Defining the targets of antiparasitic compounds

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Teaser: Targeted whole-cell screening strategies are identifying hundreds of active antiparasitic agents, but how do we determine their modes of action?

Abstract

The treatment of major human parasite infections is dependent on drugs that are plagued by issues of drug resistance. New chemotherapeutics with novel mechanisms of action (MOA) are desperately needed to combat multi-drug resistant parasites. Although widespread screening strategies are identifying potential new hits for development against most major human parasitic diseases, in many cases such efforts are hindered by limited MOA data. While MOA data are not essential for drug development, they can facilitate compound triage and provide a mechanism to combat drug resistance. Here we describe and discuss methods currently used to identify the targets of antiparasitic compounds, which may circumvent this bottleneck and facilitate the development of new antiparasitic drugs.

Background

Parasitic diseases of humans are a major global health problem causing significant morbidity and mortality, especially in less-developed countries. Each year there are hundreds of millions of people infected with disease causing parasites, particularly in tropical and subtropical regions of the world, which results in an estimated 1 million deaths [1]. Unfortunately, prevention and control of tropical parasitic diseases is challenging, compounded by a lack of vaccines, issues of parasite drug resistance and vector insecticide resistances [2-5]. This alarming situation is driving increased efforts in the discovery and development of new therapeutic agents, in particular the identification of new drug leads with novel mechanisms of actions (MOA) and subsequent progression to clinical use in order to limit potential issues of cross-resistance. As a result of recent high throughput screening (HTS) campaigns there has been enormous success in identifying new antiparasitic agents with novel chemotypes, however these studies have largely focused on whole-cell approaches. As a result, information on the target/s of most of these compounds is not available. This lack of MOA information can impact compound triage for progression to pre-clinical studies and rational strategies to improve compound potency and/or selectivity. In this review we discuss available and emerging target identification approaches for the causative agents of the major parasitic diseases malaria, leishmaniasis, schistosomiasis, and trypanosomiasis (**Box 1**), focusing on *Plasmodium falciparum*, the parasite responsible for the highest rates of malaria mortality and morbidity [3].

Challenges in antiparasitic drug discovery

Widespread resistance to commonly available drugs, the lack of licensed vaccines, complex biology, and the continuing high burden of parasitic diseases have necessitated increased efforts in the discovery and development of new antiparasitic agents. However, this drug discovery process can be extremely time consuming and expensive, which is compounded by high attrition rates of candidates at each stage of the developmental process. Even when candidates pass pre-clinical milestones, it can still take decades for individual drugs to become clinically approved. While new

formulations and combinations of existing drugs are, in some cases, helping to prolong the use of our current chemotherapeutic arsenal, very few new antiparasitic chemical classes (chemotypes) have reached the market in the past decade. For malaria, which causes the highest mortality of all tropical parasitic diseases, most new therapies are based on different combinations of known drugs or new drugs based on known pharmacophores – only a limited number of new chemotypes are currently in clinical trials [6,7]. Many different approaches, including drug repurposing, which is particularly popular at present (reviewed in [8]), are being implemented to identify new bioactive compounds with activity against parasites. However these strategies are still hampered by challenges including limited financial investment, the availability of annotated genome sequences and the suitability of models to assess compound activity.

The discovery of drugs for the treatment of infectious diseases that primarily impact developing countries has historically been hindered by limited financial investment. While the establishment of public-private partnerships (PPPs), including the Medicines for Malaria Venture (MMV) and the Drugs for Neglected Diseases initiative (DNDi; targeting malaria, leishmaniasis and trypanosomiasis) together with the injection of funds from organizations including the Bill and Belinda Gates Foundation have improved this situation, it remains a hurdle. As PPPs generally focus their activities on portfolio drug development, they provide relatively little support for early drug discovery [9]. In addition, while PPPs have had a significant impact on the antimalarial pipeline and are beginning to improve the same for leishmaniasis and trypanosomiasis there are no new anthelmintic candidates for schistosomiasis in clinical trials [10]. International partnerships have, however, played an important role in facilitating the sequencing of a number of important parasite genomes that have aided target-based drug discovery efforts.

Rational approaches to antiparasitic drug discovery became popular when genome sequences began to become available in the 2000s. While these techniques offer a mechanism to design and assess the

activity of compounds against a specific target, they often identify compounds with poor whole-organism activity [11,12]. The need to identify and validate specific parasite targets can also be problematic. Validation studies are particularly difficult when clonal organisms are not widely available or reverse genetic tools are limited as is the case with schistosomes (reviewed in [13]). While permanent transgenesis technologies to facilitate gene function studies are being development for schistosomes, these methods lag behind those available for other parasites [14].

In contrast to target-based drug discovery, phenotypic-based screening strategies are more 'holistic' and by their nature identify active antiparasitic compounds. Improvements in culture techniques and assay platforms have resulted in an increased number of phenotypic HTS campaigns of large compound libraries that have identified hundreds to thousands of small molecule inhibitors as starting points for lead identification for some parasitic diseases. This is particularly true for malaria, with >20,000 "hit" compounds being identified in recent years (e.g. [15,16]). However, these screens are largely restricted to parasite species and stages that can be easily maintained *in vitro*. Antimalarial HTS campaigns have been primarily performed with asexual stage parasites, although gametocytes screens are now becoming more routine and there are efforts to further develop assays to assess activity against liver stage parasites and hypnozoites [17-19]. In addition phenotypic screens for new antimalarial drugs have been primarily performed with *P. falciparum* as this is the only human infecting species that can be routinely maintained *in vitro*. Although the zoonotic *P. knowlesi* parasite has recently been adapted to grow in human erythrocytes *in vitro*, drug discovery efforts have yet to take full advantage of this development [20].

