Target identification and validation of novel antimalarials

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It has been recognized that new antimalarials with a novel mode of action are critical to combat the continued emergence and dissemination of drug-resistant parasites that threaten the efficacy of current malaria treatments. Thus, recent high-throughput screening campaigns have been initiated using asexual intraerythrocytic stage cell-based assays of *Plasmodium falciparum*. These have led to the unprecedented identification of over 10,000 new antimalarial compounds. Inherently, novel compounds identified by cell-based assays will have poorly defined modes of action. While some of these compounds may have recognizable targets, the majority of cell-based hits are comprised of unique chemical scaffolds usually lacking cross-resistance with known drugs. It is likely that these novel antimalarial scaffolds will reveal new targets. A challenge for the community will be to assign these small molecules to their targets. In this article, we review methodologies to assist in the determination of a compound's mode of action.

It is generally accepted that drug resistance is an inevitable fate of all malaria therapies. Unfortunately, in the absence of a vaccine, chemotherapy remains the only method to treat individuals infected with malaria. The emergence of drug-resistant *Plasmodium* parasites has already compromised the efficacy of many antimalarials leaving artemisinin-based combination therapies as one of the few treatment regimens whose efficacy is assured. Due to their clinical importance, the emergence of artemisinin (ART) resistance among parasites in the field has been closely monitored. In the last few years, field surveillance has detected parasites with a reduced ART susceptibility in western Cambodia [1–3], a locale where resistance has often emerged first [4]. This discovery has reinforced the urgent need for new antimalarials that can be deployed in the event that resistance spreads.

While much drug development energy has historically focused on improving scaffolds with known antimalarial activity, new molecular entities discovered in cell-based screens are gaining attention. Since 2008, three prominent high-throughput screening (HTS) campaigns of asexual intraerythrocytic stage *Plasmodium falciparum* have been reported [5–7]. Collectively, nearly 5 million compounds have been screened, resulting in the unparalleled discovery of thousands of new small molecule inhibitors. These screens were completed by public (St. Jude Children's Research Hospital) and private programs (GlaxoSmithKline and Novartis) and have elevated malaria drug discovery efforts to an unprecedented level. The public disclosure of these compounds and deposition in an online resource underscores the shared commitment and desire for rapid development of new malaria medicines [101]. Under the guidance of Medicines for Malaria Venture (MMV) [102] and other funding agencies, an organized and focused drug discovery pipeline has been established to foster the development of lead compounds with the goal of elevating these compounds to clinical trials.

In some cases the molecules discovered in cell-based screens have moved rapidly to the clinic. A newly discovered antimalarial class termed spiroindolones have entered Phase I clinical trials only 3 years after their discovery [8] and other compounds with novel mechanisms of action are close behind. While understanding the mode of action (MOA) of spiroindolones is not critical for their development, it will be a paramount concern if the spiroindolones fail in clinical trials. In this case, knowledge of the MOA and the molecular target(s) could help guide the development of backup compounds. Furthermore, knowledge of their target(s) could help researchers monitor patients for the emergence of drug-resistant parasites.

In light of the windfall of novel cell-active compounds identified by recent HTS campaigns, this article reviews methodologies used to determine a compound's MOA. Determination of the MOA provides a means to prioritize scaffolds

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and can assist in the optimization of leads in medicinal chemistry efforts. Just as importantly, if the target is validated then a small molecule can become a powerful chemical tool that can increase our understanding of parasite biology.

Target identification

Mode of action for licensed antimalarials

Efforts to identify the MOA for antimalarials have traditionally been met with mixed results. The target of ART, currently the most prominent antimalarial in treatment, has remained elusive. One possibility is that ART may intrinsically have a promiscuous MOA within the parasite (possible MOAs reviewed elsewhere [9]). This may be attributable to the endoperoxide bond contained by ART. This chemical moiety is essential for activity and evidence suggests that interaction with heme induces an alkoxyl radical in the artemisinin molecule [10]. Beyond ART and its endoperoxide-containing derivatives, the MOA of quinine, 4-aminoquinolines and 4-methanolquinolines are also not completely clear, but inhibition of heme detoxification is generally accepted as the primary MOA in intraerythrocytic stage *P. falciparum* [11], with some finer differences in the exact mechanism between compound classes [12]. By contrast, proguanil/cycloguanil and pyrimethamine [13], sulfadoxine [14] and atovaquone (ATQ) [15] clearly target dihydrofolate reductase (DHFR), dihydropteroate synthetase (DHPS) and cytochrome b (Cyt b), respectively.

