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The Role of Metabolomics in Antiparasitic Drug Discovery

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Abstract

Metabolomics involves the global measurement of small molecule metabolites in a biological system and allows the comprehensive investigation of metabolic pathways in pathogenic parasites. Metabolomics studies have enabled the discovery of novel aspects of parasite metabolism that constitute attractive drug targets, and have elucidated metabolic targets of antiparasitic compounds. This chapter provides an introduction to parasite metabolomics and describes metabolomics methods suitable for kinetoplastid and apicomplexan parasites. Several examples are provided describing applications of metabolomics to understand the mechanisms of action and resistance for a range of antiprotozoal compounds. This unbiased elucidation of drug mechanisms with metabolomics will facilitate the discovery and development of new drugs for parasitic diseases of global importance.

Introduction

Protozoan parasites are a diverse group of eukaryotic pathogens that are responsible for a variety of life-threatening infections in humans, including malaria (*Plasmodium* spp.), African Trypanosomiasis (*Trypanosoma brucei*), Chagas disease (*Trypanosoma cruzi*), and Leishmaniasis (*Leishmania* spp.). These diseases primarily affect tropical and subtropical regions of the world and cause significant morbidity and mortality as well as exacerbate poverty and economic hardship in developing countries. More than 1 million deaths occur annually as a result of protozoan-related diseases [1], and approximately 3.8 billion people are threatened by the risk of infection each year [2].

Control and prevention of these parasitic diseases are heavily reliant on antiprotozoal drug therapy, as there are currently no safe and highly effective vaccines. Unfortunately, the currently used antiprotozoals are severely limited

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by liabilities including poor efficacy, drug resistance, toxicity, high cost, and inadequate pharmacokinetic properties. Exacerbating this problem, the majority of antiprotozoal drugs currently in clinical use act by as-yet-unknown modes of action. This impacts our ability to optimize treatment strategies, understand parasite resistance mechanisms, and monitor drug efficacy and toxicity. In the context of these liabilities and the unlikely prospect of effective vaccines for human protozoan parasites in the near future, there is an urgent need for the discovery of new drugs that are safe, effective, and act by novel mechanisms. Despite this necessity, there has been an ongoing failure to invest into basic research and development for these diseases [3, 4]. Consequently, there are significant gaps in our fundamental understanding of the biology of these pathogens [4] and a paucity of new therapeutic products and new chemical entities being approved for the treatment of these tropical diseases [5].

The successful development of tropical medicines faces unique challenges. The ideal drug should be cheap to manufacture and distribute, be orally active, and require only once daily administration or result in a single dose cure. Furthermore, complexities in parasite biology must also be considered, such as latent parasite forms, the location of parasites within host cells, drug efflux mechanisms, and the existence of multiple life-cycle stages. Unfortunately, there is a dearth of validated molecular targets for parasitic diseases due to the lack of detailed information concerning the targets of currently used antiprotozoal drugs. These issues represent major obstacles for the success of drug discovery efforts in parasitic diseases [6].

Thousands of antiparasitic hit compounds have been identified in recent years, following the application of phenotypic screening of large compound libraries against whole organism systems [7–9]. Lead compounds that emerge from these screening programs inherently possess favorable drug-like characteristics, including cell permeability and inhibition of essential targets, which contribute to the success of this approach. The trypanocidal compounds fexinidazole and SCYX-7158 [3] and the antimalarial compounds KAE609 [10], KAF156 [11], and MMV390048 [12] have all advanced to preclinical and clinical trials after emerging from phenotypic screening programs. The phenotypic screening approach, however, forgoes mechanistic information relating to drug mode of action. As such, the elucidation of drug targets and mechanistic information from hits derived from these screens is now a primary goal [13]. An untargeted approach aimed at discerning drug modes of action, which does not rely on *a priori* knowledge of a drug's mechanism of action, will greatly improve the drug discovery process for parasitic diseases by informing rational drug design, and improve our existing understanding of the medicines currently in clinical use [14]. Untargeted metabolomics is an emerging discipline that offers a rapid, unbiased, and system-wide approach for the investigation of parasite metabolic pathways, the elucidation of drug modes of action, and the discovery of likely drug targets.

Principles of Metabolomics

The field of metabolomics is the branch of omics technology concerned with the high-throughput identification and quantification of low-molecular-weight molecules (<1500 Da) in a biological system [14–16]. The complement of small molecules in a system, known as the *metabolome*, includes a range of chemically diverse species, of varying abundance, that are produced through the interactions of larger macromolecules, including DNA, RNA, and proteins. Its close relationship with the phenotype makes metabolomics ideal for assessing the physiological state of an organism or system at a snapshot in time. Consequently, metabolomics has found applications in a diverse range of fields, such as the nutritional sciences [17], personalized medicine [18], toxicology [19], and biomarker discovery for disease diagnosis and monitoring in a variety of cancers [20–22], cardiovascular diseases [23–25], and neurological conditions [26, 27]. Metabolomics is a promising tool for antiparasitic drug discovery, and it has already demonstrated utility in the validation of known targets and the discovery of new drug targets for a range of diseases.

