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Pharmacological Metabolomics in Trypanosomes

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Abstract

Many drugs act by perturbing aspects of cellular metabolism. Human African trypanosomiasis can be treated with five drugs, depending on the causative subspecies and the stage of the disease. For only one drug, eflornithine (difluoromethylornithine), has a mode of action been ascertained – it inhibits ornithine decarboxylase, a key enzyme in polyamine biosynthesis. Modes of action remain unknown for the other drugs, which include the organic arsenical melarsoprol, the naphthalene suramin, the diamidine pentamidine, and the nitrofuran nifurtimox, although it has recently been shown that nifurtimox acts following its metabolism to a reactive trinitrile derivative. Metabolomics aims to quantify all low-molecular-weight chemicals within a given system. Metabolomic techniques have recently been developed and applied to trypanosomes, and might be expected to reveal how drugs perturb metabolism. Here, we review methods to study the trypanosome's metabolome and discuss the current knowledge with respect to the effects of anti-trypanosomal drugs on parasite metabolism.

Introduction

Human African trypanosomiasis (HAT) is caused by *Trypanosoma brucei* subspecies [1]. The disease has two defined stages. In stage 1, which follows transmission of the parasites by the tsetse fly vector, trypanosomes replicate in the hemolymphatic system, causing relatively non-specific symptoms, including general malaise, fever, and headache [1]. In stage 2, parasites have entered the central nervous system (CNS) and their presence induces various neurological sequelae, including psychological changes and disruptions to sleep/wake patterns after which the common name of "sleeping sickness" is derived [2].

In Central and West Africa the subspecies *T. b. gambiense* causes a chronic form of HAT taking around 2 years before CNS infection, followed by progressive neurological deterioration during stage 2 disease, then death [1,2]. In Eastern and Southern Africa, *T. b. rhodesiense* causes an acute disease and is fatal within months.

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Trypanosomes escape immunological destruction using a process of antigenic variation [3], which renders vaccination an unlikely proposition for HAT. Chemotherapy remains pre-eminent, although tsetse fly control can also be an effective means of intervention [1]. A decade of sustained and enhanced intervention has led to HAT prevalence declining with fewer than 7000 cases reported in 2010 [4].

However, the history of trypanosomiasis control has seen previous declines preceding resurgence, owing to the cessation of intervention programs perceived as uneconomical for a low-prevalence disease [5].

Until recently, investigations into the mode of action of trypanocidal drugs have depended upon testing inhibition of predicted enzyme targets. In the case of eflornithine, a specific inhibitor of ornithine decarboxylase, several studies confirmed this mode of action [2,6]. For other drugs, however, which were introduced empirically based on their ability to kill trypanosomes, modes of action have remained elusive [2]. The new methodologies collectively referred to as "metabolomics" now offer the means to determine how drugs perturb trypanosome metabolism in a hypothesis-free, untargeted fashion. Here, we review the published literature on the application of metabolomics technology to trypanosomes and also on what is known about actions of anti-trypanosomal drugs on metabolism.

Metabolomics – New Technologies Applied to Trypanosomes

The relative homogeneity of the chemical building blocks of nucleic acids and proteins, respectively, has enabled rapid development of generic techniques to enable the analysis of these biological constituents making genomics, transcriptomics, and proteomics relatively mature technologies. Metabolomics, the measurement of the low-molecular-weight constituents of biosystems, has been hampered by the chemical diversity and broad concentration range of different metabolites. No individual analytical platform is capable of measuring all metabolites simultaneously. However, variations on the nuclear magnetic resonance (NMR) and mass spectrometry (MS) themes have, in recent years, allowed increasing coverage of the metabolome [7–12], and these techniques are now being applied with increasing intensity to the study of trypanosomes and also drug action.