In silico assignment of MOA

One of the easiest ways to establish a hypothesis for a compound's MOA is by comparing its chemical scaffold to the scaffolds of compounds with a known drug target. For example, a scaffold, which is more than 95% identical to a known drug, is likely to bind the same target. A major drawback to computational analysis is the difficulty associated with setting reasonable similarity scores. Two very dissimilar scaffolds that share only a small pharmacophore critical to drug target specificity may not be recognized due to the scoring function. For example, cycloguanil and methotrexate both contain the diaminopyrimidine pharmacophore critical for binding to the folate pocket of dihydrofolate reductase, but have otherwise dissimilar structures (Figure 1).

In biology, network interactions can be inferred by looking at coexpression patterns or colocalization studies. Likewise, historical screening data may be used to associate compounds with drug targets or pathways. In the case of the GlaxoSmithKline compounds, data from pre-existing biochemical assays were evaluated for each chemotype resulting in 4205 compounds (31% of the cell-active hit collection) being assigned to 146 human or microbial targets with orthologs in *Plasmodium* [6]. Identified targets included inhibitors of the electron transport chain (ETC), aminoacyl-tRNA synthetases, proteases and serine/threonine kinases. A similar structure-clustering analysis performed by Plouffe *et al.* on the 8457 cell-active hits from the Novartis library revealed that only approximately 5% of compounds were found to have an annotated MOA [5]. These analyses show that, based on chemical similarity between structures, a probable MOA can be assigned to just a minority of the compounds. The remaining compounds, which represent the vast majority, emphasize the novelty of the cell-active hits and the necessity to identify the MOA using different approaches.

Stage of action

The complicated lifecycle of *Plasmodium spp* leads to numerous distinct morphological changes throughout its progression within the mosquito and human hosts. The morphology of drug-treated parasites can provide preliminary clues to the MOA. Giemsa-stained thin blood smears of synchronized intraerythrocytic stage parasites subjected to drug concentrations at or above the 90% inhibitory concentration (IC_{90}) are sufficient to determine the stage of action. In two cases the death phenotype can be highly informative: inhibitors of merozoite egress from the red blood cell and compounds that act on an unique organelle found only in apicomplexans termed the apicoplast.

The apicoplast, a relict plastid with red algal lineage (reviewed elsewhere [16]), has long been recognized as an attractive drug target due to its eubacterial ancestry and sensitivity to prokaryotic inhibitors [17]. Interestingly, apicoplast inhibitors, particularly antibiotics [18,19] have a characteristically unique 'delayed death' phenotype that make them easy to identify because it leads to parasite arrest in the second generation after treatment [20]. The mechanism of delayed death was studied in a related parasite, *Toxoplasma gondii* and revealed that apicoplast inhibitors prevent proper organellar segregation during parasite division, which results in apicoplast-deficient progeny that subsequently die [21]. Consequently, the delayed death phenotype requires a 96-h assay

window to account for the time required for *P. falciparum* to complete two complete life-cycles. Thus, the traditional isotopic assay and SYBR Green-based assay, which utilize 42-h and 72-h assay windows, respectively, cannot be used to correctly ascertain the IC_{50} value of apicoplast inhibitors [18]. For this reason, apicoplast inhibitors were selected against in the previously described HTS campaigns and apicoplast inhibitors may be under-represented in the antimalarial screening hits.

Pharmacological inhibition of *P. falciparum* calcium-dependent protein kinase (PfCDPK) 1 [22] and dipeptidyl aminopeptidase 3 (PfDPAP3) [23], along with induced degradation of transgenic PfCDPK5 [24], block merozoite egress and distinctively arrest the parasites in the late mature schizont stage. To verify that the egress mechanism was being specifically inhibited, Dvorin *et al.* ruptured the arrested schizonts by mechanical force [24]. The freed merozoites were able to reinvade new red blood cells, which confirmed inhibition was specific to egress.

Finally, it is also notable that some antimalarials may demonstrate stage-specific activity. A prime example is the work carried out by Vaughan *et al.*, which describes the essentiality of the type II fatty-acid biosynthesis pathway in liver stages, but not intraeythrocytic stages [25].