Metabolomics in Drug Discovery

Perturbations to metabolic pathways underpin many disease-state processes, and the identification of enzyme targets within these pathways may represent novel sites for therapeutic intervention. The application of metabolomics to cancer has provided further insight into potential drug targets in energy production pathways within cancer cells, which rely on aerobic glycolysis in a process known as the *Warburg effect* [28].

As untargeted metabolomics provides a global measurement of metabolic alterations within a system, this unbiased methodology can reveal changes to novel or unexplored biochemical pathways, offering a distinct advantage over traditional enzyme inhibition assays. This approach has been applied to neuropathic pain models in rats, which revealed dysregulation of sphingomyelin–ceramide metabolism [29], with the metabolite *N,N*-dimethylsphingosine (DMS) highly abundant in rats with neuropathic pain. In this case, metabolomics has revealed a previously unexplored target for therapeutic intervention, as inhibition of DMS production could serve as a potentially attractive therapeutic option for neuropathic pain [29].

In response to viral infection, metabolomics has been used to identify the change in abundance of a number of key metabolites *in vitro*. Human fibroblasts infected with influenza A virus (IVA), herpes simplex virus type-1 (HSV-1), and human cytomegalovirus (HCMV) revealed significant metabolic perturbations that were consistent with the pharmacology of known antivirals and provided insight into possible new antiviral targets [30]. IVA-infected fibroblasts showed a significant upregulation of acetylneuraminic acid, consistent with the mechanism

of action of the anti-influenza drugs such as oseltamivir, which selectively inhibit viral neuraminidase. In the case of HSV-1, changes in the 2'-deoxynucleotides, dTTP and dTMP, were seen, which is consistent with the mechanism of action of HSV-1 treatments such as acyclovir that inhibit viral thymidine kinase. Combined with metabolic flux analysis, metabolomics on HCMV-infected cells revealed changes to tricarboxylic acid (TCA) cycle intermediates and flux through fatty acid biosynthesis, via the enzyme acetyl-coenzyme A carboxylase, as the most significant perturbations [30]. This enzyme is not a current antiviral target but may represent a useful target for future intervention for the treatment of HCMV [30].

Understanding the targets of anti-infective drugs currently in use and those in the developmental pipeline is invaluable for informing rational drug design and overcoming resistance. The antimicrobial triphenylbismuthdichloride was found to inhibit the bacterial pyruvate dehydrogenase complex in a metabolomics investigation in *Staphylococcus aureus* [31]. The identification of this enzyme as an effective drug target now offers new hope in the potential treatment of multidrug-resistant bacterial pathogens [31].

In the field of antiprotozoal drug discovery, metabolomic analysis of sensitive and resistant parasites that have been treated with antiprotozoal compounds can detect drug-induced perturbations to specific parasite metabolites and pathways, offering a global, unbiased approach to discovery of potential drug targets as well as mechanisms of drug action and resistance [14, 28]. It also offers a system-wide approach to analyze parasite biochemistry and host – parasite interactions, leading to the identification of essential parasite pathways and novel enzymes that can be targeted for therapeutic intervention. This review discusses current metabolomics methodology, the application of metabolomics to antiparasitic drug discovery and summarizes the impact of antiprotozoals on kinetoplastid and apicomplexan parasite metabolism.

Metabolomics Methodology

Protozoan parasites contain complex mixtures of thousands of small molecules that vary greatly in their chemical properties and abundance. The nature of the metabolome is highly dependent on both the genome and the environment and is unique for different stages of the parasite life cycle. Therefore, metabolomics analysis requires a validated and highly robust methodology (Figure 14.1) to reproducibly measure the most comprehensive range of metabolites from each sample, in order to obtain the most reliable approximation of the organism's physiology under defined conditions [32, 33].

Study Design

The design of metabolomics experiments can vary widely depending on the aim of the study. A truly untargeted metabolomics approach is suitable for