Sample Preparation

Sample preparation is critical in metabolomics. Levels of many metabolites can change profoundly in response to external stimuli (including dramatic perturbations involved in harvesting cells for metabolite extraction). Various controls to report on deviations not related to the specific biological question addressed by an experiment are vital. Rapid quenching [13] to capture the metabolome as close to its physiological state as possible is desirable. In some microbes, including yeast, tough cell walls make them extremely robust and cells in suspension can be applied directly to solvents such as methanol at temperatures as low as $-80\,^{\circ}C$. Metabolism ceases

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instantaneously and the cells can be washed free of medium prior to metabolite extraction [14]. Unfortunately, trypanosomes lyse under such conditions. Another way to rapidly stop metabolism and separate trypanosomes from their medium has been to use a rapid filtration method followed by hypotonic lysis and extraction in aqueous solvent [15]. Although suitable for *T. brucei* procyclic forms, the method causes on-filter lysis of bloodstream forms. Metabolites can be collected by application of trypanosome suspensions directly to organic solvents including hot ethanol [16] and cold chloroform/methanol/water [17]. However, medium carries over many abundant metabolites that can swamp their intracellular counterparts. Moreover, high cell numbers are needed to provide detectable quantities of most metabolites (5×10^7 to 10^9 trypanosomes [17–19]). This "direct-squirt" method remains useful in obtaining rapid flux measurements in systems where heavy isotope precursors are followed and hence not subject to interference from extracellular metabolites (provided controls on medium at different time points are included). A rapid chilling method, where flasks of cells are cooled to 0 °C, without freezing, in a dry-ice ethanol bath before centrifugation for removal of medium was developed for analyzing polar metabolites in *Leishmania* [17–19] and is currently the method of choice in detecting changes in steady-state intracellular metabolite levels by liquid chromatography (LC)-MS in trypanosomes [20]. Metabolite extraction from quenched cell pellets is generally achieved by addition of organic solvents, such as 80% ethanol or chloroform/methanol/water (1 : 3 : 1) followed by vigorous mixing and centrifugation to remove insoluble material. Rapid enzyme inactivation and cellular disruption may be improved by using higher temperatures (80 °C) [16]. However, generally extractions should be performed below 4 °C to minimize the degradation of metabolites [19]. Samples should be stored, preferably under argon to minimize oxidation, at −80 °C prior to analysis, or may be dried by evaporation and reconstituted prior to analysis with solvents appropriate for the analytical platform (MS or NMR).

NMR-Based Metabolomics

NMR has been used for broad untargeted metabolite profiling for many years, and its reproducibility, simple sample preparation, and ability to provide absolute quantification offer some advantages [21,22]. The spin properties of atomic nuclei are detected by NMR, and in metabolite analysis ^{1}H, ^{13}C, and ^{31}P signals can be used to report on the structure and abundance of metabolites. NMR is capable of detecting only the most abundant metabolites in a given sample, which limits the approach, although it has been used successfully in unraveling the metabolic pathways operative in procyclic trypanosomes [23–28], and in detecting metabolic changes in blood and urine of trypanosome infected mice [29,30].

NMR studies on glucose metabolism in bloodstream trypanosomes under anaerobic conditions [31,32] showed that glycerol and pyruvate were major end-products of glycolysis. However, significant amounts of alanine were also detected. Alanine is produced from pyruvate using alanine aminotransferase, which has subsequently been shown to be essential to bloodstream-form as well as procyclic trypanosomes [33],

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possibly contributing to pyruvate removal if its efflux rate cannot match production [34]. ^{31}P-NMR was also used to quantify the main phosphorylated metabolites in various trypanosomatids including *T. brucei* [35]. Bringaud's systematic application of NMR to characterize secreted metabolites from procyclic trypanosomes, modified using the reverse genetic approaches, has identified roles for numerous genes [23–28]. MS-based approaches have now also been applied to determine steady state levels of metabolites in various procyclic mutants [15]. ^{13}C-NMR was also used effectively to analyze an aconitase mutant cell line, revealing that most succinate in trypanosomes is not generated within the tricarboxylic acid (TCA) cycle [36]. Proline metabolism by procyclic *T. brucei* was also probed [15,37] and shifts in metabolism noted depending on whether parasites were grown on glucose or proline as their main carbon source. [^{13}C]proline is converted into succinate if glucose is present in medium but to alanine if glucose is absent [15].

