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## Discovery of the Mechanism of Action of Novel Compounds That Target Unicellular Eukaryotic Parasites

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### Abstract

In recent years, most new candidate antiparasitic drugs have been found by screening huge numbers of compounds for their ability to kill parasites, followed by counterscreening for toxicity to mammalian cells. Several public–private initiatives have supported this, yielding many hits each for Plasmodia and Kinetoplastids. From these, candidates are selected for further investigation. Although knowledge of the precise mode of action is not necessary for successful development, detailed understanding of the drug's uptake, activation, and target can be very useful in guiding medicinal chemistry, toxicology, and pharmacology. Knowledge of the target can also provide information for further drug discovery studies and in choosing partner drugs in combinations. A multiplicity of complementary approaches can be applied to investigate the drug mode of action. Examples include selecting drug-resistant parasites and identifying the resistance-causing mutations, reverse genetics to find genes required for drug susceptibility, metabolomics, and biochemical approaches such as affinity purification. Here, we review the myriad possibilities, including numerous examples.

### Introduction

The development of new antiparasitic drugs is a necessary process, because many of the currently used drugs are unacceptably toxic and resistance is emerging [1, 2]. This review focuses in particular on compounds against *Plasmodium* spp., *Leishmania* spp., *Trypanosoma brucei*, and *Trypanosoma cruzi*. Multiple initiatives, for example, the public–private partnerships Drugs for Neglected Diseases initiative (DNDi, [www.dndi.org](http://www.dndi.org)) [3] and Medicines for Malaria Venture (MMV, [www.mmv.org](http://www.mmv.org)) [4] were founded to support discovery and approval of new drugs. There is continued discussion concerning the virtues of different drug discovery

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methods [5–7]. In the phenotypic drug discovery approach, large numbers of compounds are screened for their ability to kill the target pathogen, without regard to possible mechanism of action (MoA) [5, 7, 8]. The target-based approach, in contrast, first focuses on a particular mechanism and second considers the ability to kill the pathogen [5, 9]. A few years ago, an analysis of all approved first-in-class compounds showed that the phenotypic approach has, in practice, been more successful for the development of licensed drugs targeting infectious diseases [5]. As a consequence of these observations, efforts have been redirected toward phenotypic screens [10]. In the last few years, under the auspices of public–private partnerships, millions of chemical compounds have been tested for their abilities to kill protist pathogens without affecting the mammalian cells [11, 12]. As a result, thousands of drug-like molecules are now available for potential development.

Phenotypic screening is an agnostic approach: target, activation pathway (if relevant), and entrance route – which together are summarized as MoA – are initially not known. Indeed, drugs can achieve clinical approval even if their MoA is unknown [6, 7, 13]. Nevertheless, MoA knowledge is extremely helpful for discovery programs [6]. Target identification aids medicinal chemists, since 3D structure determination illuminates structure–activity relationships [14] and thus facilitates lead optimization [15]. MoA knowledge can also help to predict and monitor possible resistance emergence, as well as on-target side effects [16]. Even if a particular drug candidate is unsuccessful, knowledge of its MoA will suggest development of alternative compounds with the same MoA. Finally, compounds with novel MoA deepen our understanding of parasite biology [14, 16].

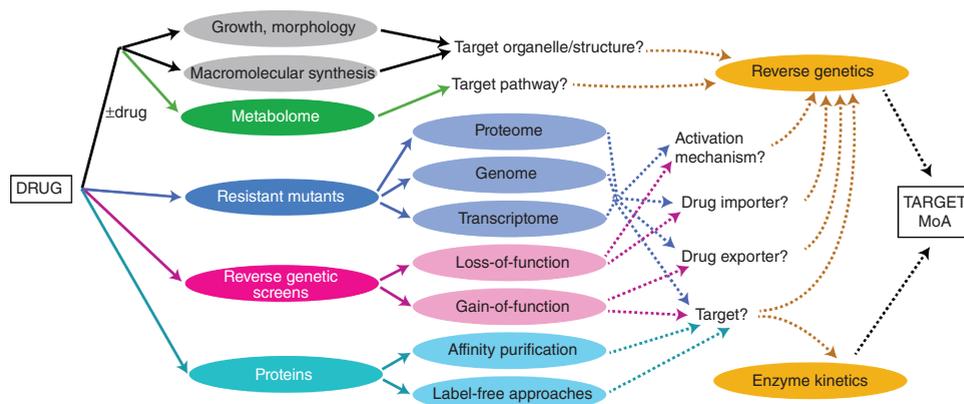
A multitude of techniques is available for target deconvolution. Usually, it is necessary to integrate several complementary approaches [16, 17]. Once a possible target has been identified, it must be validated by independent methods [18]. Here we give an overview of different method, and present examples – usually for molecules that target parasitic protists, but occasionally also other organisms (Table 1.1).

## Principles

A drug that encounters a pathogen must be taken up first, which may involve either passive diffusion or active transport [2]. In some cases, it may need to be transferred to an organelle and/or activated by pathogen enzymes [2]. The activity of the drug in the cells may involve inhibition of a single or multiple enzymes, binding to macromolecules, or less specific toxicity [6]. In the latter case, selective toxicity of the drug for the pathogen, rather than the host, must rely either on accumulation of the drug in the pathogen or a particular compartment or on activation by a pathogen-specific pathway.

Table 1.1 Overview of drugs often cited in the text.

Compound or class name	Other names – related compound	Target organism	Genes implicated in mode of entrance	Genes implicated in mode of action and resistance
Antimonials	SbV, SbIII	<i>Leishmania</i> spp.	Entrance through AQP1 [80]; export through PGPA [98]	SbV is reduced to SbIII [76–78]; nonspecific toxicity Targets phosphatidylinositol-3-kinase [105]; resistance can be caused by mutation in Kelch-13 propeller [104] Inhibits cytochrome b/c [106] Activated by type 1 NTR [57]; probably causes oxidative stress [107] Probably inhibiting hemozoin formation [37, 38]; PfMDR1 is involved in resistance [64]
Artemisinin		<i>Plasmodium</i> spp.		
Atovaquone		<i>Plasmodium</i> spp.	Possibly through TcABCG1 [100]	
Benznidazole		<i>T. cruzi</i>	PfCTR, probably in vacuoles [63, 64]	
Chloroquine		<i>P. falciparum</i> , <i>Plasmodium malariae</i>		
Difluoro-methylornithine	DFMO,	<i>T. brucei</i>	Entrance through AAT6 [55]	It targets and covalently binds ornithine decarboxylase [50]
Melarsoprol	eflornithine	<i>T. brucei</i>	Low-affinity transporter AT1 [46], most likely high-affinity AQP2 [108, 109]	Mechanism unclear; cross-resistance with Pentamidine [47]
Miltefosine		<i>Leishmania</i> spp.	Transported through LdMT [71], if in the presence of LdRos3 [72]	Probably several activity, among them increased oxidative stress [110, 111]
Nifurtimox		<i>T. cruzi</i> , <i>T. brucei</i>		Activated by type 1 NTR [57]; probably causes oxidative stress ; possibly DNA/RNA degradation [112] Mechanism unclear; cross-resistance with Melarsoprol [47]
Pentamidine	Diamidine	<i>T. brucei</i>	Low-affinity transporter AT1 [46], most likely high-affinity AQP2 [108, 109]	



**Figure 1.1** Schematic representation of the different possible approaches for target deconvolution described in the text.