Phenotypic HTS screens to identify compounds with activity against *Leishmania* and trypanosomes are problematic in that the sensitivity of these parasites to compounds can vary widely [21,22]. It is also commonly acknowledged, that to be truly representative of the *in vivo* situation, screens should assess activity against intracellular stages of parasite development [23,24]. Assessing the activity of

compounds against intracellular parasites while now possible has been challenging [23,24]. Phenotypic assays to identify compounds with activity against *Schistosoma* are available, but as studies often rely on assessing the activity of compounds against adult parasites capacity is limited by the requirement of a laboratory based life cycle facility and host animals [25].

An advantage of phenotypic screens is that no *a priori* assumptions are made about molecular targets, a factor which increases the chance of identifying novel chemotypes that have inherent cellular bioactivity. However, MOA information is not generally obtained in such screening strategies. MOA information can be advantageous for a number of reasons, including to guide the selection of partner drugs, to aid in the prioritization of candidates for further investigation and to help in rational design of more potent and/or selective compounds. Phenotypic screening campaigns are beginning to bear fruit in progressing some compounds through the drug discovery pipeline, such as the spiroindolones [6,26], and the elongation factor inhibitor DDD107498 [27], which have both entered Phase IIa clinical studies for malaria. Nevertheless in the vast majority of cases, target identification remains a bottleneck and strategies to elucidate MOA are required to improve this position.

Drug target identification approaches

Target identification in the pre-genomic era

Before the availability of parasite whole genome sequence data, target identification strategies were limited. Without sequence data and the molecular tools needed to exploit this information, studies were confined to investigating specific targets or performing more generalized phenotypic experiments to learn more about drug action. As an example, studies investigating the stage-specific activity of drugs [28-30] or the transport and location of drugs within cells [31] were often performed in an attempt to devise hypotheses on action and putative drug target/s. While some of these strategies were successful in providing important MOA information they rarely identified the target of a drug. Genomic studies, such as chromosome mapping, have also been used to analyse the progeny of

genetic crosses between drug-resistant and drug-sensitive parasites [32]. However, as these studies are complex and, at the time were limited by the lack of sequence data and tools to facilitate downstream analysis, they have not been routinely performed. Even today, with modern genotyping methods and the availability of enhanced technology and tools (see **Selected Resources**) to facilitate progeny analysis [33], genetic crosses for most parasitic species remain a challenge and are rarely performed. Notwithstanding the difficulties and costs involved, exciting advances in the malaria arena involving humanized mouse models in the generation of genetic crosses may facilitate the use of this technique going forward [34].

Genetic crosses

Genetic crosses between drug-sensitive and drug-resistant parasite clones result in recombinant progeny with different drug sensitivities that can be used for target identification and validation studies (reviewed in [35]). However, as these studies require sexual reproduction, they are generally complicated by the complex nature of parasite life cycles and the need for a suitable host. For malaria, only three *P. falciparum* genetic crosses using chimpanzees have been reported [36-38]. While progeny from each of these crosses have been used to investigate drug action it could be argued that they have been underutilized to date. However this may change now that various high-throughput genotyping tools are available. Progeny from the HB3 and Dd2 cross performed in 1990 have been used to (i) investigate the role of *P. falciparum* multi-drug resistance (*Pf*MDR) pumps 1 and 2 in mediating chloroquine resistance [37], (ii) map the chloroquine resistance locus to chromosome 7 [32], (iii) investigate quinine resistance [39] and (iv) study artemisinin resistance [40]. Progeny from the *P. falciparum* 7G8 and GB4 cross, published in 2008 [36], have been used to investigate the activity of trimethoprim, triamterene and dihydroergotamine methanesulfonate [33]. Whereas progeny from the HB3 and 3D7 cross performed in 1987 were used to provide evidence that pyrimethamine resistance is mediated by point mutations in the gene encoding dihydrofolate reductase-thymidylate synthase [41]. Similar studies investigating the action of drugs in rodent

Plasmodium species including *P. chabaudi* [42] and *P. yoelli* [43] have also been performed, however the differences between human and murine parasite species as well as host biology may limit their relevance to human disease. To circumvent the need for a mammalian host to derive *Plasmodium* crosses relevant to human disease, alternative models have been developed. For example, a recent study has shown that *P. falciparum* parasites can be crossed in "humanized" mice [34]. A human liver chimeric mouse model injected with human erythrocytes can support liver and asexual blood stage *P. falciparum* development. While this model has not yet been used to investigate drug action or resistance mechanisms, it has yielded independent progeny from three separate crosses [34]. The availability of this model, together with modern genotyping methods that facilitate progeny analysis, is likely to positively impact the use of genetic crosses as tools to investigate drug action and resistance mechanisms.

