Published in final edited form as:
J Med Chem. 2013 October 24; 56(20): . doi:10.1021/jm400325j.

Using genetic methods to define the targets of compounds with antimalarial activity

Erika L. Flannery[1], **David A. Fidock**[2,3], and **Elizabeth A. Winzeler**[1,*]

[1]Department of Pediatrics, University of California, San Diego, School of Medicine, 9500 Gilman Drive 0741, La Jolla, California 92093, USA

[2]Department of Microbiology and Immunology, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA

[3]Division of Infectious Diseases, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA

Abstract

Although phenotypic cellular screening has been used to drive antimalarial drug discovery in recent years, in some cases target-based drug discovery remains more attractive. This is especially true when appropriate high-throughput cellular assays are lacking, as is the case for drug discovery efforts that aim to provide a replacement for primaquine (4-N-(6-methoxyquinolin-8-yl)pentane-1,4-diamine), the only drug that can block *Plasmodium* transmission to *Anopheles* mosquitoes and eliminate liver-stage hypnozoites. At present, however, there are no known chemically validated parasite protein targets that are important in all *Plasmodium* parasite developmental stages and that can be used in traditional biochemical compound screens. We propose that a plethora of novel, chemically validated, cross-stage antimalarial targets still remain to be discovered from the ~5,500 proteins encoded by the *Plasmodium* genomes. Here we discuss how *in vitro* evolution of drug-resistant strains of *Plasmodium falciparum* and subsequent whole-genome analysis can be used to find the targets of some of the many compounds discovered in whole-cell phenotypic screens.

INTRODUCTION

Malaria continues to present a major health challenge in many resource-limited countries, with an estimated 219 million cases leading to estimates of between 660,000 and 1.24 million deaths in 2010.[1, 2] Most deaths result from infection with *Plasmodium falciparum*, although *P. vivax* also contributes substantially to the overall morbidity.[3–6] Infection begins when a feeding female *Anopheles* mosquito delivers *Plasmodium* sporozoites to human blood vessels in the skin, where they migrate to the liver and rapidly invade hepatocytes. After an asymptomatic phase of liver-stage replication, parasites emerge and initiate the symptomatic cycles of asexual blood-stage (ABS) infection. These ABS parasites continue to replicate in red blood cells, while a small fraction differentiate into the sexual gametocyte stage. Mature gametocytes are transmissible to *Anopheles* and are therefore responsible for sustaining the infectious cycle.[3] Clinical and modeling studies show that the most effective way to reduce the burden of malaria would be to have drugs that are both curative and prophylactic, by virtue of their action on ABS and liver-stage parasites. Ideally, these drugs would also be active against gametocytes.[7] To date, only primaquine is known to have this broad-stage activity (Table 1), however its activity *P. falciparum* ABS parasites is limited.[8]

*Corresponding author information: Elizabeth Winzeler; Phone, (858) 822-3339; ewinzeler@ucsd.edu.

Furthermore, this 8-aminoquinoline can cause dangerous levels of methemoglobinemia in patients with glucose-6-phosphate dehydrogenase deficiency (G6PDD), a common condition in malaria endemic regions.[9] Although there is much ongoing research to develop novel 8-aminoquinolines (e.g. tafenoquine (N(4)-(2,6-Dimethoxy-4-methyl-5-((3-trifluoromethyl)phenoxy)-8-quinolinyl)-1,4-pentanediamine)), which may be able to maintain or surpass the efficacy of primaquine while reducing to safe levels any G6PDD toxicity, it is not clear yet if this can be achieved. Therefore, if the malaria research and medical community is to achieve the stated goal of eliminating malaria,[10] the community needs urgently needs to identify new chemical series with broad-range activity against the distinct parasite stages developing within infected individuals. This imperative is underscored by the recent evidence that artemisinin, the core component of current first-line therapies, is starting to succumb to resistance that is emerging in Southeast Asia.[11–13]

The discovery of broad-range antimalarial chemical series will require the identification of novel antimalarial targets, as none of the current targets of available medicines exhibit activity against all developmental stages of the parasite (Table 1). For example, the 4-aminoquinolines chloroquine and amodiaquine, as well as the endoperoxide, artemisinin, interact with reactive heme-iron creating oxygen radicals that ultimately lead to cell death. Because iron is only abundant in red blood cells, these drugs more active against parasites during the intra-erythrocytic asexual replication stage. Additionally, the antifolate pyrimethamine interferes with DNA synthesis and is therefore ineffective against late-stage gametocytes which are non-replicating[14] although it does have some activity in cellular assays against other sexual forms, such as ookinetes (Table I). Recently, screens have been initiated around previously validated targets or pathways such as heme detoxification, folate metabolism or mitochondrial function (e.g.[15–17]). These efforts have successfully placed new chemical entities into late-stage development, including tafenoquine (N(4)-(2,6-Dimethoxy-4-methyl-5-((3-trifluoromethyl)phenoxy)-8-quinolinyl)-1,4-pentanediamine), OZ439 (Morpholine, 4-(2-(4-(cis-dispiro(cyclohexane-1,3′-(1,2,4)trioxolane-5′,2″-tricyclo(3.3.1.13,7)decan)-4-ylmethyl)phenoxy)ethyl)-), piperaquine (Quinoline, 4,4′-(trimethylenedi-4,1-piperazinediyl)bis(7-chloro-), pyronaridine (4-[(7-Chloro-2-methoxy-1,5-dihydrobenzo[b][1,5]naphthyridin-10-yl)imino]-2,6-bis(pyrrolidin-1-ylmethyl)cyclohexa-2,5-dien-1-one), P218 (2,4-Diamino-6-ethyl-5-(3-(2-(2-carboxyethyl)phenoxy)propoxy)pyrimidine hydrochloride) and several mitochondrial inhibitors.[18–22] Yet, because these drugs were discovered against known antimalarial targets, none of them have broad ranging activity and these compounds may succumb to already existing mechanisms of resistance. Although it may be that only a limited number of suitable antimalarial targets exist, we advocate that it is more likely that their identification has been restricted by a lack of appropriate methods, and that many new targets remain to be discovered.

A promising approach, which has recently identified several new antimalarial targets in Plasmodium, is to use chemical screening to identify novel drug scaffolds, forward genetic methods and genome sequencing to select for and discover mutations in the target and then reverse genetics to confirm the target (Figure 1A). This chemical genomics approach has been particularly successful for antimalarials that are active across multiple lifecycle stages and multiple species and that engage with parasite proteins as opposed to host factors such as hemoglobin.[23,24] The identification of these targets provides a rational basis for chemically validated target-based screens to find optimal antimalarial drugs and expands the understanding of the repertoire of viable antimalarial drug targets. In this review, we will discuss how genetic approaches can be used to discover new targets quickly and successfully.

PHENOTYPIC SCREENS TO IDENTIFY NOVEL CHEMICAL SCAFFOLDS WITH ANTIMALARIAL ACTIVITY

Recent screening campaigns have identified a wealth of compounds active against *P. falciparum* ABS parasites.[23, 25–27, 28] Additionally, screens can be conducted with the goal of identifying compounds active against *Plasmodium* liver stages. Liver-stage screens can use a similar cell-based approach as for ABS parasites, but instead of measuring drug inhibition of red blood cell infection, compounds that have known ABS activity are tested for their ability to inhibit hepatocyte infection. In the assay, freshly dissected *P. yoelii* sporozoites are added to a monolayer of hepatocytes growing in 384-well plates. High-content microscopy combined with a customized Acapella™ script allows for quantification of the number of infected hepatocytes (~1% of all cells),[27]

Similar phenotypic screens have been established to identify compounds that are also active against hepatic stages [28] or active against sexual stage parasites,[8, 29, 30] yet some of these screens are considerably lower throughput, conducted in 96-well plate format. Nevertheless, large-scale phenotypic screens are becoming accessible, and it is likely that more data will soon be available (see Avery et al. this issue).