Figure 1.1 shows a simplified version of the entire target deconvolution process. First, basic characteristics need to be established:

- 1) What effect does the drug have on parasite morphology?
  - 2) Is it cytostatic, cytotoxic, or cytolytic?
  - 3) How long does it take to act?
  - 4) What is the effect on cell cycle/at what stage in the parasite cell cycle does it act?
  - 5) How does it affect macromolecular synthesis?
  - 6) Can possible targets be predicted from the chemical structure?
- Once these basics are known, further steps can be chosen:
- 7) What are the effects on metabolites?
  - 8) Does the drug accumulate within the cell or within a particular subcellular compartment?
  - 9) Can resistant lines be generated? If so, what is the mechanism? Which genes are mutated in resistant lines? Do resistant lines show alterations in gene expression?
  - 10) A library of cells with inducible or constitutive knockdown of all genes is made and selected with drug. Which genes are no longer expressed in the resistant cells?
  - 11) A library of cells with inducible or constitutive overexpression of all genes is made and selected with drug. Which genes are overexpressed in the resistant cells?
  - 12) The drug molecule is attached to an affinity matrix and incubated with cell lysate. Which proteins selectively bind to the drug?  
Finally, potential mechanisms of action are tested:
  - 13) If a mutant gene has been identified in a resistant line: Does introduction of that mutant version into normal cells render them drug-resistant?

Reciprocally, in the resistant line, does replacement of the mutant allele with the wild-type render the cells drug-sensitive?

- 14) If a target enzyme has been identified: Does the drug inhibit that enzyme with a potency that is compatible with the intracellular drug concentration? Do the inhibitory activities of compounds in the relevant series parallel their activities against the target organism?

### Initial Investigations

Initial general tests can give substantial information. Molecules that disrupt, or form pores in, the plasma membrane rapidly cause cell lysis, as do molecules that cause irreversible chemical damage. For example, arsenical drugs that are used to treat late-stage African trypanosomiasis kill cells within half an hour even when added at low nanomolar concentrations, most likely via nonspecific covalent binding to thiol groups and resulting oxidative stress [19]. They derive selective action from accumulation within the parasites (described later). Antimalarial endoperoxides such as artemisinin and derivatives, or synthetic alternatives, are also fast-acting and cytotoxic, probably at least partially via oxidative radicals [20]. Compounds that affect biosynthesis of the plasma membrane are expected ultimately to be cytolytic [21]. In contrast, compounds that inhibit DNA synthesis – whether directly, or via nucleotide depletion – act more slowly and are likely to be cytostatic [22].

### Morphology and Stage Specificity

Some antiparasitics have very specific effects on morphology, which are observed either with live cells or on fixed ones after specific organellar staining. Kinetoplastids, such as *T. cruzi*, *T. brucei* and *Leishmania* spp., contain a large assemblage of mitochondrial DNA called the *kinetoplast*, which consists of concatenated circles (kDNA). After appropriate staining, the kinetoplast is readily seen by light or fluorescence microscopy [23]. Isometamidium – an ethidium bromide derivative that is used to treat cattle African trypanosomiasis – intercalates selectively in the kDNA, probably affecting the kDNA integrity preventing division of the network [24, 25]. In this case, the drug is itself fluorescent, so its accumulation in the kDNA network can be observed directly [25–27]. The action of drugs that affect the mitochondrial membrane potential can be detected by staining with MitoTracker. Salicylhydroxamic acid is an irreversible inhibitor of the trypanosome alternative oxidase, which is required for efficient glycolysis. The first noticeable effect of the drug is a slowing in parasite motility – presumably as a consequence of ATP depletion [28, 29]. Finally, apolipoprotein L1 – not a drug, but a protein of human serum – kills *T. brucei* via damage to the lysosome; this is visible by staining with lysosome probes or immunofluorescence targeting lysosomal proteins [30].

Apicomplexan parasites, such as *Plasmodium* spp., contain a secondary plastid-derived organelle called the *apicoplast*. This contains various pathways

and has a genome that encodes the essential proteins [31]; in the blood form, the only apicoplast-specific metabolic pathway that is required for multiplication and survival is isoprenoid biosynthesis [32]. Compounds that inhibit apicoplast replication and protein expression affect the parasites only gradually: defects in apicoplast division and segregation are seen and the cells undergo “delayed death,” after several cell divisions [33–35]. Effects at particular stages of the cell cycle are often also manifest morphologically. An inhibitor of DNA or deoxyribonucleotide synthesis will most likely cause parasites to accumulate in G1 or S-phase. Intraerythrocytic *Plasmodium* parasites obtain amino acids partially through digestion of hemoglobin in a lysosome-like organelle called the *food vacuole* [36]. The released heme is highly toxic in the soluble state, but is detoxified by biomineralization into insoluble hemozoin crystals [36]. Drugs that inhibit hemozoin formation, such as chloroquine, are not toxic to the early ring stages, but kill the trophozoites, which have very active food vacuoles [37, 38]. Such drugs also cause clear microscopically visible alterations in hemozoin accumulation [39].

### Macromolecular Synthesis

The inhibition of macromolecular biosynthesis is most readily detected by labeling with radioactive precursors followed by trichloroacetic acid (TCA) and/or ethanol precipitation: <sup>35</sup>S-methionine for protein, <sup>3</sup>H-thymidine for DNA, and <sup>3</sup>H-uracil for RNA [40]. To our knowledge, there are currently no examples of drugs that directly target these processes in the nucleus or cytosol of protists: the machineries involved may be too similar to those in the mammalian host. However, effects will be seen from inhibitors that prevent the provision of precursors.

### Structure-Based Prediction

In some cases, possible targets can be predicted from chemical structures. For example, huge numbers of inhibitors of mammalian protein kinases are already well characterized and libraries of kinase inhibitors have been published [41]. Molecules that share the same or similar scaffolds may inhibit parasite kinases (e.g., [42]). This can be tested directly in enzyme assays, if pure proteins and appropriate assays are available.

### Biochemical Methods and Candidate Genes

Simple biochemical methods can be used to test hypotheses concerning drug action – if predictions are possible. Some examples are described as follows. These methods were a mainstay of drug target identification before it was possible to sequence RNA and DNA at the genome/transcriptome level or to identify many proteins and metabolites in complex mixtures. Now, they are used mainly to confirm hypotheses that are generated based on high-throughput data.

## Melarsoprol and Diamidines

Melarsoprol, which is used against trypanosomiasis, is a highly toxic molecule containing arsenic and exhibits strong reactivity with thiols [19]. An explanation for its (rather poor) selectivity for trypanosomes – such as active accumulation – is therefore needed. To find the mechanism of drug import using biochemical approaches, one would ideally measure the drug directly, for example, using radioactively labeled drug, since the amounts from cell samples are unlikely to be sufficient for bioassay [43]. As a surrogate, however, at least for competition assays, the effect of the drug on the organism can be used. The structures of melarsoprol and the diamidines, both drugs used against African trypanosomes, bear some resemblance to adenosine. This suggested to Carter *et al.* [44, 45] that a purine transporter might be involved in drug accumulation. Indeed, adenine, adenosine, and dipyridamole compete for uptake and reduce the effect of the drugs. The responsible transporter was named the P2 transporter [44]. To find the gene encoding the P2 transporter, Mäser used *Saccharomyces cerevisiae* with a defect in adenine biosynthesis. The strain was complemented with a *T. brucei* cDNA library and selected for the ability to grow with adenine as the sole purine source. This resulted in identification of the adenosine transporter 1 (*AT1*) gene, a member of the equilibrative nucleoside transporter family [46]. TbAT1 was validated afterward: cells lacking *AT1* have reduced sensitivity to melarsoprol and diamines [47].

