino oxobutyrate, OxPhos oxidative phosphorylation. Enzymes 1: hexokinase; 2: glucose-6-phosphate isomerase; 3: phosphofructokinase; 4: aldolase; 5: triose-phosphate isomerase; 6: glycerol-3-phosphate dehydrogenase; 7: glycerol kinase; 8: glyceraldehyde-3-phosphate dehydrogenase; 9: phosphoglycerate kinase (cytosolic in the procyclic form, glycosomal in the bloodstream form); 10: phosphoglycerate mutase-enolase; 11: phosphoenolpyruvate carboxykinase; 12: glycosomal malate dehydrogenase; 13: cytosolic fumarase; 14: glycosomal fumarate reductase; 15: pyruvate phosphate dikinase; 16: pyruvate dehydrogenase; 17: proline dehydrogenase; 18: pyrroline-5 carboxylate dehydrogenase; 19: glutamine deaminase; 20: glutamate aminotransferase; 21: threonine dehydrogenase; 22: acetyl-CoA:glycine C-acetyltransferase; 23: acetate:succinate CoA transferase; 24: acetyl-CoA thioesterase; 25: methylglyoxal reductase; 26, lactaldehyde dehydrogenase; 27: alanine aminotransferase. 28: cytosolic malic enzyme

by the infected tsetse fly or, in vitro, when the environment is glucose depleted, the procyclic form metabolizes proline and uses it as its main energy source (Hendriks et al., 2000; Lamour et al., 2005). When cultured in vitro, the procyclic form preferentially metabolizes glucose, but in its absence proline catabolism increases (Lamour et al., 2005). Excreted end-products of its glucose catabolism (pyruvate, succinate, alanine, malate, α-ketoglutarate) can be taken up again and further metabolized, irrespective of the presence or absence of proline (1–2 mM). However, both the bloodstream and procyclic forms do not further catabolize acetate which is excreted by the parasite as final product or used for fatty acid biosynthesis (Villafraz et al., 2021). Depending upon the environmental conditions, the procyclic form can use other energy sources such as tricarboxylic acid (TCA) cycle intermediates (Villafraz et al., 2021; Weelden et al., 2005) or amino acids. In the latter case, threonine is converted into glycine and acetyl-CoA by threonine dehydrogenase and acetyl-CoA:glycine C-acetyltransferase, while acetyl-CoA is further converted into acetate (Linstead et al., 1977) (Fig. 2).

T. b. gambiense and *T. b. rhodesiense* mammalian infections develop in two stages. The first stage is the haemo-lymphatic stage in which the parasites multiply in the blood and lymphatic systems. This phase has mostly general symptoms (fever, headaches, joint pain, etc.) which complicate accurate diagnosis. In the absence of an effective treatment, the infection develops into the second stage named meningoencephalic phase, where the parasites penetrate the blood–brain barrier. This stage is characterized by severe neurological symptoms and sleep disturbances—giving it the characteristic name of "sleeping sickness"—that eventually lead to death if untreated (Deeks, 2019; Stoessel et al., 2016).

Research performed throughout recent years has shown that *T. brucei* can also be found extracellularly, outside the blood circulation, in organs as the adipose tissue (Crilly & Mugnier, 2021; Trindade et al., 2016). However, little is currently known about the metabolism of the parasites residing in brain and adipose tissue.

Depending on the stage of the disease, a set of six treatments (Fig. 3) is recommended by the WHO ("WHO | Human African trypanosomiasis," n.d.). For the early stages of the disease pentamidine and suramin are recommended for *T. b. gambiense* and *T. b. rhodesiense* infections, respectively. Melarsoprol, a highly toxic arsenic derivative, is used for advanced stages of *T. b. rhodesiense* infections. Other treatments, such as eflornithine or the nifurtimox-eflornithine combination (NECT) and, more recently, fexinidazole (the first all oral treatment), are used for chronic stages with a range of advantages and disadvantages, discussed later in this review. NECT and fexinidazole are both used for *T. b. gambiense*. Unfortunately, all these molecules have several limitations ranging from acute toxicity (particularly in the encephalic stage) to trypanosomal resistance (Baker et al., 2013; Franco et al., 2018; Hannaert, 2011; WHO, 2020). The need to find new selective drugs for resistant parasite strains remains high. Recently, acoziborole has been selected as a preclinical candidate for the treatment of sleeping sickness caused by *T. b. gambiense*. If approved, it would target the chronic stage of the disease with a one-day administration (Dickie et al., 2020).

Nowadays, a lack of innovative trypanosomal drugs is noticeable, as most of the recommended treatments were developed between 1910 and 1940. For many treatments several mechanism(s) of action (MoAs) on multiple intracellular targets have been described (Baker et al., 2013; De Koning, 2020; Wiedemar et al., 2020). Unsurprisingly, parasitic resistance mechanisms are associated with changes in cellular uptake and/or molecular distribution mechanisms (De Koning, 2020). Resistance factors lead to treatment failure, which hampers human disease control in Africa. It is, thus, important to understand both the drug's mode of action and the mechanism behind resistance to effectively contain the disease and continue reducing its burden.

Genomics, transcriptomics, proteomics and metabolomics are technologies able to provide robust information to address these issues. Metabolomic studies, in particular, provide a functional reading of intracellular activities. The cellular metabolome offers the most accurate image of the phenotypic functionality of a cell or organism. The metabolomic approach aims to analyze the largest metabolome using analytical chemistry, chemometrics and bioinformatics technologies. However, metabolomic analysis faces many challenges. Depending on the biological objectives, two types of metabolomic approaches are possible.