TARGET DISCOVERY OF ACTIVE COMPOUNDS

Selection for resistant parasites *in vitro*

Once leads with antimalarial activity are identified, optimization in a medicinal chemistry campaign can begin. Although this can lead directly to a new therapy, in many cases target identification is often carried out in parallel. Development of the lead can fail for a variety of reasons and gaining insight into compound mode of action can inform medicinal chemistry efforts aimed at synthesizing modified compounds within the same class as well as lead to other pathways that may have ‘drug-able’ targets. One method used to identify the target and characterize the mechanism of action of a compound of interest essentially exploits the parasite’s natural acquisition of resistance (Figure 1). Parasites are cultured in the presence of drug until they acquire resistance and then the genomes of resistant mutants are examined to identify copy number variants (CNVs) or single nucleotide variants (SNVs) that are candidates determinants of the resistance phenotype. Acquired CNVs presumably increase the number of cellular copies of a drug’s target and resistance occurs because more compound is needed to kill the parasite. SNVs that code for residue changes in the target may likewise disrupt compound binding and lead to resistant parasites. In many cases, these alleles associated with resistance map to the actual drug target although it also possible that CNVs could encode transporters that simply work to pump the drug out, or that the SNVs could be in a drug pump or a compensating protein as well. True targets discovered in this manner, as opposed to those discovered by functional genomics or genetic methods, are predicted to be more “drug-able” because their discovery was based on actual chemical inhibition of parasite growth.

To obtain resistant parasites, an *in vitro* evolution experiment is performed that allows for selection and propagation of resistant lines. Evolution experiments typically employ recently recloned *P. falciparum* Dd2 parasites (chloroquine-resistant, from Southeast Asia) but sometimes use other strains. Selection occurs in two ways, but always begins with a cloned line (the parent) that is split into flasks for multiple replicate experiments (Figure 1b). Replicates are important because whole-genome analysis may reveal a few collateral mutations, and through independent repeats, the causative allele is usually easier to identify. In the step-wise selection approach, drug pressure is applied to 2×10^9 parasites, which are exposed to the IC_{50} of the compound for 5 days. Giemsa-stained blood films are examined

daily to determine the state of the culture and small molecule pressure is increased gradually over 10–12 weeks until the cultures sustain growth at drug concentrations 2–3x the IC_{50} of the parent line. In the one-step selection method, drug pressure is applied to the same number of parasites, yet an initial drug concentration of at least 3 to 8x the IC_{50} is added. Cultures are monitored for parasite death followed by recrudescence over 10–12 weeks. In both methods, healthy asexual-stage parasites growing in the presence of drug are evidence of resistance. In the step-wise selection method, parasite lines can be tested periodically for a change in IC_{50} to monitor resistance levels and usually a minimum of a two-fold change in the IC_{50} value is required to detect genetic changes related to resistance. Indeed, low-level changes are useful, as they tend to select for CNVs, which can be easier to identify.

Identification of genetic changes associated with resistance (genome-scanning)

Sublethal compound pressure applied to a population of sensitive parasites selects for parasites that typically have acquired random spontaneous mutations in resistance genes or the target. The genomes of the resistant and sensitive parental clones are expected to differ at a handful of genomic sites, whose number is expected to be proportional to the number of generations separating the parent and progeny clones (often 3–5 nonsynonymous allelic changes after ~3 months in culture[31]). In the absence of functional complementation methods for malaria parasites, the DNA must next be analyzed at the whole-genome level, typically using tiling microarrays or whole-genome re-sequencing. For this process to work well, DNA from a clonal population of parasites needs to be obtained. Analysis of a mixed clone population could mask relevant genetic changes related to the resistance phenotype, and ultimately lead to an unsuccessful experiment.

Microarray—Historically, genetic variation has been discovered using high-density microarrays, which until recently was the gold standard approach.[23, 24, 32–34] In this approach, genomic DNA from the resistant lines and parent is isolated, digested, fragmented, and fluorescently end-labeled for detection.[35] The DNA is then hybridized to a *P. falciparum* oligonucleotide microarray, such as the “Pftiling” high density microarray (4 million 25mer oligos at ~2 base spacing), which is comprised of 5 million 25-mer, single-stranded probes derived from the *P. falciparum* genome and spaced 2–3 bp apart.[32] Fluorescence intensities are analyzed using freely available data analysis software, and because hybridization between two 25mers can be disrupted by the presence of a SNV, the array can detect most newly emerged SNVs as well as CNVs and other changes (Figure 2A). These disruptions are detected by performing z-tests with the difference in log fluorescence intensities of the reference and resistant strain hybridizations, for a sliding window of three overlapping probes, capitalizing on the fact that a SNV or small indel will cause a loss of hybridization in the resistant strain relative to the control parent. SNVs or CNVs that are detected by this method are then confirmed by conventional sequencing and quantitative PCR. Lower density oligonucleotide arrays, comprised of 75mers, have been designed. Although useful for finding CNVs[36] they have not been used, practically, as frequently for finding SNVs. This may be because a single nucleotide change may or may not change hybridization between a longer 75mer probe and its target, although newer designs are being developed.[37, 38] Until recently, an advantage of microarrays was the price and ease of use. However, the chief disadvantage of microarrays is that they may miss some proportion of newly emerged variation in coding regions, including much of the variation in multigene families. In addition these tools may have difficulty revealing more complicated genomic changes such as translocations with no accompanying copy number increase or decrease.

Whole-genome sequencing—Over the past year the cost associated with whole-genome sequencing has dropped dramatically, allowing researchers to compare the genomes of multiple resistant parasites and a parent quickly and efficiently in a laboratory. From

approximately 1 μg of DNA (for Illumina sequencing), more than enough information can be obtained from one sequencing run to determine candidate alleles of interest. A typical lane on an Illumina HiSeq yields about 500 million paired-end reads of ~100 bases, equivalent to 100× coverage for 25 *P. falciparum* or *P. vivax* genomes. There are a variety of other sequencing platforms, such as Roche454, that can be used as effectively.[39] Data analysis can be performed with freely available software that can be used on any genome[40–42] or a suite of tools (PLaTypUS) that has been adapted to the *Plasmodium* genome.[31] This software aligns the sequence reads to the reference genome and implements a series of quality control metrics that increase statistical confidence in each variant call, eliminating thousands of potential false SNVs that would be identified between the resistant lines and the reference *P. falciparum* 3D7 genome. Unlike the microarray, whole-genome sequencing returns the exact identities of the allelic differences distinguishing the parent and resistant lines; given that the genome coverage is typically very high, it is likely that all SNVs are detected (Figure 2b). Additionally, CNVs can also be detected by analyzing the depth of read coverage (Figure 2b) and recombination events that distinguish closely related parasites can also be discovered.[31] These events often physically accompany duplicated or deleted genomic regions whose effect on gene expression could modulate levels of drug susceptibility.