^{1}H-NMR applied to *T. brucei* infection in mouse urine and plasma [29] showed progressive changes to the plasma metabolome from day 1 postinfection whilst urine was clearly distinguishable in infected mice from day 7. Elevated lactate was detected in both plasma and urine of infected animals. Urine also showed increases in the branched chain amino acid metabolites 3-methyl-2-oxovalerate and 2-oxoisovalerate, and D-3-hydroxybutyrate while the branched chain amino acids leucine, isoleucine and valine themselves were diminished in plasma. Diminished quantities of hippurate were also reported in urine along with increases in trimethylamine and 4-hydroxyphenylacetic acid. In mixed infections [30] some strain-specific changes were apparent in highly parasitized rodents. The very low parasitemias that accompany *T. b. gambiense* infection in man mean that careful studies should be performed to determine whether metabolic biomarkers can also be found that diagnose human disease.

MS-Based Metabolomics

MS approaches are generally of greater sensitivity than NMR [38]. Chromatographic separation methods (including gas chromatography (GC), high-performance liquid chromatography (HPLC), ultra-performance liquid chromatography (UPLC), or capillary electrophoresis (CE) improve the technique as they separate metabolites prior to entry into the MS. Chromatography decreases problems with ion suppression (where highly ionizable chemical species can outcompete others in acquiring a charge during the ionization process), can distinguish multiple isomeric metabolites, and also provides key information on metabolite structure from the chromatographic behavior. GC-electron impact ionization (EI)-MS and LC-electrospray ionization (ESI)-MS are the most commonly used approaches in contemporary metabolomics research [38] and both approaches have been applied to the study of *T. brucei*.

GC-MS-Based Metabolomics

GC-MS is the best method to study inherently volatile metabolites and can also detect other compounds made volatile by chemical derivatization. Capillary GC can reproducibly separate hundreds of metabolites in complex biological mixtures by

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phase partitioning between a carrier gas phase and the liquid phase on the inner surface of the column. Eluted metabolites are then ionized (usually by EI) and detected using a quadrupole mass spectrometer [38]. Chemical ionization (CI) can also be used as a "softer" alternative to EI and (quantitative) time-of-flight ((Q)TOF) or high-resolution Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers have improved identification. GC-MS has been popular in metabolomics due to its capacity to detect many intermediates in central carbon metabolism (e.g., organic acids, sugar phosphates, lipids, and amino acids), and highly reproducible GC retention time and EI-generated mass spectra. Complex sample processing protocols, including the need for derivatization, and a failure to detect many polar metabolites, however, are limitations to its use. GC-MS is currently being applied to great effect in pioneering metabolomics analysis in *Leishmania* parasites [17,18,39]. In *T. brucei* GC-MS was used to identify changes to hosts (rodents) infected with trypanosomes. *T. b. gambiense* was shown to metabolize tryptophan, tyrosine, and phenylalanine to indole 3-pyruvic acid, 4-hydroxphenylpyruvic acid, and phenylpyruvic acid, respectively [40–42], and *T. brucei gambiense*-infected rodents also secreted abnormal quantities of catabolites of these aromatic amino acids [43–46].

GC-MS was also used to show that host-derived cholesterol is the predominant sterol (96% of the total sterol content) in bloodstream forms while procyclic forms have other sterols such as ergosta-5,7,25(27)-trienol [47] as a major proportion of the total sterol content. It is likely that GC-MS will be used increasingly in the analysis of *T. brucei* as metabolomic technologies become more accessible and more groups exploit their capabilities.

LC-MS-Based Metabolomics

LC separates chemicals based primarily on their lipophilicity and/or charge. Reversed-phase LC combined with MS is particularly suited to lipophilic metabolites. However, polar or charged metabolites are generally not retained on reversed-phase columns, making this approach unsuitable for separation of many polar cellular metabolites. Hydrophilic interaction chromatography (HILIC) is now preferentially used in many LC-MS-based metabolomics analyses [48,49]. ESI in positive mode (usually adding protons to metabolites) or negative mode (removing protons) in the ionization source is the most common way to generate ions. Ideally samples should be analyzed in both modes to detect as many metabolites as possible [49]. The Exactive Orbitrap (Thermo) in "switching mode" alternates between positive and negative mode thus yielding data in both modes from a single sample injection [49]. A confounding problem in LC-MS arises from the variable effect of ion suppression by coeluting metabolites, salts, or other chemicals in the ionization source, which is highly dependent on the matrix under study. It is possible to provide quantification, however, by spiking of samples with internal standards that can be obtained from, for example, *Escherichia coli* extracts in which all metabolites are labeled with ^{13}C. Metabolites of interest can then be compared to the heavy isotope spike whose quantity in the *E. coli* extract can be calculated by titration against authentic, pure standards [50].