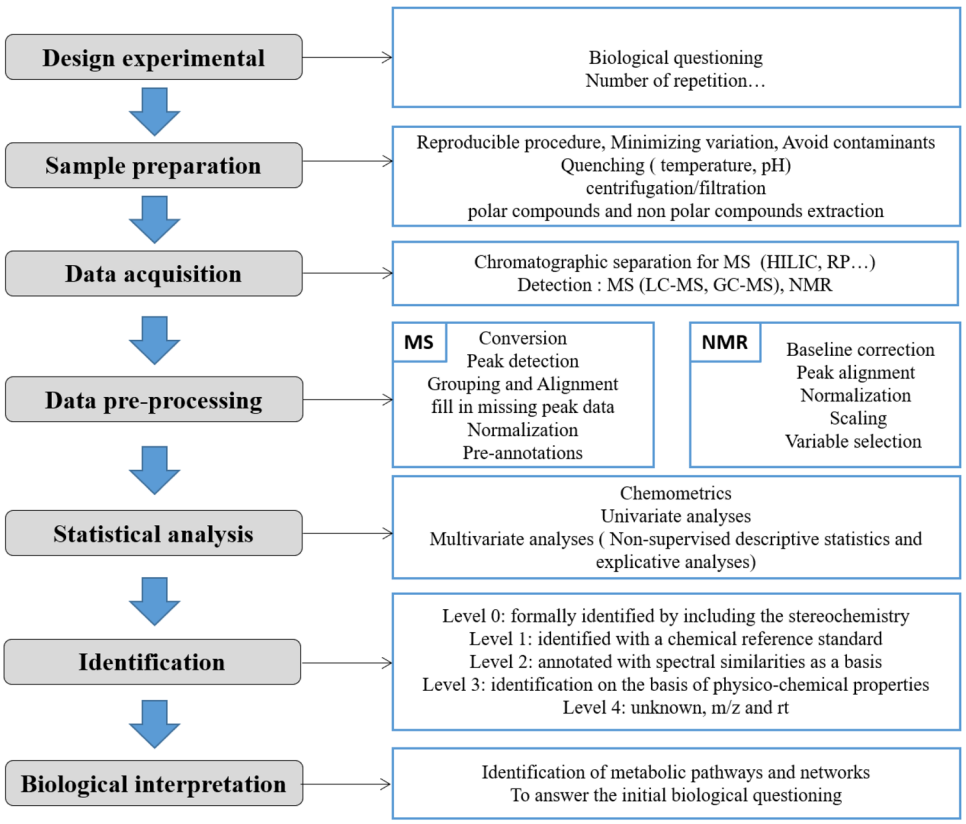


Fig. 3 Human African Trypanosomiasis (HAT) current treatments

Non-targeted metabolomics allows, without a priori knowledge of the metabolites, to analyze and obtain metabolomic signature(s) that could be correlated with a physiological or pathological status. The goal is to detect as many changes as possible in the metabolome due to a pathology, treatment or any environmental change. On the other hand, targeted metabolomics is used to specifically measure one or more selected metabolites/families or biochemically annotated metabolites. These analyses should generally be quantitative, precise and sensitive. The metabolic profile is highly variable, depending upon the investigated species, the tissue explored, the extraction method and the analytical technique employed. Some metabolites have a very high turnover rate, disappearing or becoming undetectable in seconds. Furthermore, depending on the metabolite, the concentration can vary from picomolar to millimolar within the same sample. Metabolic flux analysis (fluxomics) provides a better understanding of the turnover of metabolites and the physiological mechanisms involved in their transport, production and degradation. These studies make use of synthetic precursors enriched with stable isotopes (^{13}C, ^{2}H, ^{15}N, ^{18}O) to highlight the disturbances caused by the treatment as well as its effectiveness (Long & Antoniewicz, 2019). In fluxomic studies, ^{13}C is the most used tracer considering almost all metabolites contain carbon atoms. In these studies, the labelled substrate propagates through the metabolomic pathways and undergoes structural transformations. The isotope profiling allows the identification of the involved metabolomic pathways alongside the metabolization speed and percentage.

Parasite-based metabolomics allows the complete study of the metabolome evolution throughout the parasite's life cycle, also in case of mutations and changes in the host organism associated with the disease or the treatments. In this review, the use of parasite-based metabolomics to better understand the parasite's biochemistry, the treatment's mechanisms of action and development of resistance are discussed. Additionally, the most relevant studies performed over the last years on *T. b. brucei* metabolism and the most used in vitro methods to analyze these trypanosomes are presented.

2 Methodology for targeted and untargeted metabolomics of *T. BRUCEI*

In recent years, many in vitro targeted and non-targeted analytical methods to study *T. b. brucei* metabolism have been published. The studies cited throughout this review have been performed on *T. b. brucei* cultured in vitro, with or without addition of exogenous molecules in the standard media, and complemented with amino acids, fetal bovine serum and various reducing agents to stabilize the culture media and reduce the oxidative stress.

Primarily, the quality of the metabolomic analysis depends on the experimental design. The metabolomics operational approach is based on a series of consecutive steps, known as the workflow, that process the sample and transform it to provide reliable data. The workflow consists of sample collection, sample preparation, data acquisition, data pre-processing, statistical analysis and biological interpretation (Fig. 4). It is generally accepted that these steps directly affect the quality and quantity of the data obtained. Because of their wide range of physicochemical properties, no standard analysis method exists for all metabolites (Vincent & Barrett, 2015).

The in vitro cultured bloodstream and procyclic forms reach a maximal cell density of 5×10^6 to 2×10^7 trypanosomes/ml. Frequently, these forms were studied and subcultured using dilutions between 10 and 1000 fold every 2 to 3 days to obtain different cell concentrations suitable for metabolomic analysis (Ajoko & Steverding, 2015; Creek et al., 2015; Johnston et al., 2019; Pineda et al., 2018; Vincent et al., 2012) (Table 1).

Intracellular sampling of *T. b. brucei* requires separation of the culture medium from the parasites, usually by rapid centrifugation or filtration (Fatarova et al., 2016). Parasites are rapidly quenched before the extraction and analysis of their intracellular content. The fast and complete quenching is an important step to ensure reliable data that correlate to the metabolome, allowing also to avoid metabolic variability due to sample handling. To this purpose, several methods have been published over the years (quick filtration, quenching in cold methanol), but for the majority of protists such as *T. b. brucei*, metabolic quenching is typically done by immersing the samples in an ice/ethanol bath for approximately 20 s (Pinu et al., 2017; Vincent & Barrett, 2015).

Following the quenching step, the use of an optimal extraction solvent is necessary to extract most intracellular metabolites from the parasites. Each extraction solvent allows to extract preferentially different families of molecules depending on their polarity. For instance, hot water and boiling ethanol preferentially extract polar compounds, while hexane and chloroform extract non-polar compounds. There is no specific extraction solvent for *T. b. brucei* metabolites, but to maximize the extraction, a mixture of several solvents is often used, *e.g.* chloroform/methanol/water (1 : 3 : 1 v/v/v) or acetonitrile/methanol/water (4:4:2 v/v/v) (Creek et al., 2015; Johnston et al., 2019; Pineda et al., 2018), with the former solvent mixture most frequently used. The described cell-based metabolites extraction procedures are often based on the use of mixtures containing organic solvents, with or without water, at different temperatures and pH. The most commonly employed solvents are tabulated in Table 1. The variability of the extraction solvents is related to the necessity of adapting the protocol to the different targeted metabolites or to the technique used to analyze