Meanwhile, others followed up the hypothesis that melarsoprol might act via thiol groups and could perhaps be exported as a thiol conjugate. Trypanosomes have several homologs of the multidrug resistance protein (MRP) family, which were first discovered as exporters of anticancer drugs [48]. The major small thiol in trypanosomes is trypanothione, a conjugate of glutathione with spermidine. Prompted by the previous results with *Leishmania* (see the following section), Shahi *et al.* [49] followed a candidate gene approach. They showed that overexpression of one transporter, multidrug resistance protein A (MRPA), can cause a low degree of melarsoprol resistance, especially when coexpressed with two enzymes of trypanothione biosynthesis, ornithine decarboxylase, and gamma-glutamyl-cysteine synthase [49]. These studies, however, partially illuminated an export mechanism rather than the MoA.

## DFMO

Difluoromethylornithine (DFMO or eflornithine) is a suicide inhibitor of ornithine decarboxylase [50]. It inhibits the enzyme in mammalian cells – indeed, it was initially developed for cancer therapy [51], but it has been revived and is currently used to prevent growth of facial hair [52]. The specificity of the drug is due to its irreversible action, combined with much slower turnover of the trypanosome enzyme compared with the human one. The consequent loss of polyamine biosynthesis causes death of the parasites [53]. Although resistance to DFMO can be obtained *in vitro* through overexpression of ornithine

decarboxylase [54], when trypanosomes resistant to eflornithine were selected, no differences were found in the target enzyme [50] or in the metabolomic profile [55]. Instead, reduced drug accumulation was observed [55]. Since eflornithine is an amino acid analog, loss of an amino acid transporter was hypothesized. Therefore, the members of amino acid permease gene family were studied. One copy of *TbAAT6* was found to be absent from the resistant cells [55]. RNA interference (RNAi) on the transporter mRNA conferred a degree of resistance comparable to the selected cell lines.

### Drug Activation

Two drugs that require intracellular activation are currently in clinical use against parasitic protists. In both cases, activation results in a highly reactive and toxic free radical. Metronidazole is used to treat the anaerobes *Trichomonas* and *Giardia* and is activated by reduced ferredoxin [56]. Prior knowledge that nitroheterocyclic compounds are prodrugs, which need to be activated, led to the identification of a candidate enzyme, the mitochondrial type 1 nitroreductase (NTR) in *T. brucei* and *T. cruzi*. Reduced expression of NTR leads to resistance against nifurtimox and benznidazole [57].

### Classical Genetics and Genomics

One very successful method to identify the targets of a novel drug is to generate pathogens that are resistant to the drug. The changes responsible for resistance are then identified [2, 16]. They could be, for example, loss of a transporter that takes up the drug (loss of drug import), loss of drug activation, mutation of an enzyme target such that it is no longer affected by the drug, and increased expression of the drug target. Loss of function might be by gene deletion or mutation, or decreased expression, and increases in activities can be via mutation, gene amplification, or activated expression. However, quite often such experiments do not yield any information about the mechanism of drug action. Instead, they can reveal mechanisms for increased drug export or inactivation.

### Before the Genomes

Before the era of easy genome sequencing, identification of the causes of drug resistance relied on genetic crosses or the testing of hypotheses. Examples are listed as follows.

#### Chloroquine and Quinine

Chloroquine is one of the examples for which resistance studies revealed an export mechanism rather than a direct MoA. Genetic studies of *Plasmodium falciparum* are greatly facilitated by the fact that it has an obligatory sexual stage in the mosquito definitive host, but is haploid in the mammalian intermediate

host [58]. This means that controlled crosses can be done, and the progeny can be studied to identify genomic regions, and even single genes, linked to drug resistance [16, 59]. A landmark study using this approach was a laboratory cross between cloned chloroquine-resistant and chloroquine-sensitive *P. falciparum* field isolates [60]. Using 85 restriction fragment length polymorphisms that distinguished the resistant and sensitive strains, a 400 kb segment of chromosome 7 was linked to the transmission of chloroquine resistance to the progeny [60]. Afterward, the area was restricted to a region of 36 kb [61]. Polymorphisms were then identified and few genes proposed as candidates. To test the validity of the proposed resistance alleles, the relevant DNA segments were sequenced from field isolates of known drug sensitivities, and the authors looked for mutations that correlated with resistance. As the number of sequences increased, two initial candidates [61, 62] were successively eliminated before the real culprit, chloroquine resistance transporter in *P. falciparum* (PfCRT), was identified [63]. PfCRT is a transmembrane protein of the digestive vacuole, probably exporting the protonated version of chloroquine [64] from its site of action. Overexpression of the mutant version increased resistance of the cells to the drug and resistance selection *in vitro* led to mutations [63]. Allelic exchange was performed in sensitive strains using mutated PfCRT of different origins; this showed that mutating this gene was enough to obtain resistant cells [65].

Genetic crosses are extremely laborious and have now been made almost superfluous by the development of high-throughput DNA sequencing [16]. Nevertheless, the progeny lines from the genetic cross have been used extensively to obtain additional insights into drug action. The quantitative trait locus (QTL) approach (a statistical method that identifies stretches of DNA that correlate with a particular measured phenotype [66]) was used to identify parts of the genome apart from PfCRT that contribute to chloroquine resistance [67]. One of the identified genes was *pfmdr1* [64]. PfMDR1 is a protein of the multidrug resistance transporter class, and mutations may modulate chloroquine sensitivity [64]. Resistance to quinine, another antimalarial drug, is linked not only to *pfctr* and *pfmdr1* (multi drug resistance) genes but also to a *pfhhe* (sodium/hydrogen ion exchanger) [68]. Recently, it was shown that not only *pfctr* but also a HECT ubiquitin protein ligase (*pfut*) was a conserved trait in quinine resistance [69]. Both genes were present in chromosome 7 but in different chromosomal domains of restricted genetic diversity; therefore, they were inherited independently [69]. It was suggested that the ubiquitin action could be part of a multifactorial resistance, modulating the stability of proteins involved in survival under quinine pressure [69].

### Miltefosine

Miltefosine is an anticancer drug that is now approved for oral use against visceral leishmaniasis [70]. A candidate drug transporter (Miltefosine transporter in *Leishmania donovani* (LdMT)) was discovered by taking a resistant strain and transfecting it with a cosmid library of genomic DNA [71]. The authors then looked for

drug sensitivity. Just one cosmid restored sensitivity. By further subcloning, the gene *LdMT* was identified that encodes a protein similar to P-type ATPase APT subfamily. The retransfection of *LdMT* into a resistant strain increased the uptake of miltefosine. The resistant cell line was defective in glycerophospholipid uptake, and this was also reversed by retransfection of *LdMT* [71]. The protein was shown to be active only in the presence of another protein, LdRos3, which is presumably part of the same translocation machinery [72].

#### **Antifolates and Antimonials in *Leishmania***

*Leishmania* are susceptible to methotrexate, an inhibitor of dihydrofolate reductase. To confirm the mechanism, cells resistant to methotrexate were generated by stepwise selection in increasing concentrations of drug. The resistant cells contained specific amplified episomes. Some of these indeed encoded the target enzyme, dihydrofolate reductase [73]. However, others encoded a protein of the MRP family, which was named MDR1 [74]. *T. brucei* MRPA is the homolog of MDR1.

Pentavalent antimonials have been used against *Leishmania* for more than 65 years [75]. Similar to the arsenicals, these are nonspecifically toxic compounds, so selectivity must lie in drug activation or accumulation. The more active trivalent antimonials are produced through reduction of pentavalent antimonials, in macrophages and within *Leishmania* [76–78]. It was known that trivalent metalloid uptake can occur through aquaglyceroporins [79]. Therefore, different *Leishmania* spp. were investigated to look for homologous genes of human aquaglyceroporin 9 [80]. A gene was found that encoded aquaglyceroporin 1 (AQP1); when overexpressed in *Leishmania* cell lines, AQP1 produced hypersensitivity to antimonials [80].