CONFIRMATION OF ASSOCIATIONS BETWEEN GENOTYPE AND PHENOTYPE USING REVERSE GENETICS

Although it is relatively easy to find genomic changes in resistant clones, there can often be some ambiguity about which are causative. It is typical to observe several coding changes in each evolved strain. Although the best candidate target gene is usually the one that appears repeatedly in independent selections, there are always questions as to whether the other "background" mutations contribute to the phenotype in some ways. Therefore, ideally one would like to move the putative causative allele into a clean genetic background to demonstrate that the phenotype tracks with the allele. There are several approaches that can be used to demonstrate this, depending on the degree of altered drug susceptibility and the type of genetic change and candidate gene.

One preferred method is to introduce a second copy of the locus harboring the allele found in the resistant mutant. The allele of interest can be PCR amplified from one drug-resistant line and cloned into an expression vector that contains the serine integrase from the Bxb1 mycobacteriophage.[43] This expression plasmid can then be introduced into an engineered parasite line that contains an *attB* site-specific recombination site (Figure 3A). Recombination between the integrated *attB* site and a homologous *attP* site located on the episomally-replicating transfection plasmid is mediated by the integrase.[43] Integration results in a mutant allele co-expressed with the wild-type allele, which can be used to assess genes harboring SNPs that are implicated in reduced susceptibility. This approach can also be used to test compact genes (< 4 kb) from a chromosomal segment harboring a CNV associated with resistance, by increasing the copy number through transgene expression. Several *P. falciparum* strains are available that harbor the *attB* recombination site including Dd2, NF54 and 3D7.[8, 43, 44]

A new powerful method uses customized zinc-finger nucleases that allow highly specific and fast gene editing of the potential gene target (Figure 3B). Pairs of zinc finger proteins are designed that bind the target sequence in close proximity to the mutation of interest on separate strands of the DNA double helix. These sequences are each fused to a split FokI endonuclease that can only function as an obligate heterodimer. The endonuclease can thus cause a double-stranded break only between the two bound zinc-finger nucleases.[45] Double-stranded break repair enzymes encoded by the parasite genome will then use a mutated

donor template, provided on a second plasmid, to repair the genetic lesion and introduce the mutations of interest into the genome. This produces a transgenic parasite with specific modifications made to the gene of interest. This approach also works efficiently with single plasmid approaches that express the zinc finger proteins, coupled using a 2A 'ribosome skip' peptide that allows coordinated expression of two separate polypeptides from a single promoter, a selectable marker (such as human *dhfr*) and the donor template. Silent mutations are introduced into the donor template to prevent nuclease-mediated cleavage of the donor plasmid, resulting in the introduction of double strand breaks solely into the target genomic site that uses the donor for homology-directed repair.[46]

Finally, there are less efficient but more classical methods of allelic exchange via single-site crossover (Figure 3E), which have been successfully used to introduce SNVs from the mutant candidate allele into the parental line (e.g.[47–50]). Engineered lines that harbor the SNV observed in resistant lines would be expected to have increased levels of resistance to the compound of interest. Allelic exchange can also be employed to test the role of CNVs in resistance. For this, a single or double crossover technique is used to reduce the copy number of the amplified gene, as performed previously with *pfmdr1* where the CNV was reduced from 2 to 1 and the "knockdown" parasites acquired sensitivity to mefloquine, lumefantrine and artemisinin.[51]

CASE STUDIES

Identification of novel antimalarial targets

There are several examples of where this systematic strategy has yielded the discovery of novel antimalarial targets (Table 2). The spiroindolone, NITD609((3R,3′S)-5,7′-dichloro-6′-fluoro-3′-methylspiro[1H-indole-3,1′-2,3,4, 9-tetrahydropyrido[3,4-b]indole]-2-one), is a derivative of a natural product-like molecule, discovered in a cell-based screen of ABS parasites.[23] After 4 months of *in vitro* selection, the IC_{50} values of NITD609 increased ~10X. Genomic analysis with the Pftiling array identified several hybridation differences, most of which were found in a single gene, *pfatp4* (also known as *PFL0590c*), and the rest being mostly in randomly assorted subtelomeric or intergenic regions. Further inspection of the hybridization patterns also showed that one strain carried a CNV that surrounded *PFL0590c* – a gene encoding a cation-transporting P-type ATPase (PfATP4)[52–54] (Figure 2b). Transgenic parasites were created that showed that only mutations in *pfatp4* conferred resistance.[23] Recent functional data also indicates that PfATP4 is a sodium pump and is the likely target of the spiroindolones.[55]

Microarrays were also used to identify the likely target of cladosporin(3,4-Dihydro-6,8-dihydroxy-3-(tetrahydro-6-methyl-2H-pyran-2-yl)methylisocoumarin), a natural product that was subsequently found to inhibit lysyl-tRNA synthetase.[56] In the case of cladosporin, all three resistant lines harbored CNVs surrounding the *P. falciparum lysyl-tRNA synthetase* gene. The interaction between lysyl-tRNA synthetase and cladosporin was also evaluated in a model organism, the yeast *Saccharomyces cerevisiae*. Here, precise removal of one copy of the yeast *lysyl-tRNA synthetase* in a diploid strain resulted in an increase in sensitivity to the compound.

Finally, whole-genome sequencing of resistant clones was recently used to show that a novel class of compounds, the tetracyclic benzothiazepines (e.g. compound 1: 11-(thiophen-3-yl)-11H-benzo[b]indeno[1,2-e][1,4]thiazepin-12-ol), likely target cytochrome bc1, a well-validated target in *P. falciparum*.[57] This finding was unexpected because the tetracyclic benzothiazepines share few of the scaffold features of other cytochrome bc1 inhibitors.

Although the mechanism of action of these compounds discovered in unbiased cellular screens were almost completely unknown, in some cases the target was suspected and the technique was used primarily to ensure that the compound did not have off-target activity (e.g. causing parasite death by interacting with a cryptic target). For example, a screen for inhibitors of the endoplasmic reticulum-associated degradation pathway yielded the likely protease inhibitor, NITD731 (tert-butyl ((2S, 3R, 5R)-5-benzyl-3-hydroxy-6-(((S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)amino)-1-oxopropan-2-yl)amino)-6-oxo-1-phenylhexan-2-yl)carbamate). A resistant clone was created by growing parasites in the presence of NITD731. This line showed a 5-fold increase in IC_{50} value compared to the parent. Whole-genome sequences showed the resistant line had acquired a single nonsynonomous SNV in *pfspp*.[58] The protein encoded by this gene is a predicted signal peptide peptidase, and likely plays an important role in the degradation of unstable membrane proteins. Likewise Istvan *et al.* raised parasites resistant to suspected tRNA synthetase inhibitors and showed that one of the inhibitors specifically was directed against the cytoplasmic isoleucyl-tRNA synthetase[34] and the other against the apicoplast isoleucyl-tRNA synthetase. Genome scanning and microarray analysis confirmed that decoquinate, likely inhibits cytochrome bc1.[24] Sequencing was also used to exhaustively explore the variety of different ways that parasites become resistant to atovaquone and to confirm the sole involvement of cytochrome bc1.[31]

TARGETS OR RESISTANCE GENES?