Experimental genetic crosses with other parasites including *Schistosoma* [44-47], *Trypanosoma* [48,49] and *Leishmania* species [50] have been performed. While most of these crosses have not been used for drug action studies, some have been used to investigate drug resistance inheritance [44,47]. The progeny of crosses with drug-sensitive and drug-resistant *Schistosoma* have demonstrated that resistance to hycanthone is controlled by a single autosomal recessive gene [44-46] and that resistance to praziquantel is mediated by the inheritance of a dominant trait [47]. With regard to *Leishmania*, genetic cross studies have only recently been performed as until recently these parasites were believed to replicate only asexually [50].

Phenotypic alterations

Phenotypic alteration investigations have been and remain an important component of parasitic drug action and target identification studies. Observations including changes in morphology, biochemical alterations or identifying reduced sensitivity under certain circumstances, including the presence of another chemical or the absence of free oxygen, can suggest specific targets or pathways associated

with drug action. In addition when hypotheses concerning MOA or drug target exist, specific phenotypic studies can be used to triage targets for further investigation and validation studies. These investigations may involve many different techniques depending on the compound and parasite under investigation and the resources available. Some examples of the more widely used phenotypic assessments that can be made are discussed below.

Stage-specific activity

Parasites have complicated life cycles that involve changes in morphology and gene expression. As not all parasite proteins and metabolic pathways are required by all stages of development [51,52] the sensitivity of specific stages to drugs can provide useful drug action information (**Figure 1A**). This analysis can be particularly informative when paired with a hypothesis, expression data and knowledge of the pathways required by individual parasites at each stage of development (See **Selected Resources**). As an example, the stage-specific activity of new lead antimalarial agents is often assessed to ascertain the range of their activity and potential use [6,53]. However these studies have also provided information on drug action. For example, chloroquine, which is known to inhibit the digestion of host haemoglobin by parasites that have infected red blood cells, is primarily active against asexual stage trophozoites [54]. Stage-specific activity studies were also key in identifying that type II fatty-acid biosynthesis is essential to liver stage, but not intra-erythrocytic asexual stage, *Plasmodium* parasites [55], information that can now be used to investigate the MOA of other agents that may target this pathway. As another example, inhibition of *P. falciparum* calcium dependent protein kinase 1 blocks merozoite egress in the mature schizonts [56]. Gametocytes also appear refractory to the activity of compounds targeting the apicoplast [57]. Likewise the stage-specific activity of drugs active against *Leishmania* [28] and *Schistosoma* [29,58] have also provided information concerning drug action. For example *L. donovani* amastigotes were found to be more susceptible to the pentavalent antimony (SbV) compounds than promastigotes [28].

Activity kinetics (rate-of-action)

The time required for inhibition to be detected following drug exposure can provide information about compound MOA. Data describing antiparasitic effects, including rate-of-action, that are markedly different from clinically used drugs can highlight novel activity and help triage drugs for different indications [6,59]. Rate-of-action studies are particularly useful at identifying compounds that target the remnant, plant-like, apicoplast organelle, which is present in most Apicomplexan parasite species. Inhibitors such as the antibiotics doxycycline and clindamycin display what is commonly referred to as a "delayed death" phenotype [60] (**Figure 1B**). They are typically slow inhibitors of parasite growth, with effects not seen until parasites complete a second cycle of development [60]. To detect this phenotype, activity is assessed over two cycles of development and data compared to inhibition after one cycle exposure. For example, to detect a "delayed death" phenotype against *P. falciparum*, *in vitro* assays are performed for 96 h whereas typical drug inhibition studies are assessed for 48 or 72 h. Once a "delayed death" phenotype is identified, additional studies including isopentenyl pyrophosphate (IPP) rescue (described below) can be performed to learn more about drug action and validate apicoplast targeting.

Activity against drug resistant parasites

Drug resistant parasites have been successfully used as tools to investigate drug action for decades. The sensitivity of parasites with known drug-resistant phenotypes to compounds of interest can provide mechanistic information or indirect evidence that a compound of interest has an activity different to that of currently available drugs [6,59,61]. Those data can be used to prioritize the development of drugs less likely to be cross-resistant to clinically used drugs. This is particularly true in the malaria drug discovery field, where as a general standard, new drug leads are assessed against panels of parasites that have resistance to known and clinically used antiparasitic agents in order to triage candidates for further development [6]. While the importance of screening against multiple parasite strains is widely acknowledged in the parasitology field, the activity of drug leads for

leishmaniasis, schistosomiasis, and trypanosomiasis are less commonly assessed against drug-resistant parasites. This may be associated with funding, but in the Schistosoma arena there has also been some debate as to what constitutes praziquantel resistance given few resistant isolates have been detected in the field (reviewed in [62])

Resistant parasites can be generated by cloning culture adapted clinical isolates [63], by carrying out genetic crosses with parasites of differing resistance profiles (see above) or through *in vitro* [6,64-66] or *in vivo* selection (discussed below). As an example of the information that sensitivity studies with drug resistant parasites can provide, a recent HTS campaign involving ~3,000 compounds identified ~500 active compounds but only ~150 of these "hits" displayed activity against all seven *P. falciparum* lines (of differing drug sensitivities) included in the study [33]. Likewise, a screen of ~310,000 compounds for activity against *P. falciparum* identified ~560 validated hits (IC_{50} <2 μM) of which 58 had activity against parasites resistant to chloroquine, atovaquone, and sulfadoxine/pyrimethamine [15], potentially prioritizing these novel hits for further study.