#### **Post-Genomic Era**

The availability of protist parasite genomes, together with steadily improving techniques for reverse genetics, has enormously facilitated target deconvolution [59]. Initially, the genome sequences were invaluable in the identification of candidate genes (see the previous section). More recently, the availability of relatively cheap, high-throughput sequencing has revolutionized the way in which targets can be identified [59]. Nevertheless, screens merely yield candidates whose roles in drug action must be tested individually by more laborious methods [81].

#### **Microarrays for Analysis of Resistant Cell Lines**

High-density “tiling” microarrays are available for *P. falciparum*. Although their primary purpose was originally to study gene expression through hybridization with labeled cDNA [82, 83], they can also be hybridized with fragmented genomic DNA and thus used to find single nucleotide polymorphisms (SNPs), which lead to

decreased signals, and copy number variations (CNVs), where an increased copy number gives an increased signal [84]. The imidazopyrazines are a new class of antimalarial compounds found by phenotypic screening [83]. Resistant *Plasmodium* lines were selected and labeled genomic DNA was hybridized with the array. One gene was found to be modified consistently: it encodes phosphatidylinositol-4-OH kinase [83]. By reverse genetics, it was shown that either the mutations or the overexpression of the wild-type protein conferred resistance to the cells [83].

The same array was used to study spiroindolones [82], which are in clinical trials to cure malaria [85]. Six independent resistant clones were all found to carry mutations in the gene encoding the P-type cation-transporter ATPase4 (PfATP4) [82]. The role of the gene was confirmed by reverse genetics, as described for the imidapyrazoles.

#### Whole Genome Sequencing of Resistant Lines

Microarrays have limited sensitivity and reproducibility. At least in the case of protists with relatively small genomes, they have now been replaced for most purposes by whole genome sequencing [59]. Aminopyrazoles were found by phenotypic screening against *P. falciparum*, and sequencing of the genomes of laboratory-generated resistant lines revealed, as for spiroindolones, mutations in the gene encoding PfATP4 [59, 85]. PfATP4 maintains the Na<sup>+</sup> imbalance across the plasma membrane and the drug inhibits this [85]. It is interesting to notice how two classes of antimalaria compounds, even though chemically very different, affect the same target; clearly, PfATP4 is not only crucial for the life of *Plasmodium* but also eminently druggable [85]. This is critical information, but it is unlikely to lead to searches for further PfATP4 inhibitors for two reasons. Firstly, PfATP4 is a transmembrane protein, not amenable for recombinant protein production and inhibitor screens; and secondly, in order to avoid cross-resistance between drugs, it is important to limit the number of compounds that attack only one target.

Whole genome sequencing was used to analyze miltefosine-resistant *Leishmania major* [86]. This study showed the necessity of sequencing single clones of resistant cells, since the resistant populations showed great variability in phenotype and genotype [86]. After sequencing single resistant clones, no CNVs were found, but single point mutations in the miltefosine transporter known to cause resistance were identified [86]. Similar experiments with antimony resistant clones failed to reveal any SNPs linked to resistance, but DNA insertions and deletions were identified [87]. In particular, a deletion of the gene encoding AQP1, the protein previously identified to take up the drug [80], was found.

Linkage group selection is a method to analyze uncloned progeny of a genetic cross, comparing progeny selected or not selected with drug; genome wide markers are evaluated in a quantitative way and markers of the sensitive parent are expected to be located close to genes determining the phenotype after

selection [88]. In *Plasmodium chabaudi* and *Plasmodium vivax*, orthologs of *pfprt* and *pfmdr1* are not involved in chloroquine resistance [89]. Linkage group selection was initially performed by PCR amplification of known polymorphisms [88, 90] of three independent genetic crosses between artemisinin-sensitive and artemisinin-resistant *P. chabaudi* [90]. To improve the resolution of the analysis, a similar approach was used with crosses of parasites resistant to artemisinin [91] and chloroquine [89], coupled with whole genome resequencing of the initial cell lines [89, 91]. A deubiquitinating enzyme (encoded by *ubp1*) [90, 91] and putative amino acid transporter encoded by *aat1* [89] were thereby implicated in multidrug resistance.

Whole genome sequencing analyses are only useful if several independent drug-resistant lines are available. If these are derived in the laboratory, they are compared with the original line [85]. If, in contrast, field isolates are used, several independent sensitive isolates will be needed as controls. Work on halofuginone, which is used in Chinese medicine against malaria, illustrates some potential pitfalls of taking a simplistic approach [92, 93]. Whole genome sequencing of two independently selected resistant clones leads to the identification of nonsynonymous mutations in *cPRS*, a putative proline aminoacyl-transfer RNA (tRNA) synthetase [93]. *S. cerevisiae* are resistant to halofuginone; to test whether *cPRS* could be the drug target, the *cPRS* gene was replaced by the wild-type version of the *Plasmodium* gene, and this resulted in sensitivity to halofuginone in *S. cerevisiae* [93]. Halofuginone was shown to affect the response pathway to amino acid deprivation, but the effect was seen only when the wild-type version of the *cPRS* gene was present [93]. Therefore, Herman *et al.* hypothesized that mutations in the gene were the cause of resistance in *Plasmodium*. However, upon closer investigation, it transpired that establishment of resistance was more complex [92]. Initially, proline homeostasis was modified, then mutations in the *cPRS* gene were observed; however, these mutations lead to cells with reduced fitness. The amplification of the *cPRS* gene was actually a late, compensatory event, which increased the fitness of the resistant cells [92].

Recently, the genomes of 45 *P. falciparum* strains from Senegal were sequenced to look for genome-wide association study (GWAS) with drug resistance [94]. Genes previously known to be linked to drug resistance were identified, such as *pfmdr1*, *pfprt*, and *dhfr*. However, other genes were also found, some of which encode steps in metabolic pathways that might modulate the response to drug stress [94]. A similar approach was applied to a set of nine other *P. falciparum* isolates with different genetic backgrounds; SNPs were documented and then compared with 57 other genome sequences [95]. In addition to the previously known genes, a new locus, PF10\_0355, was identified to be connected to halofantrine resistance [95]. Overexpression of the protein encoded by PF10\_0355 conferred resistance to halofantrine and other structurally related drugs [95]. The function of this protein is as yet unknown, and we do not know whether it is a halofantrine target or somehow reduces halofantrine efficacy.

## Changes in mRNA Levels

The amounts of proteins in cells often do not correlate directly with the levels of the corresponding mRNAs [96], but nevertheless, changes in mRNA level can provide information regarding changes in the state of cells. Changes in gene expression that are an immediate response to drug treatment can offer clues about drug action if cells have feedback mechanisms that regulate expression to compensate for damage or metabolic disruption. Such adaptations do not involve any changes to the genome. So far, however, most gene expression results relevant to parasite chemotherapy are from comparisons between drug-resistant and drug-sensitive cells: in this case, differences in expression levels or pattern can reflect either gene regulation or changes in gene copy number.