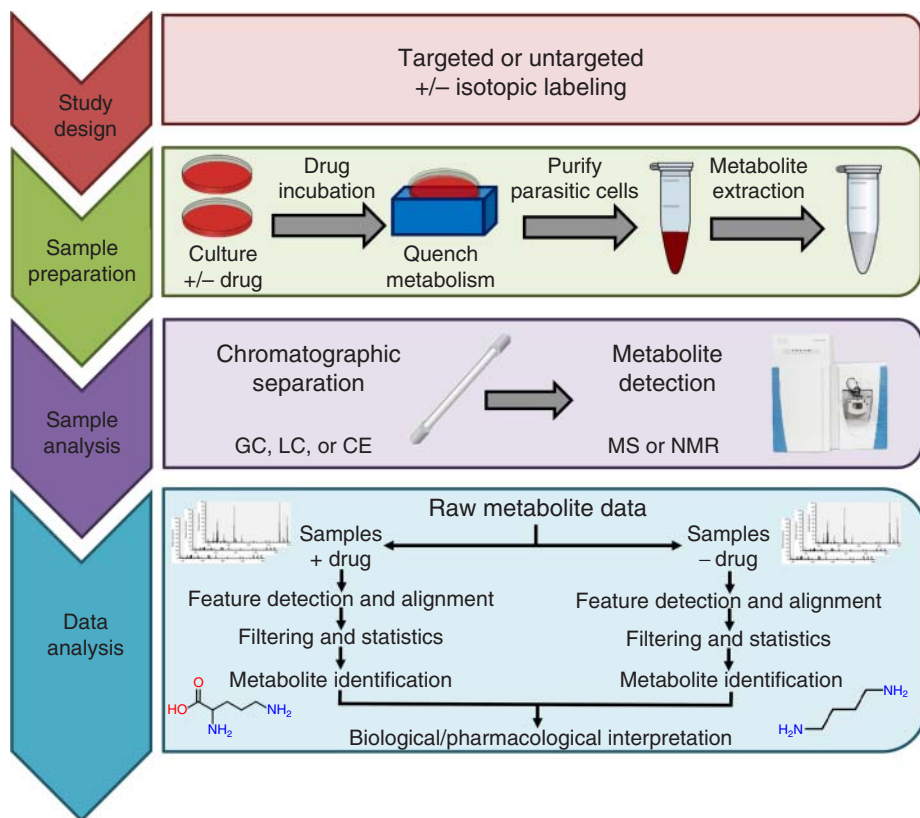


Figure 14.1 General metabolomics workflow for *in vitro* drug mechanism of action studies using protozoan parasites. Parasites are grown in culture and incubated with the test compound for the desired duration. Metabolism is then quenched by rapid cooling, and the parasitic cells may be isolated before metabolite extraction using organic solvent. Metabolite extracts are then analyzed using an appropriate detection method

such as mass spectrometry (MS) or nuclear magnetic resonance (NMR) after chromatographic separation with either gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE) after chromatographic separation. The raw metabolite data must then be processed to determine specific drug-induced metabolic perturbations responsible for parasite death.

hypothesis-free studies, such as the unbiased discovery of drug targets for novel compounds or clinical biomarker discovery. While it is not possible to measure every metabolite in a single experiment, the extraction and analytical conditions can be optimized to detect a wide range of metabolites with diverse chemical properties, and this often involves a combination of analytical techniques and data processing approaches [28, 34]. An alternative approach is to apply targeted metabolomics, whereby a predefined range of metabolites or pathways are analyzed. This approach is suitable for hypothesis-testing experiments, allows

improved sensitivity and quantification accuracy, and may be combined with stable isotope labeling to perform flux calculations [14, 35, 36].

The metabolic response of parasites to drug treatment can be measured in *in vitro* or *in vivo* systems, although host factors may complicate biochemical interpretation in the latter. *In vitro* cell culture systems allow metabolism to be studied in a controlled environment, which is important to delineate pharmacological effects from background variations in parasite metabolism. The choice of growth media is an important consideration, as nutrient availability can interfere with drug action or metabolic response, as demonstrated for the trypanocidal activity of antifolate drugs [37]. Drug incubation times and concentrations can be tuned to investigate primary and secondary effects of drugs on metabolism, and it is generally desirable to obtain samples before a generalized death phenotype is apparent. For optimal statistical power, it is important to have a minimum of four separate biological replicates, and the inclusion of time-series analysis can add further confidence and insight into temporal responses [38].

Sample Preparation

Accurate analysis of intracellular metabolism requires rapid quenching of cell metabolism to minimize the introduction of unwanted variance during sample preparation. Lowering the sample temperature to 0 °C is the most common quenching method for protozoa. Alternative quenching procedures developed in other microbes, such as filtration or freezing in cold methanol, are generally unsuitable for protozoan parasites due to metabolite leakage (DJ Creek and MP Barrett).

Once metabolism has been quenched, parasites may be washed and/or isolated from host cells, followed by extraction of metabolites and removal of macromolecules. Organic solvent extractions are generally effective and compatible with downstream mass spectrometry analysis (Table 14.1).

Sample Analysis

The combination of chromatographic separation paired to mass spectrometry (MS) allows for the most comprehensive analysis of highly complex metabolomics samples. Other techniques including Nuclear Magnetic Resonance (NMR), Fourier Transform Infra-red (FTIR), and Raman spectroscopy may also be applied, albeit with lower metabolite coverage.