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Ultra-high mass accuracy MS has revolutionized metabolomics. TOF detectors offer high mass accuracy and have been successfully deployed in metabolomic studies, but FT-ICR and Orbitrap mass spectrometers offer the best results in terms of mass accuracy (below 1 ppm) and resolution (above 100 000) allowing assignment of a putative molecular formula to each observed mass for many metabolites of interest [7].

Triple-quadrupole mass spectrometers have also found great utility in LC-MS approaches where the multiple reaction monitoring (MRM) approaches involve fragmentation of selected analytes, allowing identification of metabolite-specific fragments. The MRM approach is highly sensitive, but limited by the fact that it can only identify metabolites for which fragmentation patterns have been predetermined and thus cannot be used for untargeted metabolomics. Nevertheless, some detailed analyses of metabolism in *T. brucei* procyclic forms have shown its utility [15,51].

Targeted LC-MS approaches have been used successfully to track sugar nucleotide incorporation into glycoconjugates in trypanosomatids [52]. In a first untargeted LC-MS study, FT-ICR-MS was used to probe the metabolome of bloodstream forms of *T. brucei* [53]. Although the number of metabolites identified was not great, this study also introduced a method where metabolites identified on accurate mass can be linked to others based on the exact mass of known metabolic transformations, allowing the development of *ab initio* metabolic networks. This type of approach may prove useful in pharmacological metabolomics to facilitate the discovery of novel drug metabolites or adducts. ZIC®-HILIC chromatography, coupled to Orbitrap MS, has become a powerful way to obtain data from trypanosome samples [49]. The first untargeted metabolomic analysis of procyclic forms of *T. brucei* focused on parasites grown in either glucose- or proline-rich medium [16]. Significant changes were found in several metabolites including the precursor substrates and key metabolites of central carbon metabolism and proline catabolism consistent with those predicted using NMR analysis of end-products in various mutants [23–28].

Considerable advances have also been made in the lipidomic analysis of *T. brucei* using LC-MS approaches (reviewed in [54–56]). Phosphatidylethanolamine and phosphatidylcholine glycerolipids are abundant, while phosphatidylinositol, phosphatidylserine, and cardiolipin are relatively minor species. Ether-linked lipids are found relatively abundantly in both forms and sphingolipids are abundant too. Major changes to lipid metabolism appear to correlate to a number of major events in *T. brucei*, including differentiation and drug assault [54–56].

Data Analysis

Hundreds, or even thousands, of metabolites can now be identified simultaneously. However, the analytical runs that identify these metabolites are also polluted by tens of thousands of peaks that do not represent true metabolites (including contaminating species from solvents, buffers, plasticware, tubing within machines, and also a multitude of fragments, adducts, multicharged species, isotopomers, and clusters

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derived from all chemicals within the samples). It is crucial to distinguish true metabolites from the artifacts. In GC-MS, many common metabolites can be identified based on relatively comprehensive databases that offer specific information on retention time and EI fragmentation patterns (http://www.sisweb.com/software/ms/nist.htm). Assigning metabolite identifications from LC-MS analyses based on exact mass alone, however, often results in false-positive identifications [57]. For *T. brucei,* the Scottish Metabolomics Facility has focused largely on exact mass data from an Orbitrap mass spectrometer. A variety of filters using mzMatch [58] select and annotate the most reproducible peaks representing metabolites. Prefiltered datasets are then further filtered and represented using the Ideom software package – a Microsoft Excel-based application that allows for advanced data analysis in a user-friendly interface [59]. Caution is still needed in interpreting data and ideally any metabolites that emerge as biologically significant in a given experiment should be verified with authentic standards, using comparative chromatography and tandem MS (MS/MS or MS^n), although mass and retention times of common metabolite standards and prior knowledge of the presence (or not) of a metabolites in trypanosomes can offer reliable identification of some metabolites [60].