In vitro evolution of drug-resistant *P. falciparum*

The gene conferring chloroquine (CQ) resistance was found by mating drug-sensitive and drug-resistant parasites, and mapping sensitivity in their recombinant progeny [26], a process that took years. The availability of rapid whole genome sequencing methods or whole genome tiling arrays has in some ways made this approach obsolete. Here, *in vitro* selection of drug-resistant parasites is carried out, the resultant clones are analyzed and mutations conferring resistance are revealed using 'genome scanning' with either a high-density tiling DNA microarray or with whole genome sequencing. Although not without its shortcomings, this is the only available *Plasmodium*-driven technique to provide a 'genetic foothold' on the mechanism of resistance and will often directly reveal the drug target. The major difference between genome scanning and conventional genetic mapping has to do with strain background. In genome scanning, isogenic strains whose genome may only differ by a few base pairs are compared. However, conventional mapping between drug-sensitive and -resistant lines may reveal thousands or even tens of thousands of mutations distinguishing the genomes of the strains, necessitating genetic linkage analysis.

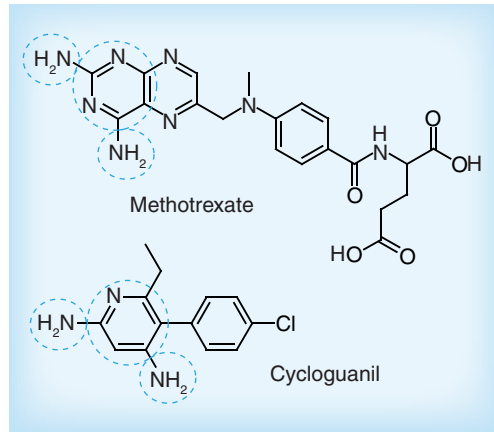

Figure 1. Cycloguanil and methotrexate. Despite the dissimilarity of these antimalarials, the diaminopyrimidine pharmacophore, indicated with dotted lines, is responsible for the specificity of these molecules to the folate-binding pocket of dihydrofolate reductase.

The technique of evolving drug-resistant *P. falciparum* parasites *in vitro* has been well established [27], but the technique was not used to determine the targets of unknown drugs until the advent of high-density microarrays to reveal newly emerged SNPs and copy number variations. Dharia *et al.* developed a software analysis platform (PfGenominator [103]) to compare the DNA microarray hybridization differences between a parental drug-sensitive clone and a drug-resistant clone [28]. The goal of the software was to make the genomic analysis user-friendly and allow someone who is untrained in bioinformatics able to easily and rapidly identify SNPs, copy number variations and small insertions or deletions acquired during resistance selection [28]. Fortunately, *Plasmodium* often acquires mutations in the drug target as a means of combating drug toxicity so this method can be very powerful for target identification. Numerous examples exist for which nonsynonymous SNPs (mutations leading to an amino acid change) in the drug-binding pocket resulted in resistance: PfDHFR [13], PfDHPS [14], PfCyt b [15,29] and *P. falciparum* protein farnesyltransferase [30]. Conversely, a gene that encodes a drug efflux pump (*pfmdr*) is amplified in response to mefloquine and halofantrine [31], and CQ resistance is acquired by polymorphisms in the gene encoding the CQ resistance transporter protein (*pfcrt*) [32]. These are well-studied examples of acquired genomic

changes in genes that are not involved in the MOA. This caveat is an important consideration when using this technique.

In theory, the detected changes in the genome of drug-resistant parasites directly reveal the mechanism of resistance. However, mutations unrelated to resistance spontaneously emerge during culturing and, therefore, must be accounted for in the analysis [28]. To assist in determining which genomic changes are significant, independent cultures are evolved in triplicate (Figure 2). It follows that those genomic changes deemed significant should be verified in genetic complementation experiments in *P. falciparum*. Various techniques are available to reintroduce the mutant allele into a drug-sensitive *P. falciparum* background to validate the conference of resistance. Anderson *et al.* thoroughly reviewed the available genetic techniques, including allelic exchange and *piggyBac* methodologies, and further discussion will not be discussed in this article [33].