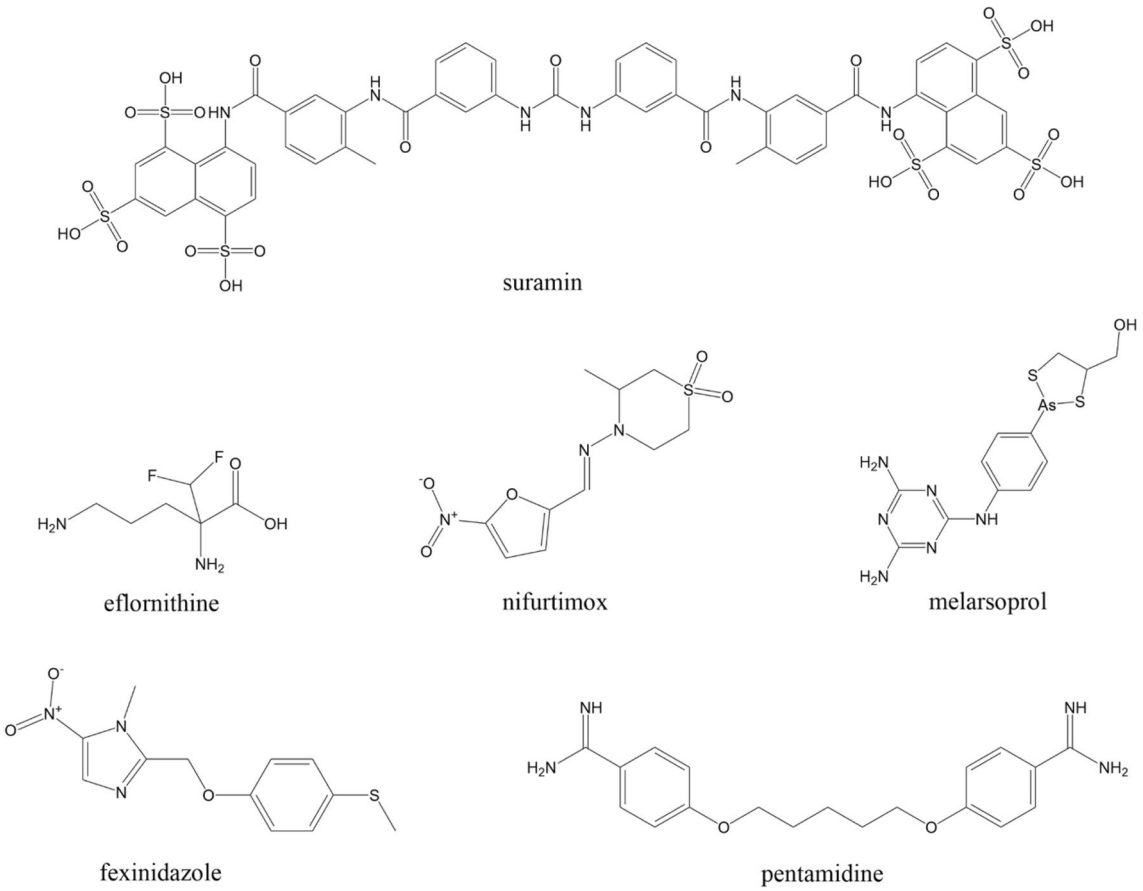


Fig. 4 General experimental workflow for analysis in metabolomics

Table 1 Analytical methodology for analysing the metabolism of *T. b. brucei*

Number of cells and form studied	Extraction solvent	Stationary phase and system	Mobile phase	Data analysis program	Reference
Bloodstream form5×10^7, 4×10^7	Chloroform : methanol : water (ratio 1 : 3 : 1)	ZIC-HILIC; HPLC-Orbitrap	A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile	mzMatch, MetaboAnalyst, IDEOM	Creek et al. (2013), Vincent et al. (2010, 2012), Kamleh et al. (2008)
Bloodstream form 5×10^7, 1×10^8	Chloroform : methanol : water (ratio 1 : 3 : 1)	ZIC-pHILIC; RSLC-Orbitrap	A: 20 mM ammonium carbonate in water, B: 100% acetonitrile	XCMS, mzMatch, Metaboanalyst, IDEOM	Johnston et al. (2019), Ali et al. (2013)
Bloodstream form 2×10^7	Acetonitrile:methanol:water (ration 4:4:2)	IonPac AS11 column; HPLC-Orbitrap	linear gradient elution of KOH	TraceFinder 3.2 software, MetaSys	Pineda et al. (2018), Burgess et al. (2011)

the samples. The extraction protocol choice is a key step of the global workflow because it greatly influences the number and the intensity of detected signals (Creek et al., 2015; Johnston et al., 2019; Vincent et al., 2010, 2012).

The two main analytical platforms that provide structural information on metabolites are nuclear magnetic resonance (NMR) and liquid or gas chromatography coupled with mass spectrometry (GC–MS and LC–MS) (Barrett et al., 2010). Between 2×10^7 and 5×10^7 cells are used for mass spectrometry (MS) analysis, while from 10^7 to 5×10^8 cells are required for NMR analysis (Creek et al., 2015; Johnston et al., 2019; Madji Hounoum et al., 2016; Mantilla et al., 2017; Vincent et al., 2012).

NMR is a fast, versatile and selective analytical technique used for detecting, quantifying and identifying molecules. It relies on the universal natural abundancy of nuclei (^{1}H, ^{13}C, ^{15}N, ^{17}O, ^{19}F and ^{31}P) to detect them through their resonant frequencies and signals, that are unique to their number and positions in a molecule. Consequently, the signal is as intense as their presence in the sample, thus NMR is inherently quantitative without requiring calibration curves with standards. NMR was introduced as the spectroscopic technique to identify and characterize naturally discovered and synthesized molecules, precisely because of the useful structural molecular information it warrants. It was subsequently successfully adapted for use in metabolomics to detect and identify metabolites, but it is not without issues. Despite not requiring extensive sample preparation, not being affected by the type and conditions of extraction as long as the sample remains stable, a metabolomics sample is complex in the variety of molecules simultaneously present.