## Candidate Genes

Before high-throughput methods were available, a “candidate gene” approach was adopted. For example, the expression of genes previously known to be involved in drug resistance could be checked in drug-resistant lines [97]. The expression of 44 candidate genes from arsenite- and antimony-resistant *Leishmania* was measured in a “mini-array” format and overexpression of mRNAs encoding the ATP-binding cassette (ABC) transporter *Leishmania* ABC transporter (PGPA) (the homolog of *T. brucei* MRPA),  $\gamma$ -glutamylcysteine synthetase, and glutathione synthetase was detected [98]. Glutathione synthetase and  $\gamma$ -glutamylcysteine synthetase are enzymes on the trypanothione synthesis pathway, and MRPA/PGPA exports antimonials and arsenicals as conjugates with trypanothione. Using the same gene array with RNA from methotrexate-resistant cells, increased expressions of the target (dihydrofolate reductase) and pteridine reductase and S-adenosylmethionine synthase were found [98]. Using a different mini-array containing all *Leishmania* genes encoding ABC proteins, three were found to be overexpressed in antimony-resistant cells [99].

## Microarrays and Sequencing

Candidate approaches are, of course, not useful for the discovery of novel modes of drug action or resistance. Genome-wide microarrays are therefore a considerable improvement – although the genes discovered have not always been novel. For example, they were used to identify overexpression of the ABC transporter TcABC1 in benzimidazole-resistant *T. cruzi* [100]; as confirmation, overexpression using a *T. cruzi* expression vector resulted in resistance to the drug [100]. In contrast, SNPs in the gene did not correlate with resistance [101]. A whole-genome microarray was also used to analyze the artemisinin tolerance of some Cambodian *P. falciparum* isolates [102]. The comparison of three resistant

and three sensitive strains revealed numerous differences. This, combined with an observed slowing of metabolism and growth but increased protein turnover, suggested wide-ranging regulatory alterations [102]. More recently, 1043 clinical *P. falciparum* isolates from 13 different endemic regions, and with different resistance levels, were analyzed by RNASeq [103]. Again, numerous changes were detected in resistant isolates, affecting 10% of the 5000 detected transcripts [103]. Most of the upregulated genes had functions in protein metabolism and quality control [103]. Downregulation of genes involved in DNA replication was also seen, which might imply developmental stalling [103]. The changes in protein quality control are consistent with genomic analyses, which found that mutations in the *P. falciparum* Kelch-13 propeller correlated with artemisinin resistance [104]. A search for inhibitors of *Plasmodium* phosphatidylinositol-3-kinase revealed that artemisinin is a potent inhibitor of enzyme activity, preventing formation of phosphatidylinositol-3-phosphate [105]. In ring stages, a range of secreted proteins is modified by phosphatidylinositol-3-phosphate, resulting in their retention in the endoplasmic reticulum; polyubiquitination and Kelch-13 binding result in degradation of phosphatidylinositol-3-kinase [105]. The Kelch-13 mutations in resistant cells result in an increased level of phosphatidylinositol-3-kinase, thus combating the action of artemisinin in rings [105]. Other MoA are, however, likely to be more important in trophozoites – which explains the complexity of resistance phenotypes.

## Reverse Genetic Screens

### Gain-of-Function Screens

One subset of reverse genetic approaches exploits the fact that overexpression of either the drug target or the proteins involved in drug metabolism or export can cause drug resistance [95, 113–115].

In *L. major*, cosmid vectors can be maintained as episomes. If the cosmid encodes a protein that can confer drug resistance, its copy number will increase upon stepwise selection [115]. After transfection of a library covering the whole genome, drug treatment will select for those cosmids that cause resistance [115]. For example, two sterol synthesis inhibitors selected for nine resistance genes. One of them was squalene synthase, which acts in the sterol biosynthesis pathway, but upstream of the previously known drug targets. It was suggested that squalene synthase overexpression increases the levels of intermediates upstream of the target enzymatic reactions, compensating by mass action for the inhibition of enzymes further along the pathway [115]. In another case, the library was transfected into antimony-resistant *Leishmania mexicana* strains, and the authors looked for parasites that had become antimony-sensitive [116]. The identified cosmid encoded AQP1, a previously known transporter of antimony [116]. It turned out that the only difference between the resistant strain and the wild-type strain was a reduced level of *AQP1* mRNA [116].

In each of these *Leishmania* experiments, the selected cosmids were recovered individually, which meant that only the cosmids that conferred the highest resistance would be recovered. High-throughput sequencing has obviated the need to do this. Recently, a protein overexpression library in *T. brucei* was created [117]. A plasmid library for tetracycline-inducible expression of mRNAs from random genomic fragments was transfected with high efficiency using a meganuclease-based system [118]. Although the library had an average of 10-fold coverage of each gene, it was limited by the fact that the inserts had an average length of about 1.2 kb [54, 117]. The trypanosome library was selected with DFMO and DDD85646 [119, 120]. After selection, DNA was prepared, the library inserts were PCR-amplified, and the resulting mix was sequenced. Comparison with the original library showed which overexpressed genes were conferring a growth advantage [54]. The only functional genes recovered were ornithine decarboxylase, the target of DFMO [50], and *N*-myristoyltransferase, which is the target of DDD85646 [119, 120]. This shows that the approach can not only identify targets but also be used to confirm drug specificity [54].

### Loss-of Function Screens

High-throughput loss-of-function screens are possible in parasites for which dominant-negative mechanisms of gene downregulation exist. So far, the best examples are for *T. brucei*, which has the RNAi pathway [121, 122]. The plasmids comprising the libraries are designed to integrate into the parasite genome. In each plasmid, a random fragment of DNA is flanked by opposing tetracycline-inducible promoters. As described earlier for the *T. brucei* overexpression library, the plasmid library is transfected into *T. brucei* and a library of parasites – each containing a different inducible RNAi fragment – was created [118]. Upon tetracycline addition, the respective inserts are transcribed into both directions, creating a double-stranded RNAs. In the absence of drug, there is selection against essential genes [122]. As described for the overexpression library earlier, plasmid inserts that were selected or lost are identified by PCR followed by deep sequencing. In the presence of drugs, it is possible to select cells with RNAi targeting any gene that is required for drug action. These could be nonessential genes, but it is also possible to select for trypanosomes that have moderate downregulation of an essential gene. In this case, the growth advantage from a decreased gene expression is overridden by the advantage that is obtained in surviving the drug pressure. Selection of a direct drug target is in contrast most improbable, since RNAi is expected to synergize with the drug. For studies of drugs against kinetoplastids, at least, such analyses seem likely to become routine.

The negative selection approach has so far proved most useful in elucidating the MoA of drugs that have relatively nonspecific action, but are selective by virtue of specific accumulation or activation. Selection of a trypanosome RNAi library with DFMO retrieved the amino acid transporter AAT6 [55] and selection with melarsoprol identified the transporter AT1 [121]; choline analogs selected for loss

of a mitochondrial carrier protein 14 (MCP14) [123]. None of these proteins is essential for cell viability, but an *MCP14* knockout led to a reduced growth rate. Downregulation of MCP14 conferred resistance to choline analogs and ectopic copy expression increased susceptibility, confirming MCP14 involvement in drug action [123].

The RNAi library approach has greatly increased our understanding of the MoA of four trypanocidal drugs that are in clinical use: suramin, pentamidine, arsenicals, and nifurtimox [108, 122, 124]. For nifurtimox, which probably acts as a free radical, loss of an activating enzyme, the NTR type 1, was detected. The MoA of suramin is unclear; one possibility is inhibition of glycolysis. Quite a large number of RNAi inserts was selected by suramin; notably, they included a selection of genes involved in endocytosis and lysosomal function [108]. One of the selected RNAi targets, invariant surface glycoprotein of molecular weight 75 kDa (ISG75), is thought to be on the surface and in lysosomes; it was demonstrated to bind suramin. This was consistent with the previously known accumulation of suramin within lysosomes. It was therefore suggested that suramin enters by receptor-mediated endocytosis, with ISG75 as the receptor [108]. It was already known that AT1 is not the only transporter for melarsoprol and pentamidine, and the RNAi library identified the culprits: two aquaporins, aquaglyceroporin 2 (AQP2) and aquaglyceroporin 3 (AQP3) [108, 109]. Treatment-refractory isolates often show loss of the AQP2 gene, or rearrangements involving AQP2 and AQP3 [125, 126], although the roles of the AQPs in resistance have been challenged and the link could be more complex [127]. When the RNAi library was used with CpdA, an inhibitor of cyclic AMP (cAMP) phosphodiesterases, reduced expression of cAMP response proteins (CARPs) was found to confer resistance [128]. cAMP response protein 1 (CARP1) is unique to kinetoplastid parasites, and CARP3 is unique to *T. brucei*; they are all believed to be part of a novel cAMP signaling pathway [128].