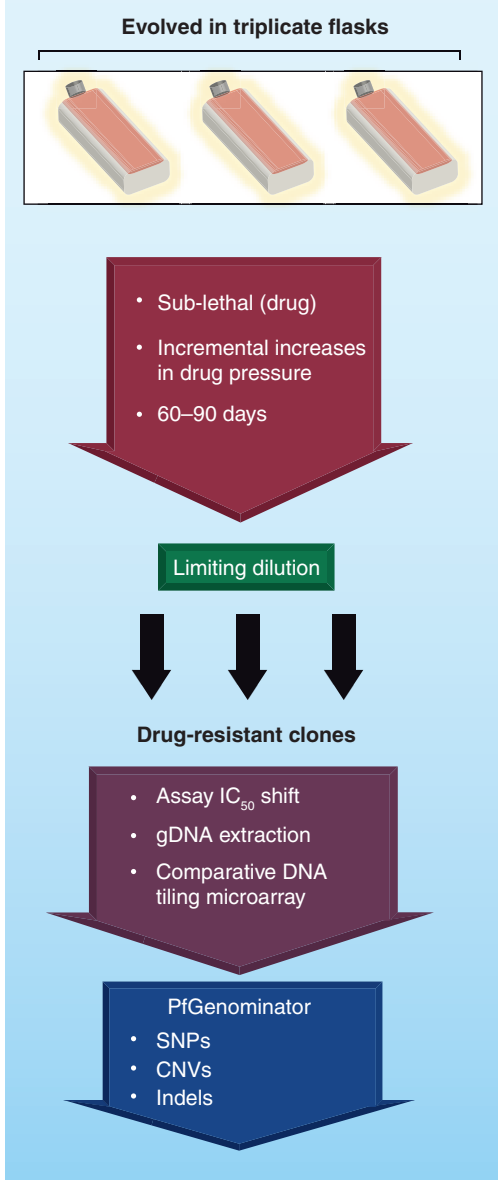

Figure 2. Evolving and analyzing *in vitro* drug-resistant parasites.
CNV: Copy number variation.

The power of genome scanning was first demonstrated by Dharia *et al.* in showing fosmidomycin-resistant parasites evolved *in vitro*-acquired gene amplifications that encompassed the putative drug target deoxyxylulose 5-phoshate reductoisomerase (PfDXR) [28]. More recently, Istvan *et al.* conclusively identified the MOA of mupirocin and thiaisoleucine based on mutations acquired in the genes of drug-resistant parasites encoding apicoplast and cytoplasmic isoleucyl-tRNA synthetases, respectively [34]. Similarly, Rottmann *et al.* also recently used this technique to reveal that mutations in a P-type ATPase cation transporter (PfATP4) confer low-level spiroindolone resistance in *P. falciparum* [35]. To date, PfATP4 has not been validated as the target of this antimalarial class, but inference based on the function of PfATP4 as a cation transporter suggests spiroindolone's MOA may involve disruption of cation homeostasis in the parasite.

Thus far, evolution of ART resistance has been challenging. However, Witkowski *et al.* have been successful in evolving a stable ART resistant line *in vitro* [36]. Application of discontinuous drug pressure over a 3-year period produced a sub-culture of parasites that overcame ART toxicity by using a quiescence mechanism. The 'resistant' parasites stall in the parasite ring stage, where they are less susceptible to ART. Once drug pressure is removed, parasite maturation is restored. It is debatable whether quiescence can be categorized as resistance and it may be best described as reduced ART susceptibility. Due to the extended duration of culturing, numerous random mutations were undoubtedly acquired and would defeat whole genome analysis. Thus, the analysis by Witkowski *et al.* was restricted to direct sequencing of select resistance gene markers and differential gene expression analysis. The latter is a less desirable method for target identification (see the section on Methodology Limitations in *Plasmodium*), but a modest down-regulation of PFE415w, a putative cell cycle regulator, may provide a clue to the quiescence mechanism. This example highlights a limitation to genome scanning of resistant parasites evolved *in vitro*.

An important question for the genes successfully identified by genome scanning is whether these *in vitro* data correlate with *in vivo* studies

of drug-resistant field isolates. Based on the limited data in hand, this would appear to be the case [37]. While differences between the SNPs observed *in vitro* versus drug-resistant field isolates have been observed, the SNPs are acquired within the same gene target and, thus, the differences could be stochastic. Consequently, genome scanning of *in vitro* evolved drug-resistant parasites can provide a likely resistance marker that can be monitored in the clinic.