For *T. b. brucei* NMR metabolomic analysis, ^{1}H-NMR is mostly used, as protons are theoretically present in all organic compounds and this technique is considered the most sensitive one for use in 1D NMR experiments (Creek et al., 2015; Mantilla et al., 2017; Pineda et al., 2018). It is noteworthy to mention that 2D NMR was only once reported in the trypanosome metabolomics field at the time of the writing of this review, which shows that its potential is still poorly explored for this parasite (Millerioux et al., 2013). Nevertheless, ^{1}H-NMR has greatly contributed to the understanding of carbon primary metabolism. Targeted ^{1}H-NMR metabolomic studies have been performed to determine metabolism of alternative carbon sources used by trypanosomes for their ATP supply when glucose levels are not high or constant. Carbon metabolic end products were thus detected exploiting the possibility to distinguish ^{13}C-enriched molecules from ^{12}C-molecules in such analyses. Notably, proton NMR is capable of not only selectively detecting and directly quantifying end products such as succinate, acetate, alanine, and pyruvate, but also the unique chemical shifts attained when these products originated from ^{13}C or ^{12}C carbon sources, and easily and successfully distinguishing them. For example, [^{13}C]-acetate originating from metabolism of a [U-^{13}C]-carbon source displayed two doublets with chemical shifts of approximately 2.00 and 1.75 ppm, respectively, whereas if derived from a ^{12}C, the central resonance of a singlet at 1.88 ppm is characteristic (Bringaud et al., 2015; Mantilla et al., 2017). Studies were performed using reverse genetics tools and [U-^{13}C]-glucose enrichment to determine the adaptability of the trypanosome to different carbon sources. Millerioux et al. investigated the reason why threonine is the most rapidly consumed amino acid by parasites in the tsetse midgut (Millerioux et al., 2013). They found that this amino acid is the main carbon source for acetyl CoA in the procyclic form, used ~ 2.5 times more than glucose, and transformed into equimolar amounts of glycine and acetate. These metabolites are then used in the mitochondrion and cytosol for lipid biosynthesis, namely sterols and fatty acids. Mantilla et al. investigated an alternative to glucose used by the procyclic form in the tsetse fly to produce ATP (Mantilla et al., 2017). Proline was supposed to be readily accessible and consumed by the trypanosome in this context, since it is the source of energy for flight by this vector. Using the same NMR advantage, the in vitro study found that *T. b. brucei* is auxotrophic for proline, contrarily to *Trypanosoma cruzi* (the parasite responsible for Chagas disease in Latin America), and that the parasite could use proline in an essential proline degradation pathway to produce energy, replicate and survive within the insect host. Bringaud et al. reviewed the possibilities to use NMR in analyzing the central metabolism of the cultured procyclic form (Bringaud et al., 2015). Recently, Pineda et al. investigated glycerol as carbon source for BSF, based on the possibility that it could be an important metabolite available in adipose tissue. ^{1}H-NMR demonstrated that both glucose and glycerol are metabolized in the same pathways, with the difference that when glycerol is the source, acetate and succinate producing branches are three times more contributive than with glucose as the substrate. Additionally, at elevated concentrations (10 mM), glycerol was shown to be incorporated into hexose-phosphates through gluconeogenesis (Pineda et al., 2018), which was confirmed at even higher concentration (20 mM) (Kovářová et al., 2018). This capability relies on this parasite's metabolic flexibility, enabling it to maintain infection in different host environments. ^{1}H-NMR was a key player in these discoveries, as it allowed straightforwardly selective detection and quantification of carbon-metabolism products.

A worthwhile alternative to NMR is MS. Frequently in metabolomics, and it is also the case for trypanosome studies, MS is coupled with LC or GC to diminish matrix effects on the detector that could compromise the sensitivity. In fact, the separative nature of the chromatographic technique increases MS sensitivity by reducing ion suppression, separating isomers and preventing artifacts arising from the fragmentation of complex mixtures simultaneously. Hence, MS is a highly sensitive method that can detect metabolites in different matrices.

For separation, a set of chromatographic columns, each with specific affinities exists. HILIC is the preferred LC technique in trypanosome metabolomics as it provides the advantage of analyzing polar molecules, the most abundant ones in the metabolome (Table 1) (Creek et al., 2015; Johnston et al., 2019; Vincent et al., 2010, 2012). Through the retention time, fragmentation pattern, *m/z* and signal intensity, chromatography coupled with MS is able to characterize metabolites with a wide range of physicochemical properties, with the exception of metabolites having the

same exact mass and retention time. Additionally, MS can be quantitative when using internal standards and proper concentration ranges. This analytical method requires long sample preparation, and needs great attention for the choice of the extraction solvent, and has the disadvantage of being sample-destructive. Moreover, the high variability between MS analyzers, acquisition modes and ionization methods alter metabolite characteristics as detected by MS, hindering the comparison to databases towards metabolite identification. This also induces low reproducibility, particularly for LC–MS, that is also susceptible to chromatographic drifts and ion suppression. Additionally, in LC–MS metabolomics, several limitations are present when detecting compounds, such as the presence of interferences or contaminants (from proteins, solvents, polymers, plastics and additives such as detergents) that can have several origins: some are related to previous equipment usage and therefore directly dependent on each laboratory; others are related to analytical conditions and may be common to all mass spectrometry laboratories.

GC–MS is used to identify and quantify small metabolites (< 650 daltons). Following derivatization, it allows to detect several families of molecules (small acids, alcohols, hydroxyl acids, amino acids, sugars, fatty acids, sterols, catecholamines, drugs, and toxins) (Fiehn, 2016). GC–MS is also an important tool in metabolomic analysis as it allows to identify isomers and volatile molecules (Fridberg et al., 2008; Kubata et al., 2000; Podolec et al., 2014). The files generated by mass spectrometry analysis contain a vast dataset. The different bioinformatic steps allow to sort and extract the most important information. To this purpose, many softwares have been developed throughout the years (Table 2). A pre-processing software uses data formats such as mzml, mzxml, cdf, etc., and allows to define regions of interest (ROI) and extract chromatographic peaks, group corresponding peaks, align retention times, and annotate adducts and isotopes. The common goal of these tools is to detect all peaks from the chromatographic analysis and associate them with MS data in order to create a two-dimensional matrix (Table 2). None of these tools is specific for *Trypanosoma* metabolomics, instead they are commonly used for cell metabolomics studies**.** Pre-processing steps in ^{1}H-NMR including baseline correction, alignment, binning and normalization, are performed when necessary. A set of software packages exists to process NMR data (Table 2).

After NMR or MS bioinformatics processing steps, data are treated with a panel of statistical methods (R, SIMCA,

Table 2 Tools available for LC–MS or ^{1}H-NMR metabolomics spectral processing and data analysis

Tool	Instrument data type	Data Processing	Statistical analysis	Identification Annotation	Website
XCMS	LC–MS, GC–MS	Yes	No	No	http://bioconductor.org/packages/release/bioc/html/xcms.html
MetAlign	LC–MS	Yes	No	No	www.metalign.nl
mzMatch	LC–MS	Yes	No	Yes	http://mzmatch.sourceforge.net/
MS-DIAL	LC–MS	Yes	Yes	No	http://prime.psc.riken.jp/compms/msdial/main.html
PiMP	LC–MS	Yes	Yes	Yes	http://polyomics.mvls.gla.ac.uk
IDEOM	LC–MS	Yes	Yes	Yes	http://mzmatch.sourceforge.net/ideom.html
AMDIS	GC–MS	Yes	No	Yes	http://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:amdis
metaMS	GC–MS	Yes	No	Yes	http://bioconductor.org/packages/release/bioc/html/metaMS.html
MSeasy	GC–MS	Yes	No	No	http://sites.google.com/site/rpackagemseasy/
MetaboliteDetector	GC–MS	Yes	No	Yes	http://md.tu-bs.de
MetaboAnalyst	LC–MS, ^{1}H-NMR	No	Yes	Yes	https://www.metaboanalyst.ca/
Workflow4Metabolomics	LC–MS, ^{1}H-NMR	Yes	Yes	Yes	http://workflow4metabolomics.org/
MZmine 2	LC–MS	Yes	Yes	Yes	http://mzmine.github.io/
BATMAN	^{1}H-NMR	Yes	No	Yes/Quantification	http://batman.r-forge.r-project.org/
PepsNMR	^{1}H-NMR	Yes	No	No	https://github.com/ManonMartin/PepsNMR
Bayesil	^{1}H-NMR	Yes	No	Yes/Quantification	http://bayesil.ca/
rDolphin	^{1}H-NMR	Yes	No	Yes	http://github.com/danielcanueto/rDolphin
ASICS	^{1}H-NMR	Yes	-	Yes/Quantification	https://bioconductor.org/packages/ASICS/
Chenomx	^{1}H-NMR	Yes	No	Yes/Quantification	https://www.chenomx.com/
NMRprocflow	^{1}H-NMR	Yes	No	No	https://www.nmrprocflow.org/