One may ask whether these experiments just reveal resistance mechanisms, because proteins involved in resistance may bypass the direct pathway on which the drug is acting. In most of the cases discussed above this approach has resulted in the identification of a likely target, although determining whether a gene identified by resistance selection is truly the direct target can be challenging. Convincingly, for cladosporin, a lysyl tRNA synthetase protein was expressed recombinantly and direct inhibition was demonstrated.[21] The half inhibitory concentration activity against the target was similar to that observed against whole-cell parasites. In other cases, the situation remains ambiguous. Resistance to the imidazolopiperazine, GNF179 (-1-(3-(4-chlorophenylamino)-2-(4-fluorophenyl)-8,8-dimethyl-5,6-dihydroimidazo[1,2-a]pyrazin-7(8H)-yl)ethanone), which is closely related to the clinical candidate, GNF156 (also known as KAF156; 2-amino-1-[2-(4-fluorophenyl)-3-[(4-fluorophenyl)amino]-5,6-dihydro-5,5-dimethylimidazo[1,2-a]pyrazin-7(8H)-yl]-ethanone)[59–61] is conferred by mutations in the *P. falciparum* cycloamine resistance locus (*pfcarl*) and studies are ongoing to assess whether the *pfcarl* product is also the drug target or merely a drug detoxification protein. In support of the former, the *pfcarl* alleles provide no cross-resistance with other compounds and are probably not acting analogously to mutations or copy number changes in the drug pumps encoded by *pfcrt* or *pfmdr1*.[62] Finally, in a few cases, it seems likely that the genomic changes confer resistance only. When parasites were subjected to piperaquine pressure (an antimalarial currently used for first-line therapies in combination with artemisinin) they acquired a CNV.[63] This 63kb amplification was found in two clones and was located upstream of the multidrug resistance gene *pfmdr1*. Deamplifications in *pfmdr1* and a novel mutation in *Pfcrt*, C101F, also arose during the selection pressure and these may have contributed to a multifactorial trait.

Alternative approaches for target identification

Because there are often concerns that genetic approaches will yield only common resistance genes, biochemical approaches such as affinity chromatography are often suggested. Here, an affinity probe is created that contains active and inactive scaffolds. The compounds with drug-like activity are modified so that they can be atttached to a column and then lysates from the organims of interest are passed over the column. Different fractions are collected

and then analyzed by mass spectrometry. This approach has yielded some successes in identifying protein kinase as the targets of small molecules in *Plasmodium*[64, 65] and other Apicomplexan parasites.[66] The problem with this approach is for *Plasmodium* parasites, large quantitites of lysate may be required and these can be heavily contaminated with red blood cell proteins—the situation is likely to be even more challenging for compounds active against transmission and liver stages. The second problem is that the deeper one looks with mass spectrometry, the more candidate proteins are identified. Sticky, abundant proteins, such as Merozoite Surface Protein 1 (MSP1) will inevitably show up at the top of the list and even in the case where one knows one is looking for protein kinases, there may be three or four credible candidates in the list that are detected. Given that it can take years to genetically validate a target through either overexpressing the protein or geneticly engineering the parasite, further proof of a gene's importance may be difficult to obtain. In addition, just because a small molecule binds a target, this does not mean that disruption of the protein's function leads to parasite killing. The approach works poorly with membrane proteins, and for proteins that may be difficult to detect by mass spectrometry. Finally, some knowledge of the likely protein-binding regions of the antimalarial is needed before a linker can be attached. It may also be more suitable for bacteria that can be grown without the help of host cells. It nevertheless, remains a valid, if high-risk approach for *Plasmodium*.

Target ID for compounds active against other stages

Discovering the target of a compound that is either not active in blood stages, or which has a different target in different parasite lifecycle stages would be expected to be difficult. It would be challenging to implement biochemical approaches given that only a small fraction of hepatocytes are infected with malaria parasites and evolution studies would also be difficult or impossible, given that there are genetic bottlenecks at the early liver schizont and sexual stages. However, it is somewhat unlikely that compounds with no blood stage activity would be considered for development, given the challenges associated with designing clinical trials for a compound with no therapeutic activity. It seems likely that in most cases the target would be shared between blood, transmission and hepatic stages, but of course, there could be exceptions. Thus, once a candidate target gene has been identified in blood stages it should be possible to evaluate its importance in other lifecycle stages. For example, parasite strains that overexpress the target could be engineered and then researchers could evaluate if the hepatic stages of the recombineered lines would be more resistant to the compound. In addition, mutations in the active site might be introduced.

CONCLUSIONS

Recent advances in malaria control have helped substantially reduce the burden of malaria in many endemic settings, and malaria has once again captured the interest of the pharmaceutical industry and the broader public health community. These fragile gains could rapidly be reversed if high-level artemisinin resistance emerges and spreads. The time is ripe to take advantage of recent high-throughput screens that have identified compounds with asexual blood- and liver-stage activity, and to use genetic methods to translate these gains into a deeper understanding of novel targets that can be leveraged to develop the future generation of drugs with a wide spectrum of activity.

Acknowledgments

EAW, DAF and ELF are partially supported by the NIH (R01 AI090141, R01 AI50234 and F32 AI102567 respectively). EAW and DAF also gratefully acknowledge support from the Bill and Melinda Gates Foundation (OPP1054480 and OPP1040399 respectively) and the Medicines for Malaria Venture.

Abbreviations

ABS	Asexual Blood Stage
Pfcrt	Plasmodium falciparum chloroquine resistance transporter
SNV	Single Nucleotide Variant
CNV	Copy Number Variant
CDUP	*Saccharomyces cerevisiae cytosine deaminase/uracil phosphoribosyl transferase*
WGS	Whole genome sequencing
dhfr	*dihydrofolate reductase*
G6PDD	Glucose 6-phosphate dehydrogenase deficiency
PCR	Polymerase Chain Reaction

References

1. Organization, W. H. World Malaria Report 2012. World Health Organization; Geneva: 2012. World malaria report 2012; p. 1-276.
2. Murray CJ, Rosenfeld LC, Lim SS, Andrews KG, Foreman KJ, Haring D, Fullman N, Naghavi M, Lozano R, Lopez AD. Global malaria mortality between 1980 and 2010: A systematic analysis. Lancet. 2012; 379:413–431. [PubMed: 22305225]
3. Greenwood BM, Fidock DA, Kyle DE, Kappe SH, Alonso PL, Collins FH, Duffy PE. Malaria: Progress, perils, and prospects for eradication. J Clin Invest. 2008; 118:1266–1276. [PubMed: 18382739]
4. Baird JK. Real-world therapies and the problem of vivax malaria. N Engl J Med. 2008; 359:2601–2603. [PubMed: 19064622]
5. Gething PW, Elyazar IR, Moyes CL, Smith DL, Battle KE, Guerra CA, Patil AP, Tatem AJ, Howes RE, Myers MF, George DB, Horby P, Wertheim HF, Price RN, Mueller I, Baird JK, Hay SI. A long neglected world malaria map: *Plasmodium vivax* endemicity in 2010. PLoS Negl Trop Dis. 2012; 6:e1814. [PubMed: 22970336]
6. Price RN, Douglas NM, Anstey NM. New developments in plasmodium vivax malaria: Severe disease and the rise of chloroquine resistance. Curr Opin Infect Dis. 2009; 22:430–435. [PubMed: 19571748]
7. malERA; Consultative Group on Drugs. A research agenda for malaria eradication: Drugs. PLoS Med. 2011; 8:e1000402. [PubMed: 21311580]
8. Adjalley SH, Johnston GL, Li T, Eastman RT, Ekland EH, Eappen AG, Richman A, Sim BK, Lee MC, Hoffman SL, Fidock DA. Quantitative assessment of plasmodium falciparum sexual development reveals potent transmission-blocking activity by methylene blue. Proc Natl Acad Sci U S A. 2011; 108:E1214–1223. [PubMed: 22042867]
9. Vale N, Moreira R, Gomes P. Primaquine revisited six decades after its discovery. Eur J Med Chem. 2009; 44:937–953. [PubMed: 18930565]
10. Feachem R, Sabot O. A new global malaria eradication strategy. Lancet. 2008; 371:1633–1635. [PubMed: 18374409]
11. Dondorp AM, Fairhurst RM, Slutsker L, Macarthur JR, Breman JG, Guerin PJ, Wellems TE, Ringwald P, Newman RD, Plowe CV. The threat of artemisinin-resistant malaria. N Engl J Med. 2011; 365:1073–1075. [PubMed: 21992120]
12. Phyo AP, Nkhoma S, Stepniewska K, Ashley EA, Nair S, McGready R, Ler Moo C, Al-Saai S, Dondorp AM, Lwin KM, Singhasivanon P, Day NP, White NJ, Anderson TJ, Nosten F. Emergence of artemisinin-resistant malaria on the western border of thailand: A longitudinal study. Lancet. 2012; 379:1960–1966. [PubMed: 22484134]