Gene annotation

A practical concern is that genome scanning of *in vitro* evolved drug-resistant parasites identifies a gene of unknown function. This is because nearly 50% of the *Plasmodium* genome remains unannotated, which would greatly complicate follow-up target validation efforts. PlasmoDB [104] provides an invaluable resource for *Plasmodium* data analysis and visualization on a gene-by-gene or genome-wide scale, including annotated genomes [38]. Unannotated genes within PlasmoDB can be further queried in OrthoMCL [105] to help identify orthologous groups from seven publicly available genomes [39]. Recently, Tedder *et al.* released PlasmoPredict [106], which integrates a wide range of functional genomics data for *P. falciparum* to aid in the gene annotation [40]. Orthologs may also provide an alternate, albeit indirect, path to study and characterize the *Plasmodium* gene product in follow-up biochemical or biological assay systems. An exemplar is the recent studies on three plastid thiol transporters from *Arabidopsis thaliana*, which are homologous to PfCRT and were demonstrated to play a significant role in regulating glutathione levels and the redox potential within the organism [41]. This study may provide long sought-after insight into the precise role of PfCRT. However, it should be recognized that the respective functions of protein orthologs may be different. Therefore, additional validation is necessary before assigning the *Plasmodium* protein the same function as the ortholog. For example, *Plasmodium* proteins with a yeast ortholog can be introduced on a complementation vector into a yeast background engineered to abolish ortholog expression under nonpermissive conditions. Kruger *et al.* used this approach to elegantly demonstrate that the introduction of PFE0485w, a *Plasmodium* gene annotated as a PI4K, into a yeast background with conditional expression of Pik1, the corresponding ortholog and an essential PI4K in yeast, can rescue yeast in the absence of Pik1 expression [42].

Target validation

Methodology limitations in *Plasmodium*

The task of identifying and validating a molecular drug target in *Plasmodium* is complicated by a number of factors:

- RNAi does not work in the parasite because it lacks the enzyme needed to degrade the dsRNA into short nucleotides [43];
- Gene expression profiling of *Plasmodium* parasites revealed that drug treatment does not induce significant changes in transcription [22,44,45], unlike other eukaryotes [46];
- Genetic interaction studies of parasite gene products are limited because nearly 50% of the *Plasmodium* genes remain unannotated [38].

Described is an experimental road map to techniques that have been and/or are expected to be effective in helping determine the MOA of cell-active hits.

Biochemical screen of a putative drug target

The most straightforward experiment to determine whether the gene identified by genome scanning of *in vitro*-evolved drug-resistant parasites is indeed the drug target is to directly assay the gene product for enzymatic inhibition. Proceeding to a biochemical assay is contingent on successful heterologous expression of the gene product and developing an assay to detect inhibition. Both of these aspects provide challenges.

Heterologous expression

Heterologous protein production of *Plasmodium* proteins has remained highly problematic and has slowed the progress of antimalarial drug target discovery [47]. *Escherichia coli* has remained the mainstay heterologous expression platform of *Plasmodium* proteins [48]. Optimization of *E. coli* expression has included the addition of fusion tags, such as glutathione *S*-transferase, to the target protein, overexpression at subambient temperatures and codon usage optimization, as well as expression trials in alternate systems, such as baculovirus and yeast [47]. These approaches are normally met with varied results and it is impossible to predict which approach will be superior.

Newer strategies, such as codon harmonization [49], have emerged and may help increase the successful expression of a protein target. Harmonization is an extension of the codon optimization strategies, but preserves translation pauses encoded in the native transcript.

This approach significantly improved the solubility and yield of vaccine candidates merozoite stage protein 1 [50] and liver stage antigen 1 [51]. In addition, the wheat germ cell-free system has garnered increased interest within the *Plasmodium* field. It has been used to successfully express *P. falciparum* S25 antigen, circumsporozoite protein and apical membrane antigen 1, notoriously difficult proteins to express, without requiring codon-optimized DNA template [52]. Although less established, the 'next door neighbor' approach is of considerable interest and reasonably suggests phylogenically related organisms may serve as the best heterologous expression hosts [53]. Finally, *Leishmania tarentolae*, a protozoan of the Moorish gecko, is commercially available system (LEXSY, Jena Bioscience) and may become a useful tool to express *Plasmodium* proteins.

Assay development alternatives

A bevy of colorimetric, fluorometric and chemiluminescence-based assays have been developed to assist in the detection of enzymatic activity. The nature of these assays are highly dependent on enzymatic substrates, products and cofactors, so assay design often varies greatly between enzyme classes. However, in the instance that an established biochemical assay has not been developed for a protein of interest, then alternative assay methods must be pursued. For example, surface plasmon resonance was used by Pandey *et al.* to characterize falcipain-2 affinity to a specific inhibitor [54], affinity chromatography was used by Kato *et al.* to selectivity purify CDPK1 from plasmodial lysates with a specific inhibitor immobilized to resin [22], Kamchonwongpaisan *et al.* utilized ultrafiltration followed by liquid chromatography–mass spectrometry to probe for inhibitors of DHFR [55], and Crowther *et al.* characterized the thermal melt profiling of 58 *Plasmodium* proteins that could be used to determine ligand–protein binding in solution [56]. The latter technique provides the simplest approach, but also requires milligram quantities of purified protein for screening purposes.