MetaboAnalyst (Chong et al., 2018), Workflow4Metabolomics (Giacomoni et al., 2015) etc.) to explore the largest amount of data generated during the analysis. Univariate (T-test, ANOVA, Wilcoxon and Kruskall-Wallis, etc.) and multivariate (PCR, PLS-DA, etc.) statistical analyses allow to highlight markers of interest which can then be identified (Fig. 4).

The statistical analysis is followed by an identification step and the annotation of statistically significant molecules. Metabolite identification remains a big challenge as it is a critical step for extracting biochemical knowledge from metabolomics datasets. Currently a putative or experimentally-driven metabolites list does not exist for many organisms. Specific repositories may help in understanding the parasite's metabolism. For instance, Trypanocyc is a curated metabolite database for *T. brucei* (Shameer et al., 2014) alongside the integrated genomic TriTrypDB which includes data from pathogens of the family Trypanosomatidae, both the *Leishmania* and *Trypanosoma* genera (Aslett et al., 2010). Leish-ExP is the currently protein sequences repository available for five *Leishmania* species (Das et al., 2020), while LeishCyc is a biochemical pathway database only for *L. major* (Doyle et al., 2009). Combining information from various model organisms and data obtained using different analytical methodologies may contribute to the metabolome annotation. According to Chaleckis et al., the annotation of metabolites consists of five levels (Chaleckis et al., 2019). Level 0 corresponds to the most reliable degree of identification including the stereochemistry of the molecules. Level 1 means a formally identified compound using an internal standard. In some research the first two levels are merged, resulting in a total of four metabolite identification levels, with the experimental data defining a metabolite identified when it matches two or more orthogonal chemical standard properties analyzed with the same analytical methods (Sumner et al., 2007) (Fiehn et al., 2007). Level 2 describes compounds whose spectra are similar to those present in public or commercial libraries for which a comparison with an internal standard was not possible. At level 3, the identification process is based on the physicochemical properties or spectral similarity within a chemical class. In other words, compounds are presumed to be characterized based on the chemical class of belonging.

Level 4 corresponds to the lowest degree of identification. The only information available at this level is the spectral data (Fig. 4) (Dona et al., 2016; Dunn et al., 2013; Viant et al., 2017). There are several databases available for the identification of metabolites: KEGG (Kanehisa & Goto, 2000), HMDB (Wishart et al., 2007), ChemSpider (Little et al., 2012) and METLIN (Smith et al., 2005). Their ultimate goal is to identify the metabolic pathways and networks of interest. MetabNet, MetaMapR and MetaboAnalayst are useful tools to this aim (Chong et al., 2018; Grapov et al., 2015; Uppal et al., 2015). New approaches to predict biological activity such as enrichment methods may also be performed, *e.g.*, the mummichog approach. Mummichog is a free Python program for analyzing data from high-throughput, untargeted metabolomics. It maps all possible metabolic correspondences with the network and searches for local enrichment. This mechanism is based on P-values resulting from the statistical analyses bypassing the peak identification step (Chong et al., 2018; Li et al., 2013).

These tools have been used in many metabolomic studies to analyze trypanosomal metabolism (Creek et al., 2015; Johnston et al., 2019; Pineda et al., 2018; Vincent & Barrett, 2015; Vincent et al., 2010, 2012).

3 Metabolomic applications

3.1 Parasite biology analysis

Over the past 10 years, considerable effort has been made to identify and quantify metabolites belonging to *T. b. brucei.* In order to better understand the parasite's metabolism and facilitate drug development, metabolomic analyses using stable isotope-enriched tracers were conducted (Creek et al., 2015; Johnston et al., 2019; Pineda et al., 2018; Vincent et al., 2012). Kim et al. performed a non-targeted flux analysis by LC–MS of *T. b. brucei* BSF metabolites by using U-^{13}C-labelled substrates from an *Escherichia coli* extract as an alternative source of isotope analogues. In this study, trypanosomes were grown in two different media: HMI-9 (Hirumi et al., 1977) or Creek's minimal medium (CMM) (Creek et al., 2013). 477 metabolites were identified belonging to different chemical families, such as amino acids, components of the carbohydrate metabolism and nucleotide metabolism (Kim et al., 2015). 43 metabolites were quantified in the *T. b. brucei* parasites cultured in both tested media. L-alanine and spermidine were the most abundant compounds detected, followed by other metabolites: pantothenate, putrescine, 3-phospho-D-glycerate, uracil, xanthine, pyruvate and a full set of amino acids (Kim et al., 2015). Creek et al. also used metabolic flux analysis on in vitro cultured *T. b. brucei* BSF to show that glucose, in addition to being catabolized in glycolysis, contributes to other metabolic processes, such as the pentose-phosphate pathway, and lipid, nucleotide and amino acid biosynthesis (Creek et al., 2015; Kerkhoven et al., 2013). These studies were conducted using different tracers to analyze the rate of metabolite consumption and production. Using ^{13}C-labelled glucose, a better understanding of glucose carbon distribution was provided (Creek et al., 2015; Johnston et al., 2019; Kim et al., 2015). These non-targeted analyses enabled the identification of molecules not previously identified in *T. b. brucei* metabolism and, simultaneously, to conduct flux

analysis which, combined with mathematical modeling, allowed to predict conversion of metabolites.

Targeted metabolomic studies on particular metabolite families were also carried out. The pyrimidine synthesis pathway of *T. b. brucei* was studied by Ali et al., with the aim of understanding the recovery and incorporation of pyrimidine analogues by trypanosomes. The purpose of this study was to analyze pyrimidines absorption in *T. b. brucei* BSF, to assess the prospect of developing a pyrimidine-based chemotherapy. Using a LC–MS metabolomics approach, the transport of all naturally occurring pyrimidines and 5-fluorouracil (an anticancer agent) by trypanosomes was investigated. Only uracil was efficiently absorbed by *T. b. brucei* BSF via a high-affinity uracil transporter (TbU3) (Ali et al., 2013).