Mass spectrometry provides sensitive detection of a wide range of metabolites, and the simultaneous identification of hundreds of metabolites has been facilitated by advances in high-resolution mass spectrometry (Time-of-Flight, Orbitrap, and FT-ICR). Sensitive detection and identification of metabolites are further improved by coupling MS to chromatographic separation techniques, such as gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE). GC produces high-resolution and reproducible separation

Table 14.1 Metabolomic quenching and extraction techniques for *Plasmodium* and *Trypanosomatid* parasites.

Parasite	Life-cycle stage	Quenching method	Extraction method
<i>Plasmodium</i> spp.	Blood stage	Not quenched	90% methanol [39]
	Blood stage	4 volumes of 100% methanol at -70°C	80% MeOH on dry ice [40]
	Blood stage	Not quenched	100% MeOH then MeOH:H ₂ O 8:2 plus sonication [41]
	Saponin extracted	Frozen -80°C	Chloroform:MeOH:ACN 2:1:1 plus steel beads, then TissueLyser [42]
	Various blood stages	Not quenched	100% MeOH on dry ice, then 80% EtOH with sonication [43]
	Blood stage	Methanol/water/chloroform 4:3:1	Biphasic liquid–liquid extraction at pH-adjusted solvents [38]
	Blood stage	Rapid cooling in dry ice ethanol bath	Chloroform:methanol:water 1:3:1 shaking 1 h [44]
<i>Trypanosomatids</i>	Blood stage	Ice-cold PBS	80% ACN [45]
	Blood stage	Rapid cooling in dry-ice ethanol bath	Chloroform:methanol:water 1:3:1 shaking 1 h [46, 47]
	Blood stage	Not quenched	80°C ethanol 2 min [48, 49]

and is ideal for volatile compounds, and has been applied to the sensitive detection of *Plasmodium*-infected blood [50]. However, nonvolatile compounds require derivatization, resulting in low throughput, sensitivity, and reproducibility [51].

LC methods are readily coupled to high-resolution MS and are ideal for the analysis of a wide range of soluble metabolites. The availability of diverse LC column chemistries allows the analytical method to be optimized for certain metabolite classes [14, 51].

Reversed-phase chromatography is the most common form of chromatography applied to global metabolite profiling studies. This method is highly robust and provides extensive coverage of lipids and nonpolar secondary metabolites, but is not appropriate for the analysis of polar compounds, which constitute the majority of metabolic intermediates in the essential central metabolic pathways.

The addition of ion-pairing reagents can improve retention and resolution of polar metabolites; however, these are often not compatible with MS detection. Hydrophilic interaction chromatography (HILIC), ion chromatography (IC), and CE are well suited for the profiling of primary metabolites, and HILIC has been extensively applied to parasite metabolomics [14].

Method validation is a key consideration when selecting the ideal method or combination of methods for a metabolomics study. While metabolite coverage is of primary concern, method reproducibility is of critical importance to allow label-free semiquantitative comparisons of treated and untreated samples in a statistically robust manner. The inclusion of multiple internal standards, and periodic analysis of matched quality control samples, is essential to monitor analytical performance and potentially correct for signal drift.

Metabolite Identification

The identification of metabolites in untargeted metabolomics studies is a key bottleneck that must be overcome to infer biological or pharmacological meaning. The Metabolomics Standards Initiative has defined four different confidence levels by which a metabolite is identified [52]. The highest level of identification confidence (level 1) requires the comparison of two or more orthogonal properties with authentic chemical standards, which have been analyzed under identical analytical conditions. In practice, this is achieved by the combination of MSMS or retention time data with high-resolution mass spectrometry. However, it is important to note that successful dereplication of isomeric species often requires optimization of the chromatographic or fragmentation parameters. In the absence of authentic chemical standards for every parasite-derived metabolite, external databases or predictive algorithms for mass, MSMS, and/or retention time may be used to provide putative identification (level 2). Unidentified metabolite features from known (level 3) or unknown (level 4) metabolite classes should also be reported, as these may represent novel endogenous or drug-derived metabolites [53, 54].

Data Analysis

Any metabolomics analysis will produce large datasets; therefore, it is essential that the data are analyzed in an efficient and meaningful way to measure the relative abundance of hundreds of metabolites [28]. Several commercial and open-source software packages are available for the detection and alignment of metabolite features (peaks) and downstream artifact filtering, metabolite identification, statistical comparison, and pathway or network modeling [55].

Metabolomics for Drug Discovery in Kinetoplastid Parasites

Kinetoplastida comprise a phylogenetically ancient group of protozoa, many of which can cause significant diseases in humans and animals. They are responsible for extensive morbidity and mortality in the developing world and also place a substantial economic burden on the countries that are affected [56, 57]. These protozoa include the South American trypanosome, *T. cruzi*, which causes

Chagas disease; the *T. brucei* subspecies, which causes human African trypanosomiasis (HAT); and multiple *Leishmania* species, which cause various forms of Leishmaniasis.