Bioassay in transgenic model

Yeast complementation

Yeast complementation studies represent the simplest screening approach of essential proteins that are otherwise difficult to express in heterologous systems or may present insurmountable assay development challenges. This generally entails replacing a yeast gene with the homologous *Plasmodium* gene by allelic exchange or using yeast mutants with episomal expression of a *Plasmodium* gene. *Plasmodium vivax* dihydrofolate reductase [57], *P. falciparum* hexose transporter 1 [58], *P. falciparum* RNA polymerase II subunits [59] and *P. falciparum* heat shock protein 90 (HSP90) [60] are recent examples of using the complementation approach to generate chemical screening platforms. Wider *et al.* also had the foresight to employ an additional isogenic yeast strain complemented with the human HSP90 to allow for parallel evaluation of *Plasmodium* and human HSP90 inhibitor selectivity [60]. Assuming near-equivalent HSP90 expression levels between strains, this will provide valuable information to assist future medicinal chemistry efforts. However, a practical concern of any chemical screen in yeast is the loss of assay sensitivity due to formidable efflux from endogenous drug pumps. Therefore, it is highly advisable that yeast strains used for chemical screens contain deletions of the major compound efflux pumps (PDR5 and SNQ2) to maximize screening sensitivity [61].

Orthologs in related organisms

Target validation can also be evaluated in orthologs within other apicomplexan parasites. As a member of the same phylum, *T. gondii* is an ideal candidate due to the evolutionary linkage and known cross-reactivity of antimalarials [62,63]. In addition, *T. gondii* has an extensive and superior repertoire of experimental techniques compared with *Plasmodium*, making it a good model organism for study [64]. An example is described in Bougdour *et al.* for FR235222, an antiparasitic inhibitor of histone deacetylase (HDAC) [65]. Their work identified FR235222's MOA as a potent HDAC inhibitor of Apicomplexa and establish HDAC as a central regulator of gene expression and stage conversion in Toxoplasma. A conserved protein, HDAC3, is likely to provide a similar function in *Plasmodium*.

Transgenic Plasmodium falciparum

Transgenic strains with altered dependencies on specific biochemical pathways have been demonstrated to be a powerful tool to identify inhibitors. Painter *et al.* ingeniously designed transgenic *P. falciparum* parasites expressing *Saccharomyces cerevisiae* dihydroorotate dehydrogenase, which circumvented the parasite's dependency on ubiquinone [66]. As a result, this transgenic line is resistant to inhibitors of the mitochondrial ETC, including ATQ. This transgenic strain has been an invaluable resource to

identify and validate inhibitors of *P. falciparum* dihydroorotate dehydrogenase (PfDHOD) as described by Patel *et al.* [67]. Furthermore, in studies by Dong *et al.* it was used to validate putative inhibitors of the ETC [68].

Metabolic labeling

Over the past 60 years, metabolic labeling has been a staple in *Plasmodium* research to study biological pathways. Traditionally, incorporation of radiolabeled cysteine and/or methionine has been used to monitor protein synthesis while nucleic acid synthesis can be investigated using radiolabeled hypoxanthine. Careful consideration and availability of a greater variety of radiolabeled biomolecules has allowed an expansion in assays designed to monitor specific pathways within *Plasmodium*.

Aside from the increased repertoire of labeled metabolites, advances in mass spectrometry have improved the analysis of endogenous metabolites leading to the identification of novel metabolites, original pathways and networks of regulatory interactions within the parasite [69]. Recent examples in the literature show the use of labeled folinic acid to assay the folate pathway [70], labeled geranylgeranyl diphosphate to confirm the presence of carotenoid biosynthesis in *Plasmodium* [71], a labeled palmitic acid analog to assist in the identification of more than 250 palmitoylated proteins in late blood-stage *P. falciparum* parasites [72] and radioactive sugar precursors and ethanolamine to discover *N*-glycan chain length is truncated in *P. falciparum* [73].