^{1}H-NMR metabolic analysis of the excreted end-products of *T. b. brucei* BSF metabolism was performed by Pineda et al. and demonstrated that glycerol and glucose, two major sources of carbon, are metabolized by the same pathways (Pineda et al., 2018). Also using ^{1}H-NMR, Mantila et al. showed that the regulated expression of proline metabolism will allow the *T. b. brucei* parasite to adapt to the nutritional environment of the tsetse fly gut (Mantilla et al., 2017). Another unique metabolic pathway of the parasite is represented by the trypanothione based redox system, which is responsible for maintaining the parasite's redox balance. Trypanothione – a trypanosomatid specific form of glutathione composed of two glutathione moieties joined by a spermidine linker – is the major redox reactive metabolite responsible for electron transfers to many key survival processes, such as peroxide detoxification and DNA synthesis. This metabolism is absent from the mammalian host, which makes it a highly promising target to design and develop new drugs. In this sense, the latest efforts in the field are focusing on critical proteins involved in the trypanothione synthesis and regeneration (Leroux & Krauth-Siegel, 2016).

Other pathways, such as the TCA cycle and succinate fermentation were investigated by metabolomics using both NMR and MS techniques (Fatarova et al., 2016). Glucose was for years considered to be the only source of energy for *T. brucei* BSF, and glucose and proline for procyclics, but recent investigations highlighted other minor pathways involved (Creek et al., 2015; Johnston et al., 2019; Linstead et al., 1977; Millerioux et al., 2013; Villafraz et al., 2021). In both parasite forms, the importance of various amino acids was investigated. By metabolomics and genomics, Milleroux et al. showed that the procyclic form consumes 2.5 times more threonine than glucose in a glucose-rich medium. In the mitochondrion, threonine is converted into acetyl-CoA from which acetate is formed which is subsequently used in the fatty acids and sterols biosynthesis in the mitochondrion and cytosol (Linstead et al., 1977; Millerioux et al., 2013). The acetyl-CoA to acetate conversion is associated with ATP production (Fig. 2). This mitochondrial ATP production, although low compared to ATP formation in the glycolytic pathway, occurs also in *T. brucei* BSF, where the acetyl-CoA may be obtained from either glucose-derived pyruvate or threonine (Mazet et al., 2013; Mochizuki et al., 2020). Glutamine is more important in the procyclic form than in the BSF for the parasite's energy metabolism. A labelled precursor of glutamate and 2-oxoglutarate production, the U-^{13}C glutamine isotope, was found to label intermediates of several metabolic pathways including TCA cycle intermediates such as succinate, malate, and citrate/isocitrate (Johnston et al., 2019). On the other hand, Besteiro et al. showed that succinate is partly produced by glucose metabolism in the *T. b. brucei* procyclic form. By metabolomic analysis they demonstrated that enzymes located in the glycosomes, such as fumarate reductase, are responsible for succinate formation (Fig. 2). Depletion of glycosomal fumarate reductase by RNA interference lowered the succinate production by 2.9 fold (Besteiro et al., 2002).

MS lipidomic analysis allowed the identification of the lipids present in both parasite forms. Lipids are collected from the local environment or synthesized de novo. Phosphatidylcholine and phosphatidylethanolamine are the major constituents and represent 45–60% and 10–20% of the phospholipidic content, respectively. Phosphatidylserine, phosphatidylinositol, phosphatidic acid, phosphatidylglycerol, and cardiolipin were also found. Sphingosines are present between 10 to 15% in *T. b. brucei* (Richmond et al., 2010; Smith & Bütikofer, 2010). It was also shown that the sterol composition varies according to parasite's developmental stage (van Hellemond & Tielens, 2006).

Metabolomic non-targeted analysis also identified other metabolic pathways operating in the BSF including those for biosynthesis of nucleotides, purine and pyrimidine bases and biosynthesis of various amino acids, all linked to the glycolytic breakdown of glucose (Table 3).

3.2 Target identification for drugs and potential active compounds

Over time, a significant number of drugs with antitrypanosomal activity have been studied and used in therapy. Finding effective treatments has become a major concern due to toxicity issues, inefficacy and bioavailability problems of current therapies (Baker et al., 2013; Hannaert, 2011). The studies cited in the previous section showed that glycolysis is an important process for the trypanosome's development. The polyamine synthesis pathway has also been described as essential for *T. b. brucei*'s growth and division (Gu et al., 2013). The enzymes involved in the synthesis and utilization of spermidine (arginase, ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (AdoMetDC), spermidine synthase, trypanothione synthetase (TryS) and

Table 3 Molecules under development and metabolic pathways affected identified by metabolomics

Method	Compound	Metabolites/Metabolic Pathways	Reference
LC–ESI/MS	Oxazolopyridine: 3-(oxazolo[4,5-b]pyridine-2-yl)anilide	Sphingolipid pathway: Significant accumulation of ceramides	(Stoessel et al., 2016)
LC–MS/MS	Benzoxaborole	Increases in S-adenosylmethionine, methyl thioadenosine, methyl lysine, dimethyl lysine, trimethyl lysine and acetyl lysine	(Begolo et al., 2018; Jacobs et al., 2011; Steketee et al., 2018)
LC–ESI/MS	1,7-bis(4-hydroxy-3-methoxyphenyl)hept-4-en-3-one AS-HK014	Reductions in glutathione-mBBr (thiol specific reagent monobromobimane), S-glutathionyl-l-cysteine and glutathionylspermidine-mBBr	(Alkhaldi et al., 2015)
Study or target			
LC–ESI/MS Untargeted metabolomic	U-^{13}C-labelled	Amino acid metabolism, biosynthesis of secondary metabolites, carbohydrate metabolism, energy metabolism, metabolism of cofactors and vitamins, nucleotide metabolism	(Kim et al., 2015)
LC–ESI/MS Untargeted metabolomic	^{13}C-labelled	Octulose 8-phosphate, nonulose 9-phosphate, d-octopine, carboxyethyl-l-ornithine, trypanothione biosynthesis pathway, arginine, cysteine, glutamine, methionine and proline	(Johnston et al., 2019)
^{1}H-NMR Targeted metabolomic	Proline in procyclic trypanosomes	TCA cycle: succinate, acetate, alanine	(Mantilla et al., 2017)
^{1}H-NMR Targeted metabolomic	[U-^{13}C]-glucose	Glycerol metabolism, alanine, fumarate, phosphoenolpyruvate (PEP), 6-phosphogluconate (6-PG) and succinate	(Pineda et al., 2018)
LC–ESI/MS ^{1}H-NMR Untargeted metabolomic	[U-^{13}C]-glucose	Glycolytic pathway, pentose-phosphate pathway, nucleotide, carboxylic acid derivatives, acetate production	(Creek et al., 2015)

trypanothione reductase (TryR)) have been investigated (Heby et al., 2007). The polyamine spermidine present in this pathway is conjugated with two molecules of glutathione to produce the active redox trypanothione metabolite. When the polyamine synthesis pathway is inhibited, the spermidine level decreases. Moreover, inhibition of the AdoMetDC has a trypanocidal effect (Heby et al., 2007). AdoMetDC catalyzes the decarboxylation of S-adenosylmethionine which acts as a donor of aminopropyl groups in the synthesis of spermidine (Heby et al., 2007).