13. Uhlemann AC, Fidock DA. Loss of malarial susceptibility to artemisinin in thailand. Lancet. 2012; 379:1928–1930. [PubMed: 22484133]

14. Vinetz, JM.; Clain, J.; Bounkeua, V.; Eastman, RT.; Fidock, DA. Chemotherapy of malaria. In: Brunton, L.; Chabner, B.; Knollman, B., editors. Goodman & gilman's the pharmacological basis of therapeutics. 12. McGraw Hill Medical; New York: 2011. p. 1383-1418.

15. Yuthavong Y, Kamchonwongpaisan S, Leartsakulpanich U, Chitnumsub P. Folate metabolism as a source of molecular targets for antimalarials. Future Microbiol. 2006; 1:113–125. [PubMed: 17661690]

16. Kelly JX, Smilkstein MJ, Brun R, Wittlin S, Cooper RA, Lane KD, Janowsky A, Johnson RA, Dodean RA, Winter R, Hinrichs DJ, Riscoe MK. Discovery of dual function acridones as a new antimalarial chemotype. Nature. 2009; 459:270–273. [PubMed: 19357645]

17. Phillips MA, Rathod PK. *Plasmodium* dihydroorotate dehydrogenase: A promising target for novel anti-malarial chemotherapy. Infect Disord Drug Targets. 2010; 10:226–239. [PubMed: 20334617]

18. Crockett M, Kain KC. Tafenoquine: A promising new antimalarial agent. Expert Opin Investig Drugs. 2007; 16:705–715.

19. Smithuis F, Kyaw MK, Phe O, Win T, Aung PP, Oo AP, Naing AL, Nyo MY, Myint NZ, Imwong M, Ashley E, Lee SJ, White NJ. Effectiveness of five artemisinin combination regimens with or without primaquine in uncomplicated falciparum malaria: An open-label randomised trial. Lancet Infect Dis. 2010; 10:673–681. [PubMed: 20832366]

20. Tshefu AK, Gaye O, Kayentao K, Thompson R, Bhatt KM, Sesay SS, Bustos DG, Tjitra E, Bedu-Addo G, Borghini-Fuhrer I, Duparc S, Shin CS, Fleckenstein L. Efficacy and safety of a fixed-dose oral combination of pyronaridine-artesunate compared with artemether-lumefantrine in children and adults with uncomplicated *plasmodium falciparum* malaria: A randomised non-inferiority trial. Lancet. 2010; 375:1457–1467. [PubMed: 20417857]

21. Charman SA, Arbe-Barnes S, Bathurst IC, Brun R, Campbell M, Charman WN, Chiu FC, Chollet J, Craft JC, Creek DJ, Dong Y, Matile H, Maurer M, Morizzi J, Nguyen T, Papastogiannidis P, Scheurer C, Shackleford DM, Sriraghavan K, Stingelin L, Tang Y, Urwyler H, Wang X, White KL, Wittlin S, Zhou L, Vennerstrom JL. Synthetic ozonide drug candidate oz439 offers new hope for a single-dose cure of uncomplicated malaria. Proc Natl Acad Sci USA. 2011; 108:4400–4405. [PubMed: 21300861]

22. Yuthavong Y, Tarnchompoo B, Vilaivan T, Chitnumsub P, Kamchonwongpaisan S, Charman SA, McLennan DN, White KL, Vivas L, Bongard E, Thongphanchang C, Taweechai S, Vanichtanankul J, Rattanajak R, Arwon U, Fantauzzi P, Yuvaniyama J, Charman WN, Matthews D. Malarial dihydrofolate reductase as a paradigm for drug development against a resistance-compromised target. Proc Natl Acad Sci USA. 2012; 109:16823–16828. [PubMed: 23035243]

23. Rottmann M, McNamara C, Yeung BK, Lee MC, Zou B, Russell B, Seitz P, Plouffe DM, Dharia NV, Tan J, Cohen SB, Spencer KR, Gonzalez-Paez GE, Lakshminarayana SB, Goh A, Suwanarusk R, Jegla T, Schmitt EK, Beck HP, Brun R, Nosten F, Renia L, Dartois V, Keller TH, Fidock DA, Winzeler EA, Diagana TT. Spiroindolones, a potent compound class for the treatment of malaria. Science. 2010; 329:1175–1180. [PubMed: 20813948]

24. Nam TG, McNamara CW, Bopp S, Dharia NV, Meister S, Bonamy GM, Plouffe DM, Kato N, McCormack S, Bursulaya B, Ke H, Vaidya AB, Schultz PG, Winzeler EA. A chemical genomic analysis of decoquinate, a plasmodium falciparum cytochrome b inhibitor. ACS Chem Biol. 2011; 6:1214–1222. [PubMed: 21866942]

25. Gamo FJ, Sanz LM, Vidal J, de Cozar C, Alvarez E, Lavandera JL, Vanderwall DE, Green DV, Kumar V, Hasan S, Brown JR, Peishoff CE, Cardon LR, Garcia-Bustos JF. Thousands of chemical starting points for antimalarial lead identification. Nature. 2010; 465:305–310. [PubMed: 20485427]

26. Guiguemde WA, Shelat AA, Bouck D, Duffy S, Crowther GJ, Davis PH, Smithson DC, Connelly M, Clark J, Zhu F, Jimenez-Diaz MB, Martinez MS, Wilson EB, Tripathi AK, Gut J, Sharlow ER, Bathurst I, El Mazouni F, Fowble JW, Forquer I, McGinley PL, Castro S, Angulo-Barturen I, Ferrer S, Rosenthal PJ, Derisi JL, Sullivan DJ, Lazo JS, Roos DS, Riscoe MK, Phillips MA, Rathod PK, Van Voorhis WC, Avery VM, Guy RK. Chemical genetics of plasmodium falciparum. Nature. 2010; 465:311–315. [PubMed: 20485428]