### Proteins and Proteomes

In the field of drug target deconvolution, high-sensitivity mass spectrometry is finding increased use in two areas: identification of proteins that bind to affinity columns and comparison of drug sensitive and resistant lines [18].

### Proteomes

CNVs can suggest that a protein might be overexpressed in a line with additional copies, but changes in expression due to changes in regulatory sequences cannot be predicted. It is therefore extremely useful to be able to compare expression directly [18, 129]. Until 10 years ago, mass spectrometry applications were characterized by limited analytical depth and quantification capability [18]. Experiments

to compare two protein preparations therefore involved initial separation of the proteins on gels, visualization by staining, followed by selection of small gel segments, which appeared to show differences. Because one-dimensional gel electrophoresis is relatively insensitive, proteins were usually separated on two-dimensional gels [130]. Since the latter is poorly reproducible, the preparations to compare were ideally prestained with two different fluorophores, then mixed before the separation was performed [131, 132].

Using 2-D electrophoresis, methionine adenosyltransferase (MAT) was found to be overexpressed in *L. major* cells with methotrexate as well as a resistant mutant [130]. Overexpression of *MAT* gene in a sensitive strain did not confer resistance, but it was demonstrated that it modified the level of resistance to methotrexate [130]. The method was also used to study the effect of doxycycline in *P. falciparum*, suggesting the mitochondrion and apicoplast as targets [131]. However, by this time, an additional method was also available to confirm the results. Isobaric tag for relative and absolute quantitation (iTRAQ). iTRAQ involves the use of different chemical labels for each polypeptide sample. Up to three samples can be pooled and then analyzed by liquid chromatography and mass spectrometry (LC-MS) [131].

Another common approach for protein labeling is stable isotope labeling by amino acids in cell culture (SILAC). In this case, one culture includes heavy nonradioactive isotopes – which are incorporated into the proteins – whereas the other does not [133–136]. Again, the samples are mixed prior to protein identification. This approach was used in *P. falciparum*, to compare the effects of chloroquine and artemisinin [133]. In this case, gel electrophoresis was avoided entirely. Instead, multidimensional protein identification technology (MudPIT) was used: an unfractionated digested protein mixture was separated by biphasic liquid chromatography and analyzed in tandem mass spectrometry. 1253 *P. falciparum* proteins were identified. In total, 22 proteins were changed after chloroquine and 23 after artemisinin treatment [133]. In both cases, just under half of the identified proteins have no known function. SILAC was also used to study the effects of amphotericin B and antimony on *Leishmania infantum* [135, 136]. No significant new information was obtained, but the antimony study at least identified the ABC transporter MRPA (the same as the *L. major* MDR1), which validates the technique [135].

### Affinity Chromatography

Chemical proteomics or chemoproteomics are terms used to define a field of research in which a small molecule is chemically modified and attached to a solid support such as beads. Protein extracts are incubated with the beads, which are then extensively washed. Afterward, the attached proteins are eluted and identified by mass spectrometry [15, 81, 107, 137–140]. The method is simple in

principle but can be limited in practice [81, 141]. First of all, the compound has to be chemically derivatized; this means using one of the chemical groups in the molecule to add a linker and bind it to a support. Adding groups to a molecule can be difficult when no suitable reactive groups are present [15, 81]. Moreover, changes in the molecule structure may affect the pharmacophore, influencing the specificity or even abolishing drug–protein interactions [142].

This approach was used to look for targets of the antimalarial quinoline drugs primaquine and hydroxychloroquine; ATP served as a control [143]. After incubation of cell extracts with the drugs, bound to sepharose, the matrix was washed. Then, the bound proteins were eluted using the relevant drug; in addition, elution with other quinolines, such as chloroquine, mefloquine, quinacrine, and quinine, were performed. Two erythrocyte proteins were found in the eluates: quinone reductase 2 (QR2) and aldehyde dehydrogenase 1 [143]. Interestingly, QR2 was potentially inhibited *in vitro* by quinoline and inhibitors targeting QR2 specifically were lethal to the parasites, probably because of oxidative stress generation [143].

Derivatives of the natural compound naphthoquinone, active against *Trypanosoma brucei rhodesiense*, *L. major*, and *T. cruzi* [144], were also used as affinity probes. This led to the identification of two proteins, glycosomal glycerol kinase and glycosomal glyceraldehyde-3-phosphate dehydrogenase; both proteins were inhibited by the compound and especially the second one seems to be part of the multitarget effect characteristic of these molecules [140].

To avoid the pitfalls associated with derivatization, different derivatized molecules can be used in parallel and the results are compared [142]. When a trypanocidal compound (4-[5-(4-phenoxyphenyl)-2*H*-pyrazol-3-yl]morpholine) was used in this way, only one of the derivatized versions identified *T. brucei* adenosine kinase as a potential target. The authors validated the interaction of this compound with adenosine kinase using circular dichroism spectroscopy and isothermal titration calorimetry, based on the principle that a protein binding to its ligand will have higher thermal stability [142]. The substrate transformation kinetics indicated that the compound was limiting the substrate inactivation pathway. The authors speculated that the parasites died because deregulation of the enzyme activity disrupted purine interconversion [142].

Another possible problem is steric hindrance – beads preventing interactions or protein complexes blocking the pores [145]. To circumvent this, it is possible to label the compound with biotin, incubate the biotinylated drug with protein extracts, and then trap the complexes on streptavidin matrices [146]. Elution can be done with an excess of biotin; alternatively, the tag can be designed to be cleavable; in this way, mild conditions can be used to elute the proteins [146].

A further advancement is the application of click chemistry; small reactive groups are added to the chemical structure and the beads are linked through this reactive group only after the drug is already bound to the proteins [147–149]. This limits the influence of bulky chemical groups and beads. Click chemistry was used with an azide group linked to compounds that inhibit a major cysteine protease active in *T. cruzi* called *cruzain* – although they may well inhibit other

cysteine proteases as well. Rather unsurprisingly, mammalian cathepsin B was identified as off-target [147].

The major issue in these approaches is the presence of a significant amount of proteins that are very abundant, prone to aggregation or stick to any matrix. This means that many of the proteins detected do not actually bind to the drug. Although this can be alleviated to some extent by changing linkers and matrices, and by including suitable negative controls, a core of contamination always persists [15, 145]. This can be defined through several independent and unrelated experiments, and lists of frequent contaminants can be generated and thus excluded from further considerations [81]. An excellent negative control is a matrix bearing an inactive version of the test compound; this can be processed in parallel with the active version [14]. However, the possibility that the inactive version can still bind to the target – even if it cannot inhibit it – cannot be ruled out [81]. A competition approach can also be used: a protein extract is first incubated with the drug in solution, to saturate the target-binding sites; afterward, the preabsorbed material is normally processed for target identification [18, 81, 141, 145]. The identified proteins are compared with those seen without preabsorption; the proteins present in a usual affinity purification experiment but missing from the competition experiment are more likely to be binding directly to the compound and not the matrix; therefore, they are candidate targets [147, 148, 150, 151].