Currently, old treatments are still in use, such as suramin and pentamidine, alongside newer ones, eflornithine, NECT and the very recently introduced fexinidazole. The therapeutic targets of all these treatments have been studied. Metabolomic analysis allowed to identify some of these targets and find new pharmacological pathways of interest for trypanocidal drugs.

Suramin was one of the first treatments used against HAT. It is a symmetrical, colourless, polysulfonated naphthalene derivative. Because of its inability to cross the blood–brain barrier, it is used to treat the first stage of the disease caused by *T. b. rhodesiense* infections (Nok, 2003). It has been shown that the drug binds to the blood's low-density lipoprotein (LDL) that is internalized in the trypanosome by endocytosis (Vansterkenburg et al., 1993). Later, another suramin uptake route was found involving the surface protein ISG75 (Alsford et al., 2012). Several hypotheses have also been proposed on the mode of action of this molecule. Suramin could inhibit glycolytic enzymes located in the glycosomes (De Koning, 2020; Willson et al., 1993) and prevent the absorption of LDL needed for parasite growth. Indeed, over the years, various enzymes of the glucose catabolism pathway, nucleoside triphosphate diphosphohydrolases and others have been defined as potential targets by using non-metabolomics methods (Wiedemar et al., 2020). The enzymes 3′-nucleotidase, protein kinase C (PKC), acid phosphatase and pyrophosphatase, that are essential to the trypanosome, may also be inhibited (Hannaert, 2011; Nok, 2003). An LC–MS metabolomics study conducted by Zoltner et al., observed alterations in mitochondrial metabolism and significantly increased pyruvate levels in the presence of suramin. The mitochondrial changes involved partial TCA

cycle activation (Zoltner et al., 2020). To date, suramin's mode of action is still not precisely explained, although its use has exceeded one century.

Pentamidine is a diamine used in the 1st stage of the disease caused by *T. b. gambiense,* as it is little effective against *T. b. rhodesiense* infections. Inhibition of trypanothione metabolism by pentamidine has also been demonstrated (Nok, 2003). A non-targeted metabolomics study provided a better understanding of pentamidine's mode of action on *T. b. brucei* in two different culture media: the standard one (HMI11) and CMM. After 48 h incubation, a decrease in certain metabolites levels (nucleotides, lipids, carbohydrates and amino acids) was observed in the HMI11 medium group. In the CMM medium group, only a decrease of phenylalanine, tryptophan and inosine was observed (Creek et al., 2013). This study indicated the better suitability of CMM for routine *T. b. brucei* culture and its significant advantage for metabolomic studies and drug activity screenings (Creek et al., 2013).

Eflornithine is an irreversible inhibitor of ODC, which, in the polyamine synthesis pathway, enables ornithine to be converted into putrescine, a precursor of spermidine and trypanothione. Non-targeted LC–MS metabolomics studies have shown that eflornithine treatment at concentrations below 20 µM causes a significant increase of ornithine and a significant putrescine decrease, confirming previous literature data. The same observations were also made for their acetylated forms (Vincent et al., 2012).

NECT, the nifurtimox-eflornithine combination therapy, was developed as a better alternative for the prolonged duration of the burdensome treatment, toxicity and expensiveness of eflornithine alone. Major eflornithine side effects are anemia, leukopenia, thrombocytopenia and gastrointestinal symptoms (Hannaert, 2011). NECT is used in the advanced stage of the disease. A non-targeted metabolomics study showed modulation of the levels of intermediates of the polyamine synthesis pathway and glycolysis, as well as those of deoxyribose and thiols. Hence, the NECT treatment acts on several metabolic pathways (Vincent et al., 2012).

For drugs such as melarsoprol, fexinidazole, and other antitrypanosomal compounds under development, mechanisms of action and metabolites of interest were determined using techniques other than metabolomics. Metabolomic analysis could then offer the opportunity to confirm and detail the role of these drugs during the treatment and help finding their currently unknown MoAs.

Melarsoprol, an inorganic arsenic compound, is used in the advanced stages of the HAT disease. Once taken up by the parasite, this molecule reacts with the dithiol form of trypanothione to form a complex known as Mel T, a competitive inhibitor of the central antioxidant enzyme, trypanothione reductase. Decreased levels of trypanothione can severely reduce the anti-oxidant defenses of trypanosomes, which is fatal to the parasite. Melarsoprol is also an inhibitor of enzymes of the pentose-phosphate pathway and glycolysis such as glycerol-3-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, pyruvate kinase, glucose-6-phosphate dehydrogenase and hexokinase (Jäger et al., 2013). A series of side effects have been shown in patients treated with melarsoprol, such as skin reactions, polyneuropathy, diarrhea and fever. Additionally, 5 to 10% of patients developed neurological attacks and reactive encephalopathy leading to death (Fairlamb & Horn, 2018; Nok, 2003). In 2013, WHO advised against melarsoprol for *T. b. gambiense* infections and promoted its use only in case of relapse (Fairlamb & Horn, 2018). No metabolomic studies have been reported for this treatment, but different targeted pathways have been described (Fairlamb & Horn, 2018; Nok, 2003).

Other potential targets and therapies are currently under investigation. Fexinidazole is the first oral-only treatment of both the first and second stage of sleeping sickness infections due to *T. b. gambiense*. Fexinidazole's MoA is not yet clear although it may act as other 5-nitro-imidazole derivates and generate reactive amines that are toxic to the parasite. Some pharmacological studies showed fexinidazole as an active pro-drug with two active metabolites, sulfoxide and sulfone, generated by fexinidazole's oxidative metabolism by a host's cytochrome P450 and flavin-containing monooxygenase (Tarral et al., 2014). Fexinidazole sulfoxide and sulfone have both trypanocidal activity with the latter being the most active (Torreele et al., 2010).

A few other molecules are in clinical or preclinical trials (Table 3). Fluoropyrimidine analogues were tested by metabolomic analysis which revealed that 5-fluoro-2′deoxyuridine and 5-fluoro-2′deoxycytidine inhibit the bifunctional dihydrofolate reductase-thymidylate synthase enzyme, causing an increased dUMP formation, the substrate required for de novo thymidylate biosynthesis (Ali et al., 2013; Vincent & Barrett, 2015).