27. Plouffe D, Brinker A, McNamara C, Henson K, Kato N, Kuhen K, Nagle A, Adrian F, Matzen JT, Anderson P, Nam TG, Gray NS, Chatterjee A, Janes J, Yan SF, Trager R, Caldwell JS, Schultz PG, Zhou Y, Winzeler EA. In silico activity profiling reveals the mechanism of action of antimalarials discovered in a high-throughput screen. Proc Natl Acad Sci U S A. 2008; 105:9059–9064. [PubMed: 18579783]
28. Derbyshire ER, Prudencio M, Mota MM, Clardy J. Liver-stage malaria parasites vulnerable to diverse chemical scaffolds. Proc Natl Acad Sci U S A. 2012; 109:8511–8516. [PubMed: 22586124]
29. Buchholz K, Burke TA, Williamson KC, Wiegand RC, Wirth DF, Marti M. A high-throughput screen targeting malaria transmission stages opens new avenues for drug development. J Infect Dis. 2011; 203:1445–1453. [PubMed: 21502082]
30. Delves M, Plouffe D, Scheurer C, Meister S, Wittlin S, Winzeler EA, Sinden RE, Leroy D. The activities of current antimalarial drugs on the life cycle stages of plasmodium: A comparative study with human and rodent parasites. PLoS medicine. 2012; 9:e1001169. [PubMed: 22363211]
31. Bopp SE, Manary MJ, Bright AT, Johnston GL, Dharia NV, Luna FL, McCormack S, Plouffe D, McNamara CW, Walker JR, Fidock DA, Denchi EL, Winzeler EA. Mitotic evolution of plasmodium falciparum shows a stable core genome but recombination in antigen families. PLoS Genet. 2013; 9:e1003293. [PubMed: 23408914]
32. Dharia NV, Plouffe D, Bopp SE, Gonzalez-Paez GE, Lucas C, Salas C, Soberon V, Bursulaya B, Kochel TJ, Bacon DJ, Winzeler EA. Genome-scanning of amazonian plasmodium falciparum shows subtelomeric instability and clindamycin-resistant parasites. Genome Res. 2010; 20:1534–1544. [PubMed: 20829224]
33. Dharia NV, Sidhu AB, Cassera MB, Westenberger SJ, Bopp SE, Eastman RT, Plouffe D, Batalov S, Park DJ, Volkman SK, Wirth DF, Zhou Y, Fidock DA, Winzeler EA. Use of high-density tiling microarrays to identify mutations globally and elucidate mechanisms of drug resistance in plasmodium falciparum. Genome Biol. 2009; 10:R21. [PubMed: 19216790]
34. Istvan ES, Dharia NV, Bopp SE, Gluzman I, Winzeler EA, Goldberg DE. Validation of isoleucine utilization targets in plasmodium falciparum. Proc Natl Acad Sci U S A. 2011; 108:1627–1632. [PubMed: 21205898]
35. Kidgell C, Volkman SK, Daily J, Borevitz JO, Plouffe D, Zhou Y, Johnson JR, Le Roch K, Sarr O, Ndir O, Mboup S, Batalov S, Wirth DF, Winzeler EA. A systematic map of genetic variation in plasmodium falciparum. PLoS Pathog. 2006; 2:e57. [PubMed: 16789840]
36. Mackinnon MJ, Li J, Mok S, Kortok MM, Marsh K, Preiser PR, Bozdech Z. Comparative transcriptional and genomic analysis of plasmodium falciparum field isolates. PLoS pathogens. 2009; 5:e1000644. [PubMed: 19898609]
37. Tan JC, Miller BA, Tan A, Patel JJ, Cheeseman IH, Anderson TJ, Manske M, Maslen G, Kwiatkowski DP, Ferdig MT. An optimized microarray platform for assaying genomic variation in plasmodium falciparum field populations. Genome Biol. 2011; 12:R35. [PubMed: 21477297]
38. Tan JC, Patel JJ, Tan A, Blain JC, Albert TJ, Lobo NF, Ferdig MT. Optimizing comparative genomic hybridization probes for genotyping and snp detection in< i> plasmodium falciparum</ i>. Genomics. 2009; 93:543–550. [PubMed: 19285129]
39. Samarakoon U, Regier A, Tan A, Desany BA, Collins B, Tan JC, Emrich SJ, Ferdig MT. High-throughput 454 resequencing for allele discovery and recombination mapping in plasmodium falciparum. BMC Genomics. 2011; 12:116. [PubMed: 21324207]
40. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Subgroup GPDP. The sequence alignment/map format and samtools. Bioinformatics. 2009; 25:2078–2079. [PubMed: 19505943]
41. Li H, Ruan J, Durbin R. Mapping short DNA sequencing reads and calling variants using mapping quality scores. Genome Res. 2008; 18:1851–1858. [PubMed: 18714091]
42. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009; 10:R25. [PubMed: 19261174]
43. Nkrumah LJ, Muhle RA, Moura PA, Ghosh P, Hatfull GF, Jacobs WR Jr, Fidock DA. Efficient site-specific integration in plasmodium falciparum chromosomes mediated by mycobacteriophage bxb1 integrase. Nat Methods. 2006; 3:615–621. [PubMed: 16862136]

44. Yu M, Kumar TR, Nkrumah LJ, Coppi A, Retzlaff S, Li CD, Kelly BJ, Moura PA, Lakshmanan V, Freundlich JS, Valderramos JC, Vilcheze C, Siedner M, Tsai JH, Falkard B, Sidhu AB, Purcell LA, Gratraud P, Kremer L, Waters AP, Schiehser G, Jacobus DP, Janse CJ, Ager A, Jacobs WR Jr, Sacchettini JC, Heussler V, Sinnis P, Fidock DA. The fatty acid biosynthesis enzyme fabi plays a key role in the development of liver-stage malarial parasites. Cell Host Microbe. 2008; 4:567–578. [PubMed: 19064257]
45. Doyon Y, Vo TD, Mendel MC, Greenberg SG, Wang J, Xia DF, Miller JC, Urnov FD, Gregory PD, Holmes MC. Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. Nature Methods. 2011; 8:74–79. [PubMed: 21131970]
46. Straimer J, Lee MC, Lee AH, Zeitler B, Williams AE, Pearl JR, Zhang L, Rebar EJ, Gregory PD, Llinas M, Urnov FD, Fidock DA. Site-specific genome editing in *plasmodium falciparum* using engineered zinc-finger nucleases. Nature Methods. 2012; 9:993–998. [PubMed: 22922501]
47. Sidhu AB, Verdier-Pinard D, Fidock DA. Chloroquine resistance in plasmodium falciparum malaria parasites conferred by pfcrt mutations. Science. 2002; 298:210–213. [PubMed: 12364805]
48. Sidhu ABS, Valderramos SG, Fidock DA. *Pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *plasmodium falciparum*. Mol Microbiol. 2005; 57:913–926. [PubMed: 16091034]
49. Lakshmanan V, Bray PG, Verdier-Pinard D, Johnson DJ, Horrocks P, Muhle RA, Alakpa GE, Hughes RH, Ward SA, Krogstad DJ, Sidhu ABS, Fidock DA. A critical role for pfcrt k76t in *plasmodium falciparum* verapamil-reversible chloroquine resistance. EMBO J. 2005; 24:2294–2305. [PubMed: 15944738]
50. Valderramos SG, Valderramos JC, Musset L, Purcell LA, Mercereau-Puijalon O, Legrand E, Fidock DA. Identification of a mutant pfcrt-mediated chloroquine tolerance phenotype in *plasmodium falciparum*. PLoS Pathog. 2010; 6:e1000887. [PubMed: 20485514]
51. Sidhu AB, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA. Decreasing pfmdr1 copy number in plasmodium falciparum malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J Infect Dis. 2006; 194:528–535. [PubMed: 16845638]
52. Trottein F, Thompson J, Cowman AF. Cloning of a new cation atpase from plasmodium falciparum: Conservation of critical amino acids involved in calcium binding in mammalian organellar ca(2+)-atpases. Gene. 1995; 158:133–137. [PubMed: 7789797]
53. Dyer M, Jackson M, McWhinney C, Zhao G, Mikkelsen R. Analysis of a cation-transporting atpase of plasmodium falciparum. Mol Biochem Parasitol. 1996; 78:1–12. [PubMed: 8813672]
54. Krishna S, Woodrow C, Webb R, Penny J, Takeyasu K, Kimura M, East JM. Expression and functional characterization of a plasmodium falciparum ca2+-atpase (pfatp4) belonging to a subclass unique to apicomplexan organisms. J Biol Chem. 2001; 276:10782–10787. [PubMed: 11145964]
55. Spillman NJ, Allen RJ, McNamara CW, Yeung BK, Winzeler EA, Diagana TT, Kirk K. Na(+) regulation in the malaria parasite plasmodiumfalciparum involves the cation atpase pfatp4 and is a target of the spiroindolone antimalarials. Cell Host Microbe. 2013; 13:227–237. [PubMed: 23414762]
56. Hoepfner D, McNamara CW, Lim CS, Studer C, Riedl R, Aust T, McCormack SL, Plouffe DM, Meister S, Schuierer S, Plikat U, Hartmann N, Staedtler F, Cotesta S, Schmitt EK, Petersen F, Supek F, Glynne RJ, Tallarico JA, Porter JA, Fishman MC, Bodenreider C, Diagana TT, Movva NR, Winzeler EA. Selective and specific inhibition of the *plasmodium falciparum* lysyl-trna synthetase by the fungal secondary metabolite cladosporin. Cell Host Microbe. 2012; 11:654–663. [PubMed: 22704625]
57. Dong CK, Urgaonkar S, Cortese JF, Gamo FJ, Garcia-Bustos JF, Lafuente MJ, Patel V, Ross L, Coleman BI, Derbyshire ER, Clish CB, Serrano AE, Cromwell M, Barker RH Jr, Dvorin JD, Duraisingh MT, Wirth DF, Clardy J, Mazitschek R. Identification validation of tetracyclic benzothiazepines as plasmodium falciparum cytochrome bc1 inhibitors. Chem Biol. 2011; 18:1602–1610. [PubMed: 22195562]
58. Harbut MB, Patel BA, Yeung BK, McNamara CW, Bright AT, Ballard J, Supek F, Golde TE, Winzeler EA, Diagana TT. Targeting the erad pathway via inhibition of signal peptide peptidase