The *T. brucei* genome [61] (and comparisons to the other TriTryps genomes, e.g., *Leishmania major* and *T. cruzi*) has enabled generation of a predicted metabolome at the Kyoto Encyclopedia of Genes and Genomes (KEGG) [62]. The MetaCyc family of databases [63] offers an alternative community-based representation of genome-predicted metabolomes. A clearly annotated version of the *Leishmania* metabolome has been assembled under LeishCyc [64] (http://leishcyc.bio21.unimelb.edu.au/). Automated reconstruction of the *T. brucei* metabolome (TrypanoCyc) has also been constructed [65], and is currently being improved with links to the TriTryp genome projects at the GeneDB [66] and TritrypDB [67] databases. Pathos (http://motif.gla.ac.uk/Pathos/index.html) [68] is an interactive web application that allows direct representation of identified metabolites and their relative abundance in the KEGG environment. MetExplore [69] (http://metexplore.toulouse.inra.fr/metexplore/index.php) has also been used extensively to analyze trypanosome metabolomics datasets in network-based representations that allows gap filling between detected metabolites in related metabolic pathways [70].

Stoichiometry-based models of metabolism have been built for many organisms, including *T. cruzi* [71] and *L. major* [72]. A model of *T. brucei* metabolism is currently being built. Such stoichiometric models have been helpful in identifying choke points; reactions that are the single route to production or consumption of key metabolites in the network that often represent potentially good drug targets, if choke point enzymes are druggable according to pharmacological criteria [73].

One of the most advanced fine-grained, dynamic models of metabolism, based on a series of ordinary differential equations reporting kinetic parameters of enzymes, has been produced for glycolysis in the bloodstream-form African trypanosome [74–76], which occurs within the glycosome, a membrane bound organelle, related to peroxisomes [77,78]. The pentose phosphate pathway has recently been added to the glycolytic model [79] and NADPH offers the potential to link glycolysis to the main redox active pathway in trypanosomes via the N^1,N^8-bis(glutathionyl)

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spermidine adduct, termed trypanothione. Measurements of protein and RNA abundance, as achieved for phosphoglycerate kinase [80], are also being put together with an ultimate aim of a full mathematical description of trypanosome metabolism or a "the silicon trypanosome" [81,82].

Metabolic Affects of Trypanocidal Drugs

An area of natural interest is metabolomic analysis of modes of action of trypanocidal drugs. Current drugs for HAT all suffer drawbacks, including toxicity, parenteral administration, and restricted efficacy [2]. For *T. b. gambiense*, pentamidine is used for stage 1 disease while eflornithine or a nifurtimox–eflornithine combination therapy (NECT) is used preferentially for stage 2. For *T. b. rhodesiense*, suramin is preferred for stage 1 and melarsoprol is the only option for stage 2 disease [1,2]. Modes of action have not been ascertained for most drugs, although eflornithine acts as an inhibitor of polyamine biosynthesis through its inactivation of the enzyme ornithine decarboxylase.

Pentamidine

Pentamidine has been used for over 60 years to treat stage 1 *T. b. gambiense* disease [1,2]. *In vitro* trypanocidal potency (IC_{50}) is of the order of 1–10 nM in a typical 3-day drug sensitivity assay [83]. Pentamidine is concentrated to high (millimolar) levels by trypanosomes using the P2 amino-purine permease and other transporters that include a high-affinity pentamidine transporter (HAPT1) and a low-affinity pentamidine transporter (LAPT1) [84]. How it actually kills trypanosomes is not certain [85,86], although diamidines bind to DNA and mitochondrial dysfunction has been associated with diamidine treatment. Pentamidine binds avidly to DNA and accumulates within the mitochondrion, leading to a disintegration of the kinetoplast. However, *Trypanosoma evansi* strains that are lacking a kinetoplast are only a few fold less sensitive to diamidines than kinetoplast containing *T. brucei*, indicating other modes of action [87].

Electrostatic interactions also mediate dicationic pentamidine binding to numerous enzymes. In one study, mice infected with trypanosomes were treated with pentamidine and the parasites then analyzed using a targeted metabolite profiling approach. The basic amino acids lysine and arginine were increased in concentration by 13- and 2.5-fold, respectively [88]. Pentamidine has been speculated to interfere with polyamine function in cells and the drug is a potent inhibitor of *S*-adenosylmethionine decarboxylase (SAMDC) when assayed in a purified form [89]. However, trypanosomes purified from pentamidine-treated mice showed no perturbations in polyamine abundance. Moreover, gene knockouts of SAMDC in *Leishmania* yielded no change in sensitivity to pentamidine [90]. Studies in the related protist *Crithidia fasiculata* revealed inhibition of nucleic acid, protein, and phospholipid synthesis, suggesting that numerous biosynthetic enzymes are

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implicated in pentamidine action [91]. Untargeted metabolomic analysis will enable direct testing of the various proposals already made on metabolic actions for pentamidine and also reveal if unexpected changes to metabolism might accompany treatment with the drug.