Several additional factors can influence the likelihood of success of these approaches. The ability of the compound to select a protein target is optimal only if binding is stable and occurs rapidly with high affinity [14]. Some proteins may be inactivated by proteolysis during cell lysis, detergents may change protein conformation, membrane proteins may not be extracted at all [14, 81]. One option here is to use cross-linkers that bind the drug to the target before lysis [152]. Covalent attachment of drug to target has the additional virtue of allowing more stringent, and even denaturing, wash conditions [14, 15, 149].

A compound, ACT-213615, with low nanomolar activity against *P. falciparum*, was developed from a screen of molecules resembling aspartic protease inhibitors, but the MoA is unknown. A derivative that retains activity was made with a photosensitive group and biotin label [14]. The compound was added to infected erythrocytes, activated, and then pulled down from cell lysates using streptavidin beads. Proteins identified using liquid chromatography coupled with tandem mass spectrometry included the well-known multidrug resistance protein 1 (PfMDR1) [153]. The derivatized molecule was also used for cell imaging, since the biotin label could be detected using fluorescent probes [14].

### Activity-Based Probes

Activity-based probes are drugs that are modified to react, not only with the solid support but also with an amino acid in the active site of the target protein [137,

141, 154]. Such probes can only be made if the target is already thought to belong to a specific protein class, such as protein kinases [148, 151] or proteases [150].

Hypothemycin is a fungal natural product that inhibits some human kinases through covalent binding to an active site thiol [155, 156]. It can also kill trypanosomes [148]. Coupling this compound to a matrix permitted purification of a subset of *T. brucei* kinases [148]; it is not yet known which of these are responsible for the *in vivo* effect. A similar approach was used to identify unknown proteins, mostly kinases, using staurosporine as an ATP-like competitive inhibitor; addition of a photoreactive group and biotin tag allowed purification of 70 *L. donovani* ATP-binding proteins [151]. K11777 is a cysteine protease inhibitor [157]; an activity-based probe based on it was used in *T. brucei*, and it identified previously expected targets, such as rodhesain and cathepsin B, but also other interesting candidates such as proteasome components [150]. Visualization of the K11777 probe was shown to be possible: interestingly, in procyclic forms, the probe was equally distributed throughout the parasite, with no specific localization, whereas in bloodstream forms, it was mainly found in lysosomes [150].

### Label-Free Approaches

Recently, new approaches have been developed using the original compound, without derivatization [18, 137]. It is hoped that in this way, artifacts will be reduced.

One of these techniques is the target identification using drug affinity responsive target stability (DARTS) [158]. This approach is based on the concept that binding of a small molecule to a protein will make the protein less flexible and therefore more resistant to proteolysis [137]. Cell extracts with and without drug are treated with limited proteolysis and then subjected to liquid chromatography coupled with mass spectrometry. For example,  $\alpha$ -ketoglutarate extends the lifespan of *Caenorhabditis elegans*; ATP synthase subunit  $\beta$  was shown to be more protease resistant in the presence of  $\alpha$ -ketoglutarate [159]. The method was recently used to study a novel anticancer compound, called RX-5902, and as a result, p68 RNA helicase was found to be the target, inhibiting the ATPase activity depending on  $\beta$ -catenin [160].

Another recently developed approach focuses on changes in stability under oxidative stress. It is called stability of proteins from rates oxidation (SPROX) [161]. Proteins or mixture of proteins are placed in the presence of a fixed amount of oxidant and increasing amounts of chemical denaturant. The more denatured the protein becomes, the more amino acids become accessible to oxidation. The oxidized residues are then detected by mass spectrometry [161]. In the presence of a ligand that stabilizes the protein folding, less oxidation occurs [161]. In a proof of concept study, yeast extracts were incubated with cyclosporin A and hydrogen peroxide, to identify proteins whose oxidation was affected by the drug [162]. Ten proteins were found to be changed and two known targets of cyclosporin A, cyclophilin A and UDP-glucose-4-epimerase, were among them [162].

These techniques are relatively new, and to our knowledge, they have not been applied yet to the study of drugs against parasites, but we think they could be useful as complementary approaches.

### Proteome Display

Protein collections of various sorts can be assayed directly for drug binding. This can be especially useful if the target of interest is of low abundance or is unstable *in vivo* [137]. Although cDNAs can be used for this, the proteins produced may be truncated or contained undesired sequences at their N-termini [163]. The ideal way to generate proteome arrays is to clone intact open reading frames. Various methods are available for protein library display. In one, using DNA templates, the mRNAs are transcribed and translated *in vitro*; they can be assayed either on microtiter plates or on solid supports [117]. Phage display technology is another possibility; in this case, the open reading frames are fused to gene encoding a phage coat protein. Each phage displays one unique protein. After affinity purification using the compound of interest, the phage can be amplified and rescreened to enhance the enrichment [164].

### Metabolomics

To determine the effect of drug treatment on metabolism, presence and concentration of many metabolites (<1400 Da) can be measured by mass spectrometry or nuclear magnetic resonance (NMR) [16, 165–167]. This provides more immediate insights into cellular homeostasis than analyses of DNA, RNA, and proteins [168–172]. The technology has yet not been used extensively in parasite drug target identification [112], although it has been used to define metabolic pathways [168, 173]. NMR detects molecules unequivocally and quantitatively, but has low sensitivity [166]. On the other hand, mass spectrometry has very high sensitivity and can identify more than a thousand different compounds in one sample, but it is less quantitative [16, 166]. It has been suggested that a combination of MS and NMR is likely to be most powerful [166, 169]. Normally, cell extracts are fractionated by liquid or gas chromatography prior to the MS analyses in order to reduce complexity. Normal-phase liquid chromatography is used to resolve polar compounds, reverse-phase columns are used for nonpolar molecules [169], gas chromatography is used for volatile metabolites [166], and capillary electrophoresis is the method used for the analyses of polar or volatile compounds [170, 174].

Targeted metabolomic experiments are hypothesis-driven, aiming to quantify a defined subset of metabolites [167]. Atovaquone inhibits the cytochrome b/c complex of *P. falciparum*, and mutations in cytochrome b result in resistance [106]. Inhibition of cytochrome b/c causes loss of membrane potential and organelle dysfunction; but the lethal result is the lack of production of intermediates for pyrimidine synthesis [175]. Quinolone lead compounds with antimalarial

activity targeting the bacterial-like type II NADH:ubiquinone oxidoreductase were also found to affect cytochrome b/c [175]. At various times after drug addition, 35 key metabolites were monitored by LC-MS and the disruption of pyrimidine metabolism was confirmed: accumulation of dihydroorotate and a reduction of downstream intermediates [175]. Similarly, fosmidomycin was thought to target *Plasmodium* deoxyxylulose 5-phosphate reductoisomerase, an enzyme involved in the production of isoprenoids [176, 177]. Five metabolites in this pathway were quantified, and it was found that in fact another enzyme, methylerythritol phosphate cytidyltransferase, was inhibited [177]. This result prompted further investigation, and indeed, overexpression of methylerythritol phosphate cytidyltransferase conferred resistance to fosmidomycin [177].