for antiparasitic therapeutic design. Proceedings of the National Academy of Sciences. 2012; 109:21486–21491.

59. Meister S, Plouffe DM, Kuhen KL, Bonamy GM, Wu T, Barnes SW, Bopp SE, Borboa R, Bright AT, Che J, Cohen S, Dharia NV, Gagaring K, Gettayacamin M, Gordon P, Groessl T, Kato N, Lee MC, McNamara CW, Fidock DA, Nagle A, Nam TG, Richmond W, Roland J, Rottmann M, Zhou B, Froissard P, Glynne RJ, Mazier D, Sattabongkot J, Schultz PG, Tuntland T, Walker JR, Zhou Y, Chatterjee A, Diagana TT, Winzeler EA. Imaging of plasmodium liver stages to drive next-generation antimalarial drug discovery. Science. 2011; 334:1372–1377. [PubMed: 22096101]
60. Nagle A, Wu T, Kuhen K, Gagaring K, Borboa R, Francek C, Chen Z, Plouffe D, Lin X, Caldwell C, Ek J, Skolnik S, Liu F, Wang J, Chang J, Li C, Liu B, Hollenbeck T, Tuntland T, Isbell J, Chuan T, Alper PB, Fischli C, Brun R, Lakshminarayana SB, Rottmann M, Diagana TT, Winzeler EA, Glynne R, Tully DC, Chatterjee AK. Imidazolopiperazines: Lead optimization of the second-generation antimalarial agents. J Med Chem. 2012; 55:4244–4273. [PubMed: 22524250]
61. Wu T, Nagle A, Kuhen K, Gagaring K, Borboa R, Francek C, Chen Z, Plouffe D, Goh A, Lakshminarayana SB, Wu J, Ang HQ, Zeng P, Kang ML, Tan W, Tan M, Ye N, Lin X, Caldwell C, Ek J, Skolnik S, Liu F, Wang J, Chang J, Li C, Hollenbeck T, Tuntland T, Isbell J, Fischli C, Brun R, Rottmann M, Dartois V, Keller T, Diagana T, Winzeler E, Glynne R, Tully DC, Chatterjee AK. Imidazolopiperazines: Hit to lead optimization of new antimalarial agents. J Med Chem. 2011; 54:5116–5130. [PubMed: 21644570]
62. Ding XC, Ubben D, Wells TN. A framework for assessing the risk of resistance for anti-malarials in development. Malar J. 2012; 11:292. [PubMed: 22913649]
63. Eastman RT, Dharia NV, Winzeler EA, Fidock DA. Piperaquine resistance is associated with a copy number variation on chromosome 5 in drug-pressured plasmodium falciparum parasites. Antimicrob Agents Chemother. 2011; 55:3908–3916. [PubMed: 21576453]
64. Kato N, Sakata T, Breton G, Le Roch KG, Nagle A, Andersen C, Bursulaya B, Henson K, Johnson J, Kumar KA, Marr F, Mason D, McNamara C, Plouffe D, Ramachandran V, Spooner M, Tuntland T, Zhou Y, Peters EC, Chatterjee A, Schultz PG, Ward GE, Gray N, Harper J, Winzeler EA. Gene expression signatures and small-molecule compounds link a protein kinase to plasmodium falciparum motility. Nat Chem Biol. 2008; 4:347–356. [PubMed: 18454143]
65. Knockaert M, Gray N, Damiens E, Chang YT, Grellier P, Grant K, Fergusson D, Mottram J, Soete M, Dubremetz JF, Le Roch K, Doerig C, Schultz P, Meijer L. Intracellular targets of cyclin-dependent kinase inhibitors: Identification by affinity chromatography using immobilised inhibitors. Chem Biol. 2000; 7:411–422. [PubMed: 10873834]
66. Gurnett AM, Liberator PA, Dulski PM, Salowe SP, Donald RG, Anderson JW, Wiltsie J, Diaz CA, Harris G, Chang B. Purification and molecular characterization of cgmp-dependent protein kinase from apicomplexan parasites a novel chemotherapeutic target. Journal of Biological Chemistry. 2002; 277:15913–15922. [PubMed: 11834729]
67. de Koning-Ward TF, Fidock DA, Thathy V, Menard R, van Spaendonk RM, Waters AP, Janse CJ. The selectable marker human dihydrofolate reductase enables sequential genetic manipulation of the plasmodium berghei genome. Mol Biochem Parasitol. 2000; 106:199–212. [PubMed: 10699250]
68. Wu Y, Kirkman LA, Wellems TE. Transformation of plasmodium falciparum malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. Proc Natl Acad Sci U S A. 1996; 93:1130–1134. [PubMed: 8577727]
69. Maier AG, Rug M, O'Neill MT, Brown M, Chakravorty S, Szestak T, Chesson J, Wu Y, Hughes K, Coppel RL, Newbold C, Beeson JG, Craig A, Crabb BS, Cowman AF. Exported proteins required for virulence and rigidity of plasmodium falciparum-infected human erythrocytes. Cell. 2008; 134:48–61. [PubMed: 18614010]
70. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. Pgh1 modulates sensitivity and resistance to multiple antimalarials in plasmodium falciparum. Nature. 2000; 403:906–909. [PubMed: 10706290]
71. Triglia T, Wang P, Sims PF, Hyde JE, Cowman AF. Allelic exchange at the endogenous genomic locus in plasmodium falciparum proves the role of dihydropteroate synthase in sulfadoxine-resistant malaria. The EMBO journal. 1998; 17:3807–3815. [PubMed: 9669998]