The advent of other postgenomic technologies in addition to metabolomics is also reporting on modes of action and mechanisms of resistance to drugs. Exploitation of the RNA interference (RNAi) pathway operative in *T. brucei* [92], for example, has enabled a comprehensive search for all genes whose loss of function can lead to diminished drug sensitivity and this, in turn, can point to modes of action [93–95]. Transformation of trypanosomes with a library of DNA fragments permitting inducible downregulation of genes by RNAi, followed by selection in drugs, allowed identification of multiple genes whose loss of function can cause resistance. This so-called RITSeq approach [95], applied to selection with pentamidine, has yielded several key observations. It has long been known that loss of the P2 adenosine transporter contributes to pentamidine resistance. The RITSeq screen also yielded an aquaglyceroporin (AQP2) [95] that might represent one of the other transporters involved in uptake whose loss relates to resistance [84] (or else regulate that second transporter). A proton ATPase was also identified, although genes encoding specific metabolic enzymes were not apparent, which might relate to the drug's activity associating principally with disruption to nucleic acid metabolism.

Suramin

Suramin is a large (molecular weight 1297) polysulfonated naphthalene derivative that was originally derived from a group of trypanocidal dyes including Trypan blue in 1916. It is administered through five slow intravenous injections of 20 mg/kg every 3–7 days for 28 days [2]. Side-effects include nausea, rashes, and circulatory problems. The *in vitro* inhibitory concentrations (50%) for suramin are in the low nanomolar range [2] and *in vivo* activity is enhanced by the long half-life (44–92 days). This large polyanionic molecule is highly plasma protein bound (99.7%) and cannot cross the blood–brain barrier (BBB), and so is ineffective for stage 2 CNS disease, although it does increase the concentration of eflornithine entering the CNS in mice [96]. Suramin was proposed to enter trypanosomes by endocytosis [97]. Low-density lipoprotein was proposed as a possible carrier [98], although this was shown not to be the case [99]. The RITSeq approach has indicated that ISG75 is the ligand that binds suramin [95]. Those experiments revealed several components of the endocytic vesicular cascade as being lost in selection of drug resistance [95]. Interestingly, downregulation of several enzymes involved in polyamine biosynthesis and *N*-acetylglucosamine biosynthesis also yielded a positive impact on growth for *T. brucei* [95]. Polyamines have recently been shown to play a role in vesicular trafficking in mammalian cells [100] so such a role is also likely in trypanosomes and *N*-acetylglucosamine loss might also perturb endocytosis.

Even with the RITSeq breakthroughs, the mode of action of suramin remains unknown. Numerous theories have sought to explain how the drug exerts its effects.

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Targeted analysis of glucose, oxygen, glycerol, and pyruvate levels suggested the trypanocidal activity resulted from inhibition of glycolysis, which could be explained by specific inhibition of numerous glycolytic enzymes in cell-free assays [101]. In *Onchocerca* spp., an inhibition of dihydrofolate reductase (DHFR) (the inhibition was 35 times more efficient on the parasite enzyme than on the mammalian enzyme) was noted [102,103]. Folate metabolism in trypanosomes has been subject to a relatively elegant targeted metabolite profiling study [104], but no evidence of suramin impact is apparent. Inhibition of thymidine kinase has also been reported, suggesting disruption of nucleotide synthesis underlies the mode of suramin action [105], but proven modes of action remain unknown.

Melarsoprol

Melarsoprol, a melaminophenyl-based organic arsenical, is extraordinarily toxic with many patients taking the drug suffering a reactive encephalopathy that frequently kills. A 3.6% solution in propylene glycol is administered, generally now over a 10-day course that superseded earlier long-term interrupted regimens. Melarsoprol converts rapidly to an active metabolite melarsen oxide *in vivo*, which shows a mean elimination half-life of 3.9 h [106]. Cyclodextrin-based formulations [107] enable oral uptake of melarsoprol in mice [108] and it is hoped that this could ameliorate some of the toxic effects associated with the drug.