If drug targets are not known, untargeted metabolomics can be used to identify candidate target pathways [167]. Taking samples at different times is important to characterize the drug effect and use of different drug combinations can also be informative. The effects of nifurtimox and DFMO on trypanosomes were studied as a proof of concept [112]. Since DFMO blocks polyamine biosynthesis, and therefore also affects trypanothione production, it was expected to cause oxidative stress. Nifurtimox, meanwhile, was believed to generate oxidative stress. Unexpectedly, the two drugs were found to be antagonistic. The authors suggested that this was because DFMO is cytostatic, while nifurtimox is active only on proliferating cells [112]. As expected, DFMO affected mainly the polyamine biosynthetic pathway [112], although a decrease in phospholipids was also detected [112]. Nifurtimox, on the other hand, caused an increase in nucleotides and nucleobases: it was speculated that this was a consequence of DNA and RNA degradation [112]. The drug combination did not result in any additional changes [112].

Both benznidazole and nifurtimox are used to treat *T. cruzi* and are thought to have similar modes of action: activation by type 1 NTR and subsequent oxidative stress [107, 178]. Metabolomic analysis of *T. cruzi* resulted in identification of about 1000 metabolites [107]. Benznidazole caused small differences, mostly in trypanothione metabolism, supporting the oxidative stress MoA; as a bonus, some benznidazole metabolites were detected [107]. An NMR metabolomic study of *T. cruzi* treated with plant extracts focused on the culture medium: a mitochondrial reductase was proposed as a target [179]. In contrast, an untargeted study of *L. infantum* promastigotes treated with miltefosine yielded so many differences in the metabolome that no obvious primary targets could be postulated; there were alterations in internal lipid metabolism and increases in alkanes, sugars, and nucleotides. The results suggested that miltefosine is characterized by multiple effects, which might include an increase in oxidative stress [110]. To increase sensitivity, it is also possible to combine different chromatographic methods [170]. This was employed to further study the MoA of miltefosine; it was showed that metabolites of the arginine and polyamine pathways in *Leishmania* were affected. The authors suggested that this supported the idea that the MoA

relates to an increase in oxidative stress [111]. The effect of miltefosine on *L. donovani* was the subject of a study that focused specifically on phospholipids, sterols, and fatty acids [180]. The authors observed a significant decrease in the amount of phosphatidylcholine and increases in lysophosphatidylcholine and phosphatidylethanolamine [180]. Lysophosphatidylcholine is thought to arise by degradation of phosphatidylcholine.

Sitamaquine is a possible drug against visceral leishmaniasis [181]. Sitamaquine is positively charged and therefore cannot cross membranes, but it was found to interact with negatively charged phospholipids. It was suggested that after this interaction, an aromatic moiety of sitamaquine can insert into the membrane [182]. Resistant cells did not show a decrease in positively charged phospholipids, which meant that reduced uptake via the proposed mechanism could not explain the resistance [183]. Nevertheless, a strong impact of the drug on lipid metabolism was seen, supporting the idea that the drug acts on such pathways: decreased sterols, reduced phosphatidylcholine, and accumulation of phosphatidylethanolamine [183].

Metabolomics is relatively fast [111] and can be used to compare many different strains and conditions. It is, however, often very difficult to distinguish specific alterations from the background of nonspecific effects that reflect growth inhibition or general metabolic disruption. Changes in oxidative stress-related molecules and lipids are a recurring theme. For example, the metabolomes of three genetically rather similar *L. donovani* strains with different levels of antimonial resistance [184] were studied in different growth and drug pressure conditions [171]. The results were rather inconclusive, although resistance appeared to be related to better protection against oxidative stress and higher membrane fluidity [171]. Another study of *L. donovani* resistant to various drugs revealed several common adaptations, such as increased resistance to oxidative stress and changes in the saturation levels of glycerophospholipids [172].

## Validation

All these approaches can result in the identification of different genes possibly involved in drug action. As we saw in many examples, hypotheses generated by one method need to be validated using others. Overexpression of a putative target protein or export transporter should increase the  $EC_{50}$  of the drugs [71, 100, 123]. If mutations are thought to influence drug action, the mutated gene can be expressed in the cells – or the wild-type gene replaced – to assess the effect on drug sensitivity [63, 65, 101]. Drug targets are expected to be essential, so downregulation should inhibit cell growth and increase drug sensitivity, and gene knockout should either cause severe growth inhibition or be impossible [123].

When an enzyme is suspected to be involved in drug action, effects of the drug on kinetics and binding to the enzyme can be tested [142]. If a transporter is

suspected to be the route of entrance or to be blocked by a compound, channel inhibitors can be used to test this hypothesis, evaluating the influence on drug action [44].

## Conclusions

Phenotypic drug discovery is now revealing new promising compounds to fight diseases caused by protists. Very often, nothing is known about their MoA. We surveyed different methods that have been used more or less successfully for drug target deconvolution. In some cases, one method was enough to identify specific targets or causes of resistance. However, in many cases, the MoA is complex and several approaches are needed. There is no golden rule about which is the best method to be used, every drug is different and has different effects on the cells. Therefore, the strategy chosen to assess the MoA needs careful consideration and fine-tuning.

## Abbreviations

AAT1	Amino acid transporter 1
AAT6	See TbAAT6
ABC	ATP-binding cassette
ABCG1	ATP-binding cassette subfamily G member 1
AQP1	Aquaglyceroporin 1
AQP2	Aquaglyceroporin 2
AQP3	Aquaglyceroporin 3
AT1	See AT1
cAMP	Cyclic AMP
CARP	cAMP response proteins
CARP1	cAMP response protein 1
cDNA	Complementary DNA
CNVs	Copy number variations
cPRS	Cytosolic proline aminoacyl-tRNA synthetase
DARTS	Drug affinity responsive target stability
DFMO	Difluoromethylornithine or eflornithine
DHFR	Dihydrofolate reductase
DNDi	Drugs for neglected diseases initiative
EC <sub>50</sub>	Half maximal effective concentration
GWAS	Genome-wide association study
ISG75	Invariant surface glycoprotein of molecular weight 75 kDa
iTRAQ	Isobaric Tag for relative and absolute quantitation
kDNA	Kinetoplast DNA
LC	Liquid chromatography mass spectrometry
LdMT	Miltefosine transporter in <i>L. donovani</i>

MCP14	Mitochondrial carrier protein 14
MDR1	Multidrug resistance protein 1
MMV	Medicines for Malaria Venture
MoA	Mechanism of action
mRNA	Messenger RNA
MRP	Multidrug resistance protein
MRPA	Multidrug resistance protein A
MS	Mass spectrometry
MT	See LdMT
MudPIT	Multidimensional protein identification technology
NMR	Nuclear magnetic resonance
NTR	Nitroreductase
PfATP4	P-type cation-transporter ATPase4 in <i>P. falciparum</i>
PfCRT	Chloroquine resistance transporter in <i>P. falciparum</i>
PfMDR1	Multidrug resistance protein 1 in <i>P. falciparum</i>
PfNHE	Sodium/hydrogen ion exchanger in <i>P. falciparum</i>
PfUT	HECT ubiquitin protein ligase in <i>P. falciparum</i>
PGPA	<i>Leishmania</i> ABC transporter
PROSC	Proline synthetase co-transcribed homolog
QR2	Quinone reductase 2
QTL	Quantitative trait locus
RNAi	RNA interference
RNAseq	RNA high-throughput sequencing
SILAC	Stable isotope labeling by amino acids in cell culture
SNPs	Single nucleotide polymorphisms
SPROX	Stability of proteins from rates of oxidation
TbAAT6	Amino acid transporter gene in <i>T. brucei</i>
TbAT1	Adenosine transporter 1 in <i>T. brucei</i>
TCA	Trichloroacetic acid
TcABCG1	ABCG1-like transporter in <i>T. cruzi</i>
TRiC	TCP-1 Ring Complex
tRNA	Transfer RNA
UBP1	Ubiquitin-specific protease 1

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