Pathway rescue

Chemicals that interfere downstream in a molecular pathway to "rescue" the effect of an inhibitor thought to act on the same pathway at an upstream point have been effectively used *in vitro* to examine drug action and to aid in the identification of drug targets (**Figure 2**). This pathway rescue approach has also been used to demonstrate the importance of different metabolic processes to parasite survival. Pathway rescue was recently used to validate non-mevalonate isoprenoid synthesis in the apicoplast as the target of fosmidomycin in *P. falciparum* [67]; with studies showing that cultures supplemented with IPP are protected from drug action. Interestingly IPP also protected *P. falciparum* from the activity of "delayed death" antibiotics and was integral in establishing non-mevalonate isoprenoid synthesis as the only essential function of the apicoplast in asexual blood-stage *Plasmodium* parasites. [67]. More recently, IPP rescue has been used to determine that one of the compounds from the

Medicines for Malaria Venture "Malaria Box" (MMV008138) specifically targets the apicoplast and non-mevalonate isoprenoid synthesis [68]. Additional rescue strategies that have been used to investigate the action of compounds against *Plasmodium* parasites include the use of hypoxanthine, coenzyme A, *para*-aminobenzoic acid (PABA), folate and Na^+. Hypoxanthine rescues the effect of transition state analogue inhibitors of purine nucleoside phosphorylase (PNP), an enzyme that catalyses inosine phosphorolysis to hypoxanthine [69], coenzyme A rescues the inhibition of its own synthesis [70] and folate and PABA interfere with the activity of anti-folate agents including sulfamethoxazole [71]. Low concentrations of Na^+ on the other hand were recently shown to protect *Plasmodium* parasites from the action of pyrazolemide PA21A050 [61].

In *Trypansoma brucei rhodesiense*, rescue experiments have supported the hypothesis that farnesyl pyrophosphate synthase is the principal target of nitrogen-containing bisphosphonates [72]. In those studies data showed that risedronate-induced growth inhibition of bloodstream-form trypomastigotes is reversed by the addition of exogenous farnesyl pyrophosphate or farnesol, both used downstream in the protein prenylation pathway and in the production of dolichols, ubiquinones, and sterols [72]. As another example, inhibition of *T. brucei* blood stream forms by 1-[(*n*-oct-1-ylamino)ethyl] 1,1-bisphosphonic acid, an inhibitor of solanesyl diphosphate synthase (*Tb*SPPS) in the ubiquinone (UQ) synthesis pathway, is abrogated by addition of UQ10 (UQ with 10 isoprenyl subunits) [73]. Likewise the activity of DL-alpha-difluoromethylornithine (DFMO or elflornithine) against *T. brucei* is inhibited by putrescine providing evidence that ornithine decarboxylase is the target of this inhibitor [74].

For *Leishmania*, studies have shown that the effects of DFMO can be abrogated by the addition of putrescine [74] and bis(benzyl)polyamine analogues that target polyamine biosynthesis [75]. More recently the inhibitory effect of the antidepressant drug ketanserin has also been shown to be partially

rescued by supplementing growth media with ergosterol, but not cholesterol, indicating some involvement in the ergosterol biosynthetic pathway [76].

Drug combinations

In an effort to combat the development of drug resistant parasites, the treatment of parasitic diseases is becoming increasingly dependent on drug combinations. While not always indicative of efficacy *in vivo*, the *in vitro* activity of drug combinations can help triage potential drug partners and identify drug resistance reversal agents. In addition to this potentially useful information, studies examining the interaction of drugs, whether *in vitro* or *in vivo*, can provide useful mechanistic information. This is particularly true if the action of the drug chosen as the partner is known. To provide evidence that HIV aspartic proteases target one or more *Plasmodium* parasite aspartic proteases known to be involved in haemoglobin digestion, the activity of these drugs in combination with other agents known to inhibit haemoglobin was investigated [77,78]. These studies provided evidence that the action of these drugs, while perhaps complicated by multiple activities including the inhibition of the chloroquine resistance transporter (PfCRT) [79], is unlikely to involve a *P. falciparum* digestive vacuole aspartic protease (plasmepsin). Subsequent studies using additional techniques such as transgenic parasites have supported these observations [78,80]. Similar studies have been performed with other protease inhibitors to examine their action against *Plasmodium* parasites [81]. Additional combinations such as those that include atovaquone (known to inhibit electron transport) [82], the nitroxide radical spin trap, 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) [83], and the ergosterol biosynthesis inhibitors terbinafine and ketoconazole [84,85], have also been used to provide drug action information. As a word of caution, data from such studies can often be confounded by off-target effects and should be interpreted with care and in conjunction with additional supportive studies.

Other phenotypic investigations

Additional phenotypic studies can also provide evidence for drug action and, similar to combination studies, can support drug target hypotheses (**Figure 3A**). Depending on the parasite species, drug induced changes in morphology may be easily assessed and may provide MOA information, although this may sometimes be complicated by downstream off-target treatment consequences. The distinct digestive vacuole abnormalities induced by the cysteine protease inhibitor E64 in *Plasmodium* parasites is a particularly good example of a morphological change directly associated with drug action. As an inhibitor of cysteine proteases, E64 inhibits digestive vacuole haemoglobin digestion in *P. falciparum* and causes a distinct swelling in these organelles post exposure [81]. The morphology of *S. mansoni* treated with miltefosine has also been shown to result in severe surface membrane damage [86], an observation that correlates well with its hypothesized antineoplastic activity [87]. In addition, praziquantel has been reported to cause severe muscle contraction in these parasites [86], an observation that fits well with this drugs ability to disrupt Ca^{2+} homeostasis [88].