Rapid uptake via the P2 (*Tb*AT1) aminopurine transporter and at least one other carrier (probably the HAPT1 transporter [109]) contributes to the selective activity of the drug and loss of transport relates to resistance [110]. The *Tb*AT1 and the aquaglyceroporin gene found in the pentamidine screen also emerged in the RITSeq melarsoprol screen [95]. Roles for several protein kinases and reduced expression of both trypanothione synthase and trypanothione reductase pointed to the previously identified melarsen oxide–trypanothione conjugate (MelT) [111] as contributing to toxicity to the parasites. This, however, is the opposite of the situation in *Leishmania* treated with the related heavy metal (antimony), where overexpression of enzymes (ornithine decarboxylase and γ-glutamylcysteine synthetase) [112] relates to resistance via production of excess trypanothione to bind the toxic heavy metal.

The mode of action of melarsoprol remains to be definitively elucidated. Dithiols are structurally important for many proteins and are also key residues in many enzyme active sites, which explains the large number of metabolic enzymes that are inhibited by melarsoprol and related arsenicals. Melarsoprol inhibition of several trypanosomal glycolytic enzymes has been demonstrated, including glycerol-3-phosphate dehydrogenase [113], 6-phosphogluconate dehydrogenase [114], pyruvate kinase [115], glucose-6-phosphate dehydrogenase, malic dehydrogenase, and hexokinase [116], although cell death precedes significant inhibition of glycolysis and ATP levels are not significantly decreased at the time of cell lysis [117]. The application of metabolomics to pharmacological evaluation will require great care to distinguish specific drug-induced metabolic changes from non-specific changes associated with cell death.

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Trypanothione, an essential dithiol antioxidant molecule in trypanosomes, binds to melarsoprol and has been proposed as the molecular target [111]. Accumulation of the melarsen–trypanothione adduct (MelT) may be responsible for increasing the intracellular concentration of melarsen, but trypanothione levels are not significantly depleted by melarsoprol. Although trypanothione reductase is inhibited by melarsoprol (and by MelT) *in vitro*, parasites with altered expression of this enzyme did not show altered sensitivity to melarsen oxide [118]. One suggestion is that trypanothione sequestration of melarsoprol may be a protective mechanism [119] although the RITSeq data [95] was more consistent with MelT toxicity. Lipoic acid and lipoamide have been proposed as alternative targets, due to formation of drug-dithiol adducts that are 500-fold more stable than MelT [120].

Trivalent arsenicals have also been studied for their action against leukemia cell lines, demonstrating induction of apoptosis. Proteomic analysis of As_2O_3-treated cells revealed changes to levels of more than 50 proteins, including a number of enzymes [121]. Whilst the modes of action of melarsoprol and As_2O_3 are not identical, it is assumed that multiple biochemical changes will also be associated with melarsoprol action.

Eflornithine

Eflornithine (D,L-difluoromethyl ornithine (DFMO)) is an analog of the amino acid ornithine and inhibits the polyamine biosynthetic enzyme ornithine decarboxylase (ODC). The drug is active against the *T. b. gambiense* trypanosome subspecies, but less active against the related *T. b rhodesiense*. The usual treatment regimen involves intravenous infusions of 100 mg/kg body weight at 6-h intervals (i.e. 400 mg/kg/day) for 14 days. This intensive dosage schedule is necessary, because the compound is only weakly active against *T. brucei* bloodstream stages when compared to the other licensed drugs (IC_{50} values of 20–40 μM rather than the low nanomolar range) and *in vivo* activity depends upon an additional contribution from the host immune system [122].