Human African Trypanosomiasis

HAT, also known as *sleeping sickness*, is caused by infection of the host with *Trypanosoma brucei rhodesiense* or *Trypanosoma brucei gambiense* [58, 59] following a bite by the tsetse fly vector. In the first stage of the disease, known as the hemolympathic phase, nonspecific symptoms such as headache and fever are most common. Stage 2 is considered the neurological phase and occurs when the parasite crosses the blood–brain barrier, inevitably resulting in death in the absence of effective treatment [60]. Although reported HAT mortality has significantly declined in recent years [2], there are serious concerns regarding drug resistance [49], cost, and toxicity for the currently available arsenal of trypanocidal medicines. Treatment options for stage 1 of the disease include suramin and pentamidine, both of which must be administered by injection [61, 62], while stage 2 treatment utilizes melarsoprol or eflornithine, either as monotherapy or in combination with nifurtimox [63–65].

A metabolomics approach has been used to explore the mechanisms of action of several trypanocidal compounds in *T. b. brucei* [66, 67]. Untargeted metabolomic analysis of eflornithine has confirmed its mode of action. Eflornithine treatment induced predictable changes in the levels of polyamine pathway intermediates, resulting in significant accumulation of ornithine and depletion of putrescine, mediated by the inhibition of ornithine decarboxylase. Due to the untargeted nature of the method, the analysis also revealed the absence of arginase activity as well as drug-induced changes in N-acetylated ornithine and putrescine and depletion of the downstream polyamine pathway product spermidine [67]. Polyamines serve an important role in trypanosomes, combining with glutathione to form trypanothione, the major intracellular antioxidant molecule [68–70]. Oxidative stress was also thought to be involved in nifurtimox action [65, 71]. However, metabolomic analysis revealed additive, but not synergistic, metabolic perturbations for eflornithine and nifurtimox, which supports the lack of trypanocidal synergism observed *in vitro* [67]. The untargeted metabolomics analysis of nifurtimox treatment confirmed metabolic activation by nitroreductase to form a reactive nitrile intermediate [72, 73] and revealed changes in endogenous metabolites involved in nucleotide and carbohydrate metabolism [67].

Untargeted metabolomics applied to pentamidine-treated parasites showed nonspecific changes in metabolite levels that could not be directly related to a specific metabolic pathway [37]. Rather, the drug may impact nonmetabolic parasite targets, by interacting with nucleic acids or may even disrupt the mitochondrial membrane potential [74, 75].

Metabolomics has also been utilized for drug target discovery for novel antitrypanosomal compounds. Halogenated pyrimidines have demonstrated high affinity for the trypanosomal uracil uptake transporter, leading to incorporation of 5-fluorouracil into pyrimidine nucleotides and RNA [46]. The analogs 5-fluoro-2'-deoxyuridine and 5-fluoro-2'-deoxycytidine demonstrated prodrug activity, with the active metabolite 5F-dUMP inhibiting thymidylate synthase activity. Overall, these studies showed that halogenated pyrimidines interfere with pyrimidine salvage in *T. b. brucei* by distinct mechanisms [46]. Curcumin analogs bearing a monoene linker motif show highly potent trypanocidal activity. Untargeted metabolomics revealed significant depletion of intracellular thiols, such as trypanothione and glutathione. This was most likely mediated through the rapid formation of drug-thiol adducts and was not apparent in drug-resistant parasites, confirming the link between thiol depletion and trypanocidal activity [53].

Chagas Disease

Chagas disease, or American trypanosomiasis, is caused by infection with the parasite *T. cruzi*, following the bite of an infected triatomine bug. Up to 10 million people are infected [2] with the disease manifested by low-grade fever, lymphocytosis, and hyperthermia [76]. Although the acute phase may remain asymptomatic, it can progress to a chronic phase and cause death by cardiac failure [76]. Benznidazole and nifurtimox are the only licensed medicines with proven efficacy against Chagas disease; however, drug resistance has been identified [77, 78].

Multiple drug-specific metabolomic perturbations were observed in *T. cruzi* following benznidazole treatment [54]. Benznidazole is believed to be activated by an unusual type I nitroreductase, leading to the formation of a dihydroxy-dihydroimidazole derivative, which decomposes to give toxic glyoxal [73, 79]. A metabolomics study identified depletion of low-molecular-weight thiols by covalent binding of activated benznidazole, which is believed to be responsible for the drug's toxicity [54]. This is consistent with the drug's proposed mechanism, involving covalent modification of lipids, DNA, and proteins [80, 81]. However, key oxidation products of nucleotides could not be detected [54].