Drug-induced changes in morphology can be particularly informative if partnered with drug localization or specific organelle tagging or staining techniques. As an example, transgenic parasites with chimeric proteins effectively tagging organelles can be used in conjunction with drugs to assess drug-induced changes in morphology [89]. Alternatively, or in addition, the location of an inherently fluorescent drug can be examined. For example, studies with the fluorescent inhibitor, 2,5-bis(4-amidinophenyl)furan (DB75) have shown that this agent co-localizes to the mitochondria in trypanosomes where it disrupts membrane potential and inhibits cellular respiration [31]. Other phenotypic changes that can be used to provide MOA related information include those that demonstrate a drug associated change in parasite metabolism or gene expression (See **Figure 3**). As an example the presence of undigested haemoglobin has been used to validate the inhibition of haemoglobin digestion by E64 and other protease inhibitors [81].

Target identification in the post-genomic era

The genomic era and associated technological advances have revolutionized antiparasitic drug target identification strategies. Although drug target investigation methods used before the genomic era are still used today and continue to provide data to support drug target identification, many of those techniques have been modified to provide whole-organism data rather than specific information on a particular pathway or target. Techniques not previously possible in the absence of the relevant technology and genomic data are now available and are being widely implemented to provide unprecedented information on the action of currently used and experimental antiparasitic agents (discussed below).

Genomic strategies

Drug-resistance and whole-genome analysis (Genome-Wide Association Studies; GWAS)

As discussed above, drug-resistant parasite lines are a powerful tool in the antiparasitic target identification arsenal. They have been widely used to investigate drug action and the advent of rapid genome sequencing methods and bioinformatic tools means that it is now feasible to directly select for parasite clones with reduced drug sensitivity and perform multiple whole genome sequencing to identify putative drug targets in multiple resistant parasites [90,91] (**Figure 3B**). Alternatively genomic differences can be identified using high density tiling arrays and comparing the hybridization data of parasite clones [6,92]. For *P. falciparum*, software to analyse DNA microarray hybridization differences between drug-sensitive and drug-resistant parasites and to assess and compare deep coverage sequence reads are freely available online [92,93] (See **Selected Resources**). As parasites often evolve mutations in drug targets as a means of combating toxicity, such techniques are very powerful at identifying possible targets (specific examples discussed in [94]). However, as drug resistance can result from mutations not associated with drug action, this technique may not always provide the desired information [95]. Arguably the biggest difficulty associated with this method of target identification is accounting for the spontaneous mutations that are not associated with drug resistance [94]. To account for these unrelated mutations it is often necessary to perform

multiple independent selections and comparisons so that common changes in resistant parasites can be identified (**Figure 3B**). In recent years these techniques have been used to identify the putative targets of drugs including fosmidomycin [92], decoquinate [96], mupirocin [97], pyrazolemide compounds [61], MMV008138 [64], cladosporin [98] the spiroindolones [6] and DDD107498 [27] in *P. falciparum*. *Leishmania* parasites resistant to 5-fluorouracil [99] and miltefosine [100] and *Trypanasoma* parasites resistant to DFMO [101] have also been generated in order to better understand the mechanisms of resistance and the targets of these agents.

Resistant parasites are most often generated *in vitro* [6,61,100]. However, *in vivo* selection [102,103] and historical crosses [33] have also been used. The *in vitro* selection of parasite lines resistant to antiparasitic agents usually involves drug pressure, however this process can also involve the use of a mutagen [105]. The generation of resistant parasites via transposon mutagenesis, while not widely employed at this stage (primarily as a result of poor saturation) may also provide important drug target identification data in the future [106]. Different drug exposure methods can be used to select for parasite resistance, however standard reference clones with known phenotypes and genotypes should be used as it is far easier to tease out the underlying changes responsible for drug resistance when parental genetic and phenotypic information is available [91,95].

Resistant parasites can also be generated *in vivo* and those studies are most often performed using established murine models, which have been particularly useful in the generation of *Plasmodium* parasites with resistance to antimalarial drugs [103]. Similar to *in vitro* selections, the time required to generate resistant murine *Plasmodium* parasites largely depends on the compound being studied with reports suggesting chloroquine resistance is detectable in 6 to 77 days [107,108] whereas quinine resistance develops in 38 days [107], atovaquone within 10 days [102] and artemisinin resistance occurs in $\geq$300 days [102]. *In vivo* drug resistance selection protocols have also been successfully applied to *Schistosoma* [109,110] and *Trypanosoma* parasites [111,112]. A caveat to this approach

however, is that there may be differences in compound susceptibility of human versus animal infecting parasites, and hence, such strategies may not always be suitable for all agents in the context of downstream target identification studies.