Modifications of existing drugs are being studied to avoid resistance mechanisms of commercially available antitrypanosomal drugs. Among them, diamine derivatives, oxazolopyridine or thiazole-2-ethylamine. N-myristoyltransferase (NMT) has as well been indicated as potential therapeutic target (Berninger et al., 2017). In *T. brucei*, the N-myristoylation of proteins is due to the activity of the single-gene encoded NMT, whose inhibition has a predictable pleiotropic effect on the parasite's biology. NMT inhibition or its depletion by RNA interference resulted in parasite growth arrest (Price et al., 2003). A TbNMT inhibitor model (DDD85646) showed multiple substrate implications in the in situ killing of *T. brucei* BSF, both in vitro and in vivo, and a possible endocytosis disturbance as a potential mechanism of action. Despite the promising selectivity and potency, this model presented some limitations as the narrow window between human NMT and TbNMT and its impossible

CNS penetration (Frearson et al., 2010). Spinks et al. developed two TbNMT enzyme inhibitor series, thiazolidinones and benzomorpholinones, which were validated as potent hits for TbNMT in the HAT drug discovery implementation (Spinks et al., 2015). More recently, high-throughput screenings identified highly potent, selective, and CNS penetrant TbNMT inhibitors based on quinoline and indazole scaffolds. Further research on these inhibitors is ongoing to assess their potential as leads in drug development for treatment of both stages of HAT (Harrison et al., 2018).

Metabolomics has also allowed to reveal resistance mechanisms. In the case of eflornithine, targeted metabolomics showed that in a *T. b. brucei* resistant line, eflornithine levels were significantly reduced. Genetic analysis of eflornithine resistant subspecies showed an absence of the *TbAAT6* gene, which encodes an amino acid transporter. However, in this study, no metabolic changes were found to be significant, even for the polyamine synthesis pathway, contrary to what had been expected (Vincent et al., 2010).

T. brucei is auxotrophic for a number of amino acids which are needed, not only for functional protein synthesis, but also across its different lifecycle stages for different cellular functions (Marchese et al., 2018). During a trypanosomal infection, aromatic amino acids (tryptophan, phenylalanine and tyrosine) are depleted from the host plasma, forcing the parasite to use exogenous sources to generate the respective α-ketoacids from which these amino acids are produced by transamination (Cockram et al., 2020). Given the importance of amino acid uptake and metabolism for *T. brucei* survival and virulence, amino acid metabolism is an additional validated target for HAT treatment. Eflornithine represents an established example of a non-natural amino acid analogue that targets amino acid metabolism by inhibiting ODC through covalent modification (Grishin et al., 1999). Furthermore, some non-natural tryptophan analogues have shown significant trypanocidal activity without clearly identifiable targets, but with confirmed disruption of transamination processes within the parasite (Cockram et al., 2020). Future studies on amino acid metabolism and functional processes can be used to help future new antitrypanosomal drugs development.

Products from plants and fungi with anti-trypanosome activities have also been studied through metabolomics. Fungal endophytes offer diverse and unique secondary metabolites which form a source of interesting active compounds. Extracts of the endophytic fungus *Aspergillus flocculus* from the medicinal plant *Markhamia platycalyx* have been reported as effective anti-trypanosome agents by Tawfike et al. (Tawfike et al., 2019). The bioactivity-guided isolation showed significantly enhanced antitrypanosomal activity of 3-hydroxymelin and diorcinol compared to other compounds identified by MS, such as ergosterol, campesterol, kojic acid, etc. This study revealed that antitrypanosomal activity could be due to synergistic activity of several compounds. Metabolomics was used to define the metabolites mediating the bioactivity of the fungal fermentation culture. A set of metabolites with level 4 identification were found using LC–MS. The combination of NMR and GC–MS allowed the identification of 19 antitrypanosomal metabolites. This example shows that metabolomics simplifies the active principle's identification process in natural extract components (Tawfike et al., 2019).

Extracts of the endophyte *Lasiodiplodia theobromae* from fresh healthy leaves of *Vitex pinnata* also showed significant activity. Metabolomic analysis revealed that the following metabolites were responsible for this activity: cladospirone B, desmethyl-lasiodiplodine and R-(-)-melamine (Kamal et al., 2017).

Likewise, non-targeted metabolomics confirmed polyamine synthesis as a target of interest. The use of non-targeted metabolomics permits to identify the metabolites modulated by a compound with trypanocidal activity (Table 3).

4 Conclusions and future directions

Parasite metabolomics is a rapidly expanding field. In order to have robust metabolomic information and cover the entire metabolome, it is important to combine several analytical techniques. LC–MS provides more information than NMR but requires considerably longer sample preparation time. Both techniques are generally presented as complementary methods that allow to cover a broader range of metabolites. An important consideration would be to standardize the extraction and metabolomic analysis methods using internal standards which can be, as mentioned above, labelled molecules. An analytical limitation to metabolomic analysis of parasites relies on the lack of dedicated databases. To date, "Trypanocyc" (http://www.metexplore.fr/trypanocyc/) is the most extensive database dedicated to *T. brucei* metabolism (Shameer et al., 2015). Nonetheless, metabolomic analyses applied to trypanosomes have revealed potential markers of pharmacological interest. This allowed the identification of therapeutic targets, such as the polyamine synthesis pathway in addition to glycolysis, the most studied metabolic process. More metabolomics research should be performed on *T. b. brucei* as it has been the case for *Plasmodium* and *Leishmania* in recent years. This would allow new drug targets identification to address the resistance problems shown by most drugs, as well as to confirm resistance mechanisms identified in other studies, *e.g.* involving genetics and proteomics. For instance, in *Plasmodium falciparum*, metabolomics has made it possible to highlight important metabolomic pathways, such as the biosynthesis of isoprenoids, fatty acids and folate (Allman et al., 2016). In *Leishmania,* a large number of metabolites belonging to

several metabolic pathways were found to be interesting for the characterization of metabolic events (Atan et al., 2018; Saunders et al., 2021). Bloodstream forms of trypanosomes can be observed in two morphologically and metabolically different forms: the long slender trypomastigote and short stumpy trypomastigote, with the short stumpy trypomastigotes having more metabolic similarities, although still being different, with the insect-stage procyclic form. It is therefore important to define the appropriate parasite life cycle stage when performing metabolomics studies. The combination of metabolomics with fluxomics allows a better understanding of the central carbon distribution within a metabolism, and similarly, provides information on the production, levels and turnover rates of the different *T. b. brucei* metabolites.

To determine the mode of action of drugs or identify therapeutic drugs targets, metabolomics has emerged as an effective methodology. Nowadays, most existing treatments available for *T. brucei* infections are complicated by several resistance mechanisms and toxic side effects. Other molecules are under development and metabolomics is today the most versatile and fastest method to study their efficacy on trypanosomal metabolism. Metabolomics may also help to rapidly identify the active molecules present in extracts from plants with trypanocidal activity and obtain further information on their targets/mode of action. Application of metabolomics appears thus very promising in the fight against parasitic diseases, including trypanosomiasis.

Funding Laura Schioppa is a Research Fellow of the Fonds de la Recherche Scientifique – FNRS.

Declarations

Conflict of interest The authors declare no conflict of interest.

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