Leishmaniasis

Infection with *Leishmania* spp. can cause a complex disease state that may manifest as visceral, cutaneous, or mucocutaneous clinical forms. The parasite is transmitted by the bite of a sandfly and, once inside the host, largely resides in macrophages [82]. Drugs currently available for the treatment of Leishmaniasis include miltefosine, amphotericin B [83], and pentavalent antimonials (Sb^V) [84]. Unfortunately, they are restricted by less than ideal pharmacokinetics, toxicity, and poor efficacy. The mode of action for miltefosine has been proposed to involve inhibition of protein kinase B [85], influencing lipid metabolism, GPI anchor

synthesis, and signal transduction [86]. It has also been suggested to interact with lipid monolayers [87], membrane phospholipids [88], and choline transport [89] and induce apoptotic responses including DNA fragmentation and chromatin condensation [90]. Untargeted metabolomic analysis identified increased alkene fragment release, which is proposed to be synchronized with the production of reactive oxygen species [91]. This study also identified signs of DNA damage by detecting changes in thiols and polyamines, loss of cell membrane integrity, and intact native membrane phospholipids [91]. Amphotericin B is believed to inhibit glucose uptake and compromises the permeability barrier to small metabolites in *L. donovani* promastigotes by affecting membrane sterols [92]. Sb^V is believed to inhibit trypanothione reductase activity in the parasite [93] and stimulate macrophages to kill their intracellular invaders [94]. An untargeted metabolomic approach has also been used to investigate the mechanisms of resistance to antimonial drug combinations in *L. donovani*. Experimentally generated resistant lines demonstrate metabolic adaptations that protect against drug-induced and external oxidative stress, in addition to changes in membrane fluidity and drug uptake [84].

Metabolomics for Drug Discovery in Apicomplexan Parasites

The *Apicomplexa* phylum contains several human pathogens that are responsible for significant morbidity and mortality worldwide, including five species of *Plasmodium* that cause human malaria. The *Plasmodium* parasite has a complex life cycle and is transmitted by the bite of the female *Anopheles* mosquito during a blood feed. Patients experience symptoms during the parasite's asexual replication within host red blood cells, and current treatments act on this stage. However, resistance has now emerged to the first- and last-line therapeutic agents, the artemisinins, which threatens to make the disease untreatable [95]. Use of other antimalarials, such as quinolines and antifolates, is already limited by widespread resistance, highlighting the urgent need for new antimalarials with novel modes of action.

Antimalarial Drug Mechanisms

Several approved antimalarial medicines act by perturbing parasite metabolism. Metabolomics, as an approach to detect drug-induced changes in parasite metabolism, has been validated with studies of the antimalarial atovaquone [96]. Atovaquone acts by inhibiting the cytochrome bc₁ complex in the mitochondrial electron transport chain, consequently leading to the dysfunction of dihydroorotate dehydrogenase (DHODH). DHODH dysfunction perturbs pyrimidine synthesis, which is ultimately fatal to the parasite [97, 98]. As expected, metabolomics analysis of atovaquone-treated *Plasmodium falciparum*-infected red blood cells (iRBCs) showed an accumulation of the substrate of DHODH,

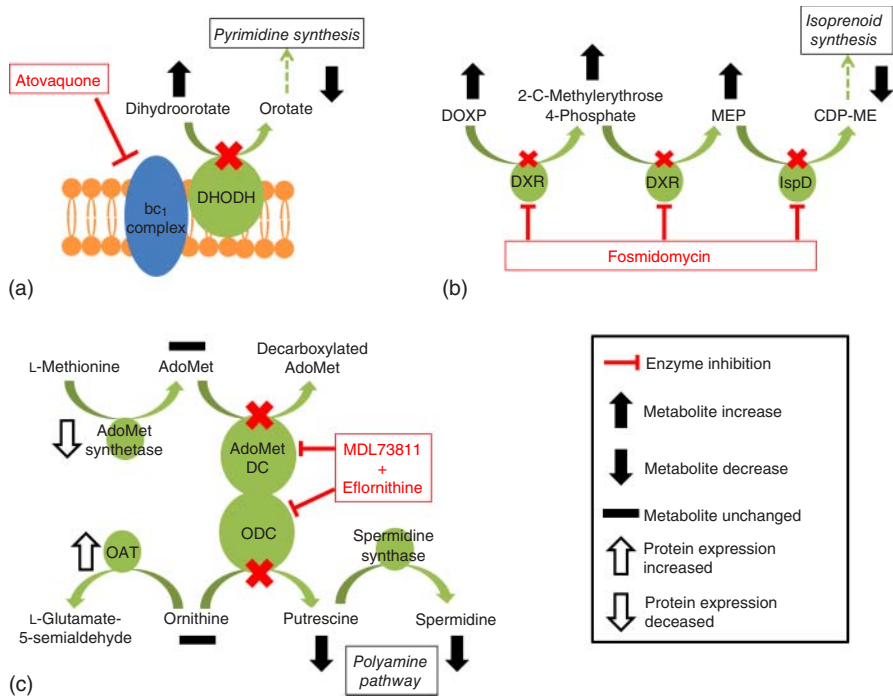


Figure 14.2 Schematic of metabolic mechanisms of action of three anti-apicomplexan compounds. (a) Atovaquone inhibits the cytochrome bc₁ complex in the mitochondria. This inhibits oxidation of the ubiquinone cofactor resulting in dysfunction of dihydroorotate dehydrogenase (DHODH) such that dihydroorotate accumulates and pyrimidine synthesis is disrupted [96]. (b) Fosmidomycin inhibits deoxyxylulose phosphate reductoisomerase (DXR) causing accumulation of 1-D-deoxyxylulose 5-phosphate (DOXP). It also inhibits methylerythritol phosphate cytidyltransferase (IspD) causing the accumulation of 2-C-methylerythrose and methylerythritol phosphate (MEP) and the depletion of cytidine diphosphate methylerythritol (CDP-ME). This disrupts