Genome-wide RNA interference screening

RNA interference (RNAi) is a process that regulates gene expression. It is driven by the presence of double-stranded RNA (dsRNA) and cellular machinery that process the RNA in a fashion that results in the regulation of genes with complementary sequences. Double-stranded RNA processing requires Dicer, an enzyme that cleaves the RNA into small double-stranded fragments known as small interfering RNAs (siRNA; ~20 nucleotides). These siRNAs are then unwound so that antisense strands can be incorporated into a multi-protein complex (RISC; RNA-induced silencing complex) that recognizes complementary sequences and cleaves them, effectively preventing them from being translated. The phenomenon of RNAi has been revolutionary, providing scientists with a powerful tool that can be harnessed to study the impact of specific gene knock-downs on various phenotypic characteristics. Almost all eukaryotes have the cellular machinery for RNAi (dicer and RISC), with *Plasmodium* parasites being an obvious exception [113,114]. While effective in Schistosomes, *T. brucei*, and *L. (V.) braziliensis* this process is not functional in *T.cruzi*, *L. major* and *L. donovani* [114].

RNAi has been used to effectively investigate gene function in *T. brucei* [115] and schistosomes [116]. More relevant to this review however, is the relatively recent use of this tool as a method to investigate the role of genes in mediating a phenotype on a genome wide-scale [117,118]. While studies with the parasites of interest to the current review have been largely restricted to *T. brucei*, this work has demonstrated the power of RNAi in identifying genes that contribute to drug susceptibility and parasite drug resistance. Those studies have been used to investigate the resistance/action of tubercidin [118], nifurtimox, benznidazole and DFMO [117,119] against *T.*

brucei. In essence genome-wide RNAi involves the construction of an RNAi plasmid library to produce dsRNA and gene silencing in parasites. Parasites with differential sensitivities to drug candidates can then be selected and analysed to identify processes and associated genes involved in drug action and resistance [117,118]. An obvious limitation of this process, however, is the potential detrimental effect of knocking-down essential genes [117]. In addition off-target and partial knock down as a result of this strategy has the potential to complicate target identification.

Transcriptomic strategies

In this approach drug-induced changes in transcription are assessed to identify putative drug targets. The transcription profile of parasites exposed to drug is compared to that of parasites left untreated (**Figure 3A**). As global changes in transcription and protein expression can vary considerably between stages of parasite development, these studies must be careful to control for such developmental changes. In addition, secondary responses as a result of stress, parasite death or life cycle progression, must also be filtered from data if this approach is to be useful. The use of high drug concentrations and long exposure times can compound these issues [120] and must be carefully considered and controlled.

The global transcription of *Plasmodium* parasites exposed to different antimalarial agents including chloroquine and pyronaridine has been examined using microarrays [120]. In this process, cDNA generated from treated and untreated parasites is labelled and hybridized to a microarray [120]. As the data derived from these studies are often complex, they have, to date, been unable to discern compound-specific MOAs. However, they have highlighted numerous responses to drug exposure that may be useful when taken together with additional target identification strategies. This approach may also be useful in identifying drugs with different MOAs [120]. Examples of this approach being used to investigate the action of compounds against Schistosomes are available [121,122] and demonstrate that while complex, data derived from these studies can be useful in identifying groups

of candidate targets or pathways. The greater power of RNAseq (reviewed in [123,124]), which permits the unbiased detection of novel and less abundant transcripts, together with enhanced bioinformatics tools may further improve this position in the future.

In addition to the examination of drug-induced changes in gene expression studies, investigating drug action have also compared transcriptional changes in drug-sensitive versus drug-resistant parasites (**Figure 3B**). Similar to drug treated parasites the transcriptional changes in drug-resistant versus drug-sensitive parasites have been assessed using microarrays. As examples, the resistance of *Leishmania* parasites to methotrexate and *Plasmodium* parasites to artemisinin has been investigated using this strategy [125,126]. Both of these studies identified numerous transcriptional changes in drug-resistant versus drug-sensitive parasites, shedding further light on mechanisms of drug resistance that fit-well with current knowledge. Of particular note the *Leishmania*/methotrexate study successfully identified changes in dihydrofolate reductase-thymidylate synthase (DHFR-TS) expression when parasites were exposed to this antifolate and DHFR-TS inhibitor.

Proteomic strategies

Activity-based Protein Profiling (ABPP)

ABPP has recently matured as a tool to identify unknown targets, as well as putative off-targets, of small molecules in mammalian systems. The approach directly interrogates the native proteome of the organism in question (either whole cells or lysates thereof) using active probes designed to label specific target bio-molecules [127]. Probe molecules comprise (i) a target binding group to specifically interact with the target/s, (ii) a reactive moiety to covalently link the probe to the protein target/s, and (iii) a reporter group to facilitate visualization and/or purification of the probe-labelled target/s [127]. The success of ABPP hinges on probe design, as structural modifications to introduce the linker and reporter groups must not interfere with target binding. This concern can be addressed by the use of sterically benign, latent tags that are subsequently modified in a second step (e.g. using

click chemistry) to incorporate the reporter group [127]. Identification strategies for probe-labelled target/s are numerous but commonly employ Western blot and liquid chromatography-tandem mass spectrometry [128]. ABPP is relatively new to the field of parasitology but this technique has proven useful in the identification of inhibitor targets.