The continued advancement of metabolic labeling methodologies are best exemplified by studies elucidating the mechanistic details of the tricarboxylic acid (TCA) cycle in *P. falciparum*. Olszewski *et al.* traced ^{13}C-labeled compounds using mass spectrometry and elegantly demonstrated that this pathway is noncanonical and has independently evolved within the parasite to generate a dedicated pool of acetyl-coenzyme A to be used in histone acetylation [74]. Meanwhile, Bulusu *et al.* utilized the purine salvage pathway for both radiolabel uptake studies and ^{13}C-nuclear magnetic resonance spectroscopy to follow the conversion of fumarate, a product of TCA metabolism, to aspartate [75]. This work led to the identification of functional roles for fumarate hydratase, malate quinone oxidoreductase and aspartate aminotransferase in fumarate conversion. Furthermore, Bulusu *et al.* utilized ATQ as a chemical tool to demonstrate that the ETC was linked to fumarate conversion [75]. This underscores that novel, specific inhibitors of *Plasmodium* with a defined MOA can be used as powerful chemical tools to extend our knowledge of parasite biology.

Metabolic assays can be extended to comparative studies to evaluate incorporation differences between untreated and drug-treated parasites. However, it is incumbent on the investigator to distinguish whether a difference in incorporation is a reflection of altered metabolite uptake or directly attributable to inhibition of the biosynthetic pathway in question. A significant drawback in this methodology, for drug discovery purposes, is that pre-existing knowledge of the drug-targeted pathway is required to direct metabolic analyses.

Proteomics

There are only a limited number of proteomic studies dedicated to the elucidation of the MOA for antimalarials in *P. falciparum*. In general, drug-treated parasites have revealed a relatively low number of deregulated proteins. Briolant *et al.* used two complementary proteomic approaches, 2D fluorescence difference gel electrophoresis and isobaric tagging reagents for relative and absolute quantification (iTRAQ), to show doxycycline-treated parasites perturb the expression of 32 and 40 proteins, respectively [76]. While mitochondrial and apicoplast-localized proteins were identified, no clear target or pathway was revealed. In addition, a large-scale comparative study by Prieto *et al.* investigated the differential expression of proteins treated with ART or CQ using mass spectrometry and, respectively, found 41 and 38 uniquely upregulated proteins [77]. Finally, a 2D electrophoresis and tandem mass spectrometry identification study performed by Radfar *et al.* established that CQ treatment of parasites result in the oxidation of proteins involved in protein folding, proteolysis, energy metabolism, signal transduction and pathogenesis [78]. These early results suggest that specific drugs may produce a 'fingerprint' of uniquely deregulated protein expression based on the MOA. It will be interesting to see whether the continued analysis of novel antimalarials and those with a known MOA will confirm these observations and reaffirm a greater role for proteomics in MOA studies.

Conclusion

It may be presumptuous to declare the current advances in malaria drug discovery a success until one or more clinically approved antimalarials emerges from the pipeline. The earliest OZ439, currently in early Phase II trials, could qualify for drug approval is 2016 [79],

and NITD609, currently in Phase I, is farther behind. Statistically, 22% of all new molecular entities submitted for neglected diseases led to clinical approvals [80]. While this is better than the 16% clinical approval success rate for mainstream drugs, the odds are stacked against the successful approval of a drug [80]. For this reason, it is important to push as many clinical candidates into the pipeline as possible.

The path from compound hit to preclinical drug requires an intense medicinal chemistry effort with the infrastructure to rapidly and concurrently determine the antimalarial activity, cytotoxicity and pharmacokinetic properties of newly synthesized analogs. In general, academic laboratory are not equipped with the combined chemistry expertise, *in vitro* and *in vivo* screening facilities and personnel to independently drive a project from compound hit to preclinical candidate. Fortunately, MMV excels at creating collaborations to assist in advancing drug development of promising leads. In addition to identifying lead compounds, the cell active hits can be exploited as powerful chemical tools to assist in furthering our understanding of parasite biology. In turn, elucidation of *Plasmodium* biology with novel compounds will also likely identify novel targets, which will support continued drug discovery.