isoprenoid biosynthesis [42]. (c) Together, eflornithine and MDL73811 inhibit the bifunctional enzyme, *S*-adenosylmethionine decarboxylase/ornithine decarboxylase (AdoMetDC/ODC), which leads to the depletion of polyamines and is ultimately fatal to the parasite. The substrate of AdoMetDC, AdoMet, does not accumulate as expected upon AdoMetDC inhibition. This is due to the decreased expression of AdoMet synthetase, as revealed by transcriptomics and proteomics, such that less AdoMet is synthesized. Similarly, the substrate of ODC, ornithine, does not accumulate as expected upon ODC inhibition. This is due to the increase in ornithine aminotransferase (OAT) expression, which degrades ornithine [41].

dihydroorotate, and its precursor, carbamoyl-L-aspartate (Figure 14.2a). Complementarily, the products of DHODH activity, downstream pyrimidine nucleotides, were depleted [45, 96]. Antifolate drugs kill malaria parasites by inhibition of either dihydrofolate reductase (DHFR) or dihydropteroate synthase (DHPS), perturbing folate metabolism and consequently depriving the pyrimidine synthesis pathway of essential intermediates [99]. This depletion of thymidine nucleotides

by cycloguanil has been confirmed using metabolomics methodology [45]. Another antifolate, proguanil, mediated dysregulation of arginine metabolism, suggesting that a novel mechanism may be involved in the antimalarial mechanism of this drug. Other antimalarials including dihydroartemisinin and chloroquine induced perturbations in hemoglobin-derived peptides, consistent with their proposed mechanisms of action involving the parasite digestive vacuole [45].

Plasmodium Drug-Resistance Mechanisms

Resistance has emerged to all available antimalarial drugs, and resistance mechanisms must be considered during the development of new antimalarials. Genomic sequencing is effective at detecting the genetic mutations associated with drug resistance, but does not always elucidate the mechanisms responsible for resistance. Chloroquine (CQ) resistance is primarily associated with mutations in *P. falciparum* CQ-resistance transporter (*pfcr*t) [100]. However, the molecular mechanisms by which point mutations in PfCRT facilitate CQ resistance remain unclear. Metabolomics revealed an accumulation of many hemoglobin-derived peptides in lines of *P. falciparum* expressing the CQ-resistant form of PfCRT compared to lines expressing the CQ-sensitive form [39]. This suggests impaired hemoglobin digestion in CQ-resistant parasites. Indeed, there was a fitness cost attributed to the expression of the CQ-resistant form of PfCRT [39], which could explain the reemergence of CQ-sensitive parasites in the field following cessation of widespread CQ use.

Novel Anti-Apicomplexan Compounds

Based on the success of atovaquone, differences in human and parasite electron transport chains have been investigated as potential drug targets for malaria. It was found that *P. falciparum* lacks a canonical NADH dehydrogenase and instead possesses a bacterial-like type II NADH:ubiquinone reductase (PfNDH2) situated upstream of the bc₁ complex in the electron transport chain [101]. Biagini *et al.* [96] led a drug discovery initiative to find drugs with activity against PfNDH2 that could kill atovaquone-resistant parasites. Upon treatment of iRBCs with the novel quinolone compound, CK-2-68, metabolomics detected perturbations consistent with DHODH dysfunction. This confirmed that the mechanism of action of CK-2-68 was via disruption of the electron transport chain [96].

Investigation of host and parasite metabolism led to the discovery that, unlike humans, malaria parasites possess a nonmevalonate isoprenoid biosynthesis pathway [102]. Several antibiotics target this pathway in bacteria, and when tested, it was found that these antibiotics had antimalarial activity [103]. Fosmidomycin was one such drug, which blocks isoprenoid synthesis in *Escherichia coli* via inhibiting deoxyxylulose phosphate reductoisomerase (DXR) [104]. Targeted metabolomics of fosmidomycin-treated *P. falciparum* showed the anticipated

accumulation of DXR's substrate, 1-D-deoxyxylulose 5-phosphate (DOXP), but did not detect the expected depletion of its product, methylerythritol phosphate (MEP). In fact, MEP levels increased. There was depletion in metabolites further downstream in the isoprenoid pathway, including cytidine diphosphate methylerythritol (CDP-ME), the product of the enzyme methylerythritol phosphate cytidyltransferase (IspD) (Figure 14.2b). This suggests that fosmidomycin may also target IspD, which was supported by *in vitro* inhibition of recombinant IspD. Supplementation of fosmidomycin-treated parasites with a downstream isoprenoid was able to rescue *P. falciparum*, suggesting that fosmidomycin exclusively targets the isoprenoid pathway [42]. Fosmidomycin is currently in clinical trials in combination with clindamycin [105, 106] for uncomplicated malaria.