Targets of chloroisocoumarin (the essential *P. falciparum* serine protease *Pf*SUB1) and bestatin (the *P. falciparum* aminopeptidases *Pf*A-M1 and *Pf*LAP) were identified using biotin-based activity-based probes [128,129]. An E-64 activity-based probe, DCG04, has been used to demonstrate that this broad spectrum cysteine protease inhibitor recognizes *P. falciparum* falcipain-1, calpain-1 and calpain-2, together with several cysteine proteases, in multiple *Schistosoma* species [130-132]. Another notable example includes the use of the vinyl sulfone probe VS-1 to identify the putative cellular targets of the anti-*Trypanosoma brucei* agent K11777 [133]. Collectively, all these examples demonstrate the potential usefulness of this technique in identifying antiparasitic inhibitor targets.

To eliminate the need for the large number of cells that are required for affinity purification experiments with activity-based probes, it is possible to substitute cell lysates with libraries of expressed proteins or peptides. While to our knowledge this technique has not yet been published for the identification of antiparasitic drug targets, it has been used to identify drug-target interactions in other systems including humans [134,135]. These methods of target identification could be of interest for parasites as it is often difficult to obtain sufficient parasite material for affinity purification experiments. This is particularly true from experiments involving stages of parasite development that are difficult to propagate *in vitro*. *P. falciparum* gametocytes, for example, while inducible in *in vitro* culture systems are terminally differentiated and difficult to amass in high numbers for these experiments. It is also currently not possible to effectively propagate many of the human *Plasmodium* parasites species *in vitro*.

Protein/peptide libraries can be generated using several different techniques including cell-based and cell free systems [136,137]. Yeast three-hybrid and phage display systems can also be used in activity-based probe studies and offer distinct benefits by permitting repeated rounds of interaction and amplification and the facile identification of the interaction partner. While a yeast three-hybrid system has yet to be used to investigate the action of an antiparasitic agent, a modified mammalian three-hybrid system has been used to investigate the interaction of *P. falciparum* type 2 methionine aminopeptidase with fumagillin [138]. Many eukaryotic cDNA libraries have been constructed in phage. These libraries can be used to search for protein-protein, peptide-protein and protein-antibody interactions, however, there are also numerous examples of their use in drug-target interaction studies, including the identification of binding partners of ~30 small molecules including FK506 [134] and the curcumin analogue, HBC [139]. Phage display libraries have been used in the parasitology field to identify protein-protein interactions [140], but they are rarely exploited as tools to identify the targets of antiparasitic agents. Nevertheless this technique has demonstrated potential with one study illustrating its use to examine the activity of praziquantel against *S. mansoni* [141].

Drug Affinity Responsive Target Stability (DARTS)

In a similar fashion to ABBP, DARTS involves the interrogation of whole-cells or cell lysates for small molecule targets. DARTS relies on the theoretical principle that a protein once saturated with a specific ligand becomes thermodynamically stable and resistant to proteolysis. In essence the process involves the incubation of either whole cells or protein lysates with compound at increasing concentrations, proteolysis of the sample (negative enrichment) and follow-up protein identification strategies [142]. Small molecules require no chemical modification and the ligand-protein stability phenomenon appears widespread [143]. While not yet used to identify the targets of antiparasitic agents DARTS has been used to confirm small molecule-protein interaction in other systems (reviewed in [143]) and warrants further investigation in this arena. Additional methods such as cellular thermal shift assay (CETSA) [144] that rely on the same stability phenomenon but different

approaches to measure or assess protein stability as a result of ligand binding are also in use to investigate drug targets, but may be better placed as target validation tools.

Inhibitor-induced proteome changes

In this approach, inhibitor-induced protein expression changes are assessed in order to glean MOA information (**Figure 3A**). Proteins expressed by parasites exposed to drug are compared to those expressed by parasites not exposed to drug [145]. However, studies may also compare the protein expression of parasites with different sensitivities to drugs of interest (**Figure 3B**) [146]. Protein samples can be compared using techniques including, 2D electrophoresis [146] or 2D Difference Gel Electrophoresis (2D-DIGE) married with in-gel trypsin digest and subsequent mass spectrometry [145] or isobaric tag for relative and absolute quantitation (iTRAQ) labelling followed by nano-liquid chromatography tandem mass spectrometry [145]. Once proteins with altered expression have been identified specific parasite processes that may be linked to drug action can be identified. The potential impact of these whole-proteome assessment techniques in providing drug action information in the parasitology field has been demonstrated by several studies investigating the activity of the antimalarial agents including doxycycline [145], quinine [147] and mefloquine [147].

While currently providing a fingerprint of drug-induced changes and implicating pathways involved in drug action, this target identification strategy has yet to pinpoint a specific target of a drug. It is also limited by the fact that a large proportion of putative parasite proteins currently have no known function (reviewed in [148]). In the study performed by Briolant et al. (2010) 20% of the proteins differentially expressed could not be assigned a function [145]. In addition it is clear that non-specific changes or responses to drug treatment (including changes in the expression of proteins involved in redox metabolism) need to be considered in the overall analysis [145].