Eflornithine's target in trypanosomes has been confirmed by HPLC-based analyses that show decreases in putrescine (the direct product of ODC) and other polyamines, spermidine and trypanothione, and increases in ornithine (the direct substrate of ODC) and adenosylmethionine levels after 48 h of treatment in rats [123,124]. The knockout of the gene could only be obtained *in vitro* if using putrescine-supplemented medium to bypass the lesion in the polyamine pathway [125]. An RNAi knockdown of ODC expression was also achieved, which mimicked the effects of eflornithine treatment and could be rescued with exogenous putrescine, but not spermidine [126]. In a first global metabolomics approach using the HILIC-Orbitrap approach to the determination of drug action, eflornithine was shown to induce the expected changes to ornithine and polyamine levels (and also revealed unexpected acetylated derivatives) as the most significant drug-induced perturbations [127]. A lack of off-target effects observed during sublethal drug exposure suggested that additional metabolic alterations only become apparent subsequent to the onset of membrane lysis and cell death.

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The specificity of the drug against trypanosomes may relate to the enzyme target being far less rapidly turned over in trypanosomes than in mammalian cells, hence ODC inactivation leads to long-term loss of polyamine biosynthesis in *T. b. gambiense* trypanosomes while enzyme activity is continuously replenished in mammals. In bloodstream forms of the parasite, the drug enters the cell via a transporter-mediated process and recent evidence points to loss of a particular amino acid transporter, *Tb*AAT6, as underlying eflornithine resistance [93,94,128]. This transporter was shown to be lost during selection of resistance to eflornithine and sensitivity to eflornithine was restored when the gene was reintroduced to the resistant trypanosome's genome [128]. Interestingly the RITSeq approach identified *Tb*AAT6 as the only gene whose knockdown related to improved survival in eflornithine [95].

NECT

Recently it was recommended that eflornithine be given with nifurtimox in a combination therapy (NECT) [129] where eflornithine is given by intravenous infusion at 200 mg/kg every 12 h for 7 days (rather than 100 mg/kg every 6 h for 14 days as in monotherapy), in addition to 15 mg/kg nifurtimox orally every day for 10 days.

A rationale behind the combination was that eflornithine leads to depletion of spermidine, a key component of the trypanosome-specific redox active metabolite trypanothione (N^1,N^8-bis(glutathionyl)spermidine). Since nifurtimox was proposed to generate reactive oxygen species, a synergistic effect with eflornithine in redox-sensitive parasites could be inferred [130,131].

Curiously, eflornithine and nifurtimox are not synergistic in *in vitro* assays [127], possibly because nifurtimox's mode of action involves reduction by an unusual type I nitroreductase in *T. brucei* [132] followed by further metabolism to a highly reactive nitrile derivative [133], which may react with macromolecules by acting as a Michael acceptor [133]. In addition to the nitroreductase itself, the RITSeq approach yielded a flavokinase implicated in the synthesis of FMN, the cofactor of the nitroreductase enzyme [95]. Downregulation of proteins of the ubiquinol biosynthetic pathway also yielded improved viability in nifurtimox, pointing to a physiological role for the nitroreductase in the ubiquinol reduction pathway. The recent untargeted metabolomic analyses provide evidence for this theory with increases in nucleotides and nucleobases suggestive of DNA and RNA breakdown after nifurtimox treatment *in vitro* [127]. Of note too is that metabolomic changes with eflornithine and nifurtimox are additive rather than synergistic.

Conclusion

The biochemical investigation of drug mechanisms has long interested parasitologists, and previous studies have supported numerous hypotheses regarding the mechanisms responsible for the trypanocidal activity of existing drugs. Recent

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advances in metabolomics technology provide a new tool to investigate the metabolic impact of each drug from an untargeted (hypothesis-free) perspective, and a recent proof-of-concept study has revealed the mode of action of eflornithine and confirmed a metabolic activation of nifurtimox [127]. Knowledge of the mechanisms of existing drugs will support optimal utilization of these drugs by consideration of potential resistance mechanisms, drug interactions, adverse effects, and contraindications. Perhaps more importantly, elucidation of the mode of action of existing drugs will allow rational design of new compounds that retain optimal trypanocidal activity, but overcome the existing drawbacks with respect to pharmacokinetics and toxicity (an approach recently demonstrated for peroxide antimalarials based on the action of artemisinin [134]).

The potential role of metabolomics in pharmacology extends beyond the investigations of drug mechanisms described here, including biomarker discovery, which has enormous potential for diagnostic applications and monitoring of treatment response. Metabolomics may also provide improved methods for detection of drug toxicity and understanding of the mechanisms responsible for host toxicity and adverse effects.

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