Eflornithine, when coadministered with a bis(benzyl)polyamine analog, can cure mice of the rodent malaria strain, *Plasmodium berghei* [107]. Together, eflornithine and the bis(benzyl)polyamine analog, MDL73811, inhibit the bifunctional *S*-adenosylmethionine decarboxylase/ornithine decarboxylase (AdoMetDC/ODC) enzyme, blocking polyamine synthesis [108, 109]. To investigate polyamine depletion as an antimalarial strategy, a combined transcriptomics, proteomics, and metabolomics study was performed [41]. Metabolomics confirmed that polyamine depletion is induced by eflornithine-MDL73811 (Figure 14.2c). Surprisingly, the substrate of AdoMetDC AdoMet (*S*-adenosylmethionine), did not accumulate as expected, upon inhibition of the enzyme. Proteomics and transcriptomics detected a decrease in AdoMet synthetase such that less AdoMet would be synthesized, revealing a compensatory feedback mechanism in *P. falciparum*. Furthermore, the substrate of ornithine decarboxylase (ODC), ornithine, did not accumulate upon drug treatment as occurs in trypanosomes. This can be explained by the increase in ornithine aminotransferase (OAT) expression, which degrades ornithine.

Summary

Due to the lack of information on the mechanisms of action of current drugs, there are very few well-defined clinically relevant drug targets for protozoan parasites. Therefore, recent high-throughput screens of large compound libraries belonging to pharmaceutical companies [7, 110] have been invaluable for identifying novel antiparasitic compounds from a diverse range of chemical classes. However, there is a significant bottleneck in the development of these compounds for clinical use due to a lack of information regarding their mechanism of action [13]. Metabolomics represents a novel approach for understanding parasite biology and provides a valuable way to capture a global, untargeted perspective on drug-induced changes to parasite metabolism, which can then generate hypotheses regarding mechanisms of drug action. This will support the rational design of novel antiparasitic compounds that overcome issues relating to drug toxicity, resistance, and poor pharmacokinetic properties. It will also aid in the optimal utilization of the current repertoire of drugs by better guiding combination

therapies and the monitoring of efficacy and resistance. Metabolomics has already revealed the modes of action of multiple antiparasitic drugs [53, 67, 91, 96] and has uncovered many fundamental aspects of parasite biology such as isoprenoid synthesis in *P. falciparum* [42] and pyrimidine metabolism in *T. brucei* [46] that could be targeted for therapeutic intervention.

Metabolomics has been driven forward by the recent advances in analytical technologies that allow for greater metabolite coverage from complex biological matrices with increased sensitivity and resolution [111]. This will continue to improve in coming years, and the ability to efficiently analyze and biochemically interpret omics scale data will likely be the limiting steps in the progression of metabolomics in the drug discovery field. However, the improvement and accessibility of a variety of freely available data analysis packages are beginning to overcome this hurdle. The interpretation of metabolomics data, and the ability to link changes in metabolite levels with drug mechanisms, largely relies on our knowledge of parasite biochemical pathways and their interactions with host cell metabolism, which is unfortunately not completely understood [14]. Metabolomics can facilitate traditional biochemical approaches to more comprehensively annotate and characterize parasite metabolites and pathways, thus enhancing our ability to interpret drug-induced metabolic perturbations.

The integration of metabolomics data across multiple levels of systems biology, including the proteome, transcriptome, and genome, also offers huge potential for drug discovery. It will allow mechanisms of drug action to be discerned in more detail and allow researchers to better understand modes of resistance from a system-wide perspective, across multiple molecular levels. The advent of metabolomic ^{13}C -flux analysis also provides further in-depth investigations of drug mechanisms and potential biochemical targets by identifying choke points in parasite metabolism [112]. When combined with computational approaches, network analysis can help explain therapeutic effects and aid in the development of predictive *in silico* models for drug action, which may offer extensive financial savings during the drug discovery process [113, 114].

Metabolomics is not only limited to the investigation of drug mechanisms of action and the identification of drug targets, but also has applications across the entire drug discovery pipeline, including identification of biomarkers and the analysis of off-target actions that could explain toxicity and adverse effects. Metabolomics has already demonstrated utility in drug discovery for parasitic diseases and will play an important role in its future, fostering the development of novel drugs to combat these life-threatening infections.

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