Untargeted metabolomics

This approach involves the investigation of global drug-induced changes in cellular metabolism and in essence involves quantifying the presence of hundreds of small molecules in the biological system of interest pre- and post-inhibitor treatment (**Figure 3A**). Changes in parasite metabolism as a result of inhibitor exposure can then be detected and used as a mechanism to identify individual metabolites or pathways associated with activity [149]. Metabolomics has been used to identify the target/s of many antimicrobial agents including some antiparasitic agents (reviewed in [149]). The inhibition of ornithine decarboxylase by the trypanocide DFMO [150] and the inhibition of cytochrome bc1 by the antimalarial drug atovaquone [151] have been confirmed using untargeted metabolomics. Additional examples include the identification of the mevalonate isoprenoid synthesis pathway, particularly deoxyxylulose phosphate reductoisomerase and methylerythritol phosphate cytidyltransferase as the target of fosmidomycin in *Plasmodium* parasites [152] and the identification of cytochrome bc1 as the target of the novel antimalarial CK-2-68 [151].

Whole cell metabolome studies are typically carried out *in vitro* as environmental factors can be controlled. However, care must be taken to ensure that culture medium do not impact drug activity. Multiple inhibitor concentrations and time-point assessments are also important when performing these studies as off-target changes can be seen following prolonged exposure at high concentrations. Samples are usually assessed by mass spectrometry or nuclear magnetic resonance (NMR) spectroscopy and data subsequently analysed using open-sourced software [149]. While this method of target identification is limited to compounds that target a metabolic pathway and can be hindered by off-target responses, the primary bottle-neck preventing the widespread use of this technique is arguably our current knowledge of parasite metabolism. As mentioned previously, many parasite genes have not yet been annotated [148] and as a result many metabolic processes are not yet understood.

Overexpression libraries

This technique involves generating a shotgun plasmid library and transfecting it into a parasite population so that various proteins/peptides are over-expressed. It works on the premise that the overexpression of a targeted protein would protect parasites from the activity of a drug and allow it to be selected from the population permitting the target to be identified. This process has not been widely implemented in the field, but was recently used to identify the target of DFMO in *T. brucei* [153]. While potentially powerful, this method is not without its pitfalls in that is unlikely to express a full complement of fully-functional proteins and hence may not be successful in every application. The expression levels of some proteins may also be tightly regulated by parasites. Given these limitations the overexpression concept is arguably better utilized for target validation purposes.

In silico *approaches*

Experimental approaches to investigate drug targets are often expensive. As an alternative to experimental investigations, *in silico* analyses (aka *in silico* target fishing) can be used to minimize costs and prioritize putative compound targets for further investigation. The availability of complete genome data (see **Selected Resources**) together with drug databases linked to bioactivity data in user-friendly platforms (see **Selected Resources**) has facilitated these approaches of drug target identification.

Putative drug targets can be identified *in silico* by cross referencing drug sensitivity data with suspected biological targets via genome mining. When a small molecule has been assessed for activity in multiple biological assays these data, combined with interactive genomic databases can aid in the identification of potential target pathways/proteins. This process of target identification is particularly useful when the small molecule in question has a defined target in another organism, as may be the case in drug-repurposing strategies. The histone deacetylase (HDAC) inhibitors and the antiretroviral aspartic proteases inhibitors are good examples since both inhibitor classes have antiparasitic activity and known targets in other systems that have guided target identification strategies in parasites

[77,154]. Once identified putative targets can be further examined by mining for additional data, including expression profiles, which may provide further evidence for further experimental studies. *In silico* docking experiments to test hypotheses concerning protein-small molecule interactions can also be performed if structural data are available (see **Selected Resources**). If X-ray data are not available, the target of interest can be modelled *de novo* or by using a chosen template (homology modelling). Homology modelling was used to identify hexokinase as a putative target of selected dialkylphosphorylhydrazones in *L. braziliensis* [155]. However further validation studies were not performed.

An alternative method of *in silico* target fishing involves comparing the structures of compounds with unknown MOAs to the structure/s of compounds with known MOA. However the scoring method chosen and the pharmacophore responsible for activity must be carefully considered if using these comparisons. If biological data are available it appears more prudent to cluster and compare the activity profiles of compounds with known MOAs to the activity profiles of unknown small molecules. This technique is particularly useful when large bioactivity data sets are available and has been used to identify folate synthesis/DHFR-TS as the putative target of 11 hit compounds in a screen for antimalarial agents [156].

Conclusion

In the current climate of increasing parasite drug resistance it is becoming increasingly important to understand the MOA of agents that target the major parasitic diseases that cause significant health, social and economic impacts. This knowledge can be used to triage the development of bioactive compounds that have different targets or MOA to currently available drugs, suggest tools to monitor the development of resistant parasites, and seed development of new analogues with improved pharmacodynamic and/or pharmacokinetic profiles. Methods to identify drug targets and understand small molecule inhibitor MOA have historically been limited and complicated by a lack of whole-

organism data and the tools required to assess organism-wide responses. However the '-omics era' has provided researchers with novel tools and resources to derive hypotheses in relation to drug action. Although understanding antiparasitic drug action can still be complicated by factors including bioactive promiscuity and poor gene annotation, multiple techniques to facilitate drug target identification are now available. These methods, together with additional target-validation strategies (beyond the scope of the current review) are facilitating an unprecedented understanding of drug action. The genome wide-analysis of drug-resistant parasites has been particularly productive in this regard and is arguably the current method of choice to establish a gene-driven MOA hypothesis in the parasitology field.

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