The challenges of discovering a compound's MOA and subsequent validation of the macromolecular target of the drug, have driven innovative approaches. This is exemplified by the application of genome-wide analysis to *in vitro* evolution of drug-resistant *P. falciparum*. This singular technique has become the gold-standard technique to establish a gene-driven hypothesis on a compound's MOA. The annotation and characteristics of the gene product verified in resistance will dictate the secondary assays used to validate the target. Target-based screens are preferable because assays utilize a purified gene product. However, due to the complications in expressing *Plasmodium* proteins, chemical screens in related organisms or model systems are required. Metabolomic and proteomic approaches are continually improving and currently their strength is providing a broader view of the affected biosynthetic pathways. Successful validation of a drug target will rely on as many of these techniques as possible to give high confidence in the identification of a novel drug target.

Future perspective

High-throughput platforms have played a significant role in the identification of small molecule inhibitors of *P. falciparum*. A similar high-throughput approach will be required to accelerate the characterization of these inhibitors. Haploinsufficieny profiling and click chemistry are newer technologies that appear primed to assist the *Plasmodium* drug discovery field.

Click chemistry has revolutionized the biologist's toolbox and provides an alternative HTS-friendly approach [81,82]. This borrows from the same basic concept from metabolic labeling that biomolecules can be rationally incorporated into proteins, sugars, DNA, RNA or lipids, except that detection is contingent on labeling after incorporation. A nonperturbing 'chemical handle' within the incorporated biomolecule allows for selective attachment of a fluorophore or affinity tag, which will allow for detection of the incorporated biomolecule. It remains to be determined whether assay sensitivity and reagent cost will be prohibitive factors.

Haploinsufficiency profiling (HIP) is a chemogenomic assay that is ideally suited to directly identify the target of a drug and has continued to be an underutilized technique in the *Plasmodium* field. HIP is founded on the principle that reducing the expression of a single gene will hypersensitize the gene product to any drug that acts upon it [83]. This is achieved by eliminating one of two copies in diploid yeast to form a heterozygote. The heterozygote strains, each possessing a unique 'genetic barcode', are pooled, grown competitively in the presence of drug and analyzed in parallel by a high-density DNA microarray to determine the relative strain abundance. Strains most sensitive to drug often carry deletions in genes that encode the drug target and provides hypotheses for the MOA of any compound of interest. As a follow-up, drug-resistant yeast can be rapidly selected and DNA sequencing techniques can readily identify acquired mutations conferring resistance.

Homozygous deletion profiling comprises a library of diploid strains in which both copies of nonessential genes have been deleted. Growth defects in these strains after drug treatment reveals genes that are related to the drug target pathway. These genes typically comprise other pathway components and/or genes involved in multidrug resistance (e.g. drug transport, detoxification and metabolism). The homozygous deletion profiling results can be used to validate the gene(s) observed in the HIP profile.

Drawbacks to this approach include a lack of cross-reactivity to *S. cerevisiae* targets due to evolutionary divergence and/or the simple absence of *Plasmodium*-specific targets. However, IC_{50}

concentrations as high as 150 μM are acceptable in HIP profiling and analysis by this approach is in the order of weeks, whereas genome scanning of drug-resistant evolved parasites takes approximately 4–5 months of dedicated culturing. Finally, the HTS format of this assay makes it ideal for screening small libraries. We see this combined approach as a fantastic platform to significantly advance MOA studies of novel antimalarials.

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Executive summary

High-throughput screening summary

- Over 10,000 antimalarials have been recently identified from cell-based high-throughput screens of intraerythrocytic stage *Plasmodium falciparum*, representing a vast library of cell-active hits with an unknown mode of action (MOA).

Target identification

- *In silico* approaches can successfully identify and cluster compounds with similar chemical structures. Clusters containing a compound with a known MOA would infer a similar MOA for the entire cluster of structurally related antimalarials.
- Genome-wide analysis of drug-resistant parasites evolved *in vitro* identifies genomic mutations that confer resistance. A gene-driven hypothesis on the MOA of the antimalarial can be established for target validation studies.

Target validation

- Biochemical screening of the target is desirable to assay for direct inhibition by the antimalarial.
- Challenges in heterologous expression of *Plasmodium* proteins can be combated by transgenic expression of the putative drug target into model systems, such as yeast and *Toxoplasma gondii*.
- Metabolomics and proteomics can reveal pathways within the parasite affected by drug treatment.

Conclusion

- The collection of cell-active hits represents a valuable library of chemical tools to elucidate *Plasmodium* biology.

Future perspective

- Metabolites utilizing 'handles' based on click chemistry and haploinsufficiency profiling in yeast provide high-throughput avenues to accelerate the assignment of MOA to novel antimalarials.

Bilbiography

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