72. Lelievre J, Almela MJ, Lozano S, Miguel C, Franco V, Leroy D, Herreros E. Activity of clinically relevant antimalarial drugs on plasmodium falciparum mature gametocytes in an atp bioluminescence “transmission blocking” assay. PLoS One. 2012; 7:e35019. [PubMed: 22514702]

73. Shekalaghe S, Drakeley C, Gosling R, Ndaro A, van Meegeren M, Enevold A, Alifrangis M, Mosha F, Sauerwein R, Bousema T. Primaquine clears submicroscopic plasmodium falciparum gametocytes that persist after treatment with sulphadoxine-pyrimethamine and artesunate. PLoS ONE. 2007; 2:e1023. [PubMed: 17925871]

74. Coleman RE, Clavin AM, Milhous WK. Gametocytocidal and sporontocidal activity of antimalarials against plasmodium berghei anka in icr mice and anopheles stephensi mosquitoes. Am J Trop Med Hyg. 1992; 46:169–182. [PubMed: 1539752]

75. Tekwani BL, Walker LA. 8-aminoquinolines: Future role as antiprotozoal drugs. Current opinion in infectious diseases. 2006; 19:623–631. [PubMed: 17075340]

76. Delves MJ, Ramakrishnan C, Blagborough AM, Leroy D, Wells TN, Sinden RE. A high-throughput assay for the identification of malarial transmission-blocking drugs and vaccines. Int J Parasitol. 2012; 42:999–1006. [PubMed: 23023046]

77. van Pelt-Koops J, Pett H, Graumans W, van der Vegte-Bolmer M, van Gemert G, Rottmann M, Yeung B, Diagana T, Sauerwein R. The spiroindolone drug candidate nitd609 potently inhibits gametocytogenesis and blocks plasmodium falciparum transmission to anopheles mosquito vector. Antimicrobial agents and chemotherapy. 2012; 56:3544–3548. [PubMed: 22508309]

78. Ploemen IH, Prudêncio M, Douradinha BG, Ramesar J, Fonager J, van Gemert GJ, Luty AJ, Hermsen CC, Sauerwein RW, Baptista FG. Visualisation and quantitative analysis of the rodent malaria liver stage by real time imaging. PLoS One. 2009; 4:e7881. [PubMed: 19924309]

79. Crockett M, Kain KC. Tafenoquine: A promising new antimalarial agent. Expert Opinion on Investigational Drugs. 2007; 16:705–715. [PubMed: 17461742]

80. Coleman RE. Sporontocidal activity of the antimalarial wr-238605 against plasmodium berghei anka in anopheles stephensi. American Journal of Tropical Medicine and Hygiene. 1990; 42:196–205. [PubMed: 2180334]

81. Huang W, Luo M, Zhou M, Pan X. Study on the treatment of plasmodium cynomolgi infections of the macaque with ketotifen]. Yao xue xue bao= Acta pharmaceutica Sinica. 1987; 22:409. [PubMed: 3450138]

82. Mazier D, Renia L, Snounou G. A pre-emptive strike against malaria’s stealthy hepatic forms. Nat Rev Drug Discov. 2009; 8:854–864. [PubMed: 19876040]

Biographies

Erika Flannery is a Postdoctoral Fellow in the laboratory of Dr. Elizabeth Winzeler at the University of California San Diego School of Medicine. She received her Bachelor of Science in Cellular and Molecular Biology in 2004 from the University of Michigan. She earned her M.P.H. in Hospital and Molecular Epidemiology and her Ph.D. in Epidemiology from the University of Michigan School of Public Health where she studied horizontal gene transfer in the laboratory of Dr. Harry L.T. Mobley and host colonization rates with multi-drug resistant organisms with Dr. Betsy Foxman and Dr. Lona Mody. She is currently a Ruth L. Kirschstein NRSA award recipient. Her research interests include combining genomics and epidemiology to effectively identify pathogen factors contributing to antimalarial drug resistance in *Plasmodium* species.

David Fidock is a Professor of Microbiology & Immunology and of Medical Sciences in Medicine at the Columbia University Medical Center in New York. He received his Bachelor of Mathematical Sciences with Honors from Adelaide University in Australia in 1986, and his Ph.D. in Microbiology from the Pasteur Institute in Paris in 1994. Following postdoctoral research at UC Irvine with Dr. Anthony James and the NIH with Dr. Thomas Wellems, he started his independent group at the Albert Einstein College of Medicine in New York. He moved to Columbia University in 2007. His research focuses mostly on using genetic tools to elucidate the genetic and molecular basis of antimalarial drug resistance in

P. falciparum, antimalarial drug discovery, genetically attenuated parasite vaccines and parasite lipid metabolism.

Elizabeth Winzeler Ph.D. received her Bachelor of Arts degree in Natural Sciences and Art from Lewis and Clark College in 1984. She obtained a Ph.D. 1996 from the Department of Developmental Biology at the Stanford University School of Medicine. This was followed by a postdoctoral fellowship in the Department of Biochemistry. In 1999 she moved to San Diego to take a joint appointment at the Scripps Research Institute and the Genomics Institute of the Novartis Research Foundation (GNF). At GNF she developed a malaria drug discovery program and screening methods that have resulted in the identification of several novel antimalarial chemotypes. She recently moved to the University of California, San Diego, School of Medicine where she is a professor in the Department of Pediatrics.

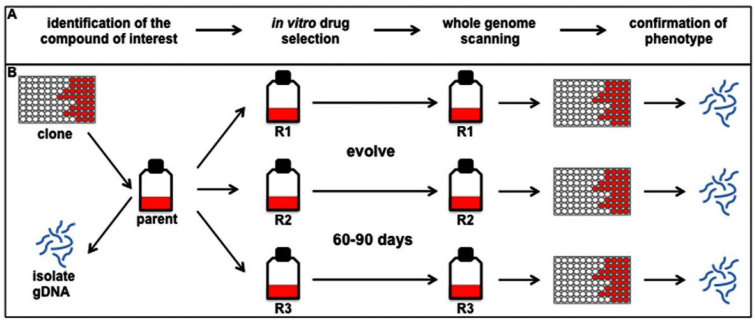

Figure 1. A) Procedure for identification of targets of novel compounds with antimalarial activity

Novel chemical classes are identified using cell-based, high-throughput phenotypic screens of parasites at any of the three lifecycle stages of *Plasmodium* in the vertebrate host (liver, ABS, gametocyte). In order to elucidate the target of the antimalarial compound, parasites are grown *in vitro* in the presence of the compound of interest until the emergence of resistant lines. The genetic determinants associated with resistance are identified using microarrays or whole-genome sequencing with the goal of ascertaining the target or at least genes involved in resistance. Once the potential target is identified, reverse genetic approaches can be used to show that the phenotype is caused by the identified mutations.

Selection of compound-resistant clones. In order to have a clean genetic background for the identification of genetic variants in compound-resistant clones, the parent culture is cloned by limiting dilution and genomic DNA is isolated and stored. The parent is then split so it can be used to generate three independent lines and compound pressure is applied to each independent culture, using either a one-step or multi-step method, until resistant parasites emerge. The independent resistant lines are cloned and genomic DNA is isolated to compare the genomes of the parent with resistant lines, either by microarray or whole-genome sequencing. IC_{50} values for the resistant lines are defined in a dose-response format to measure the fold gain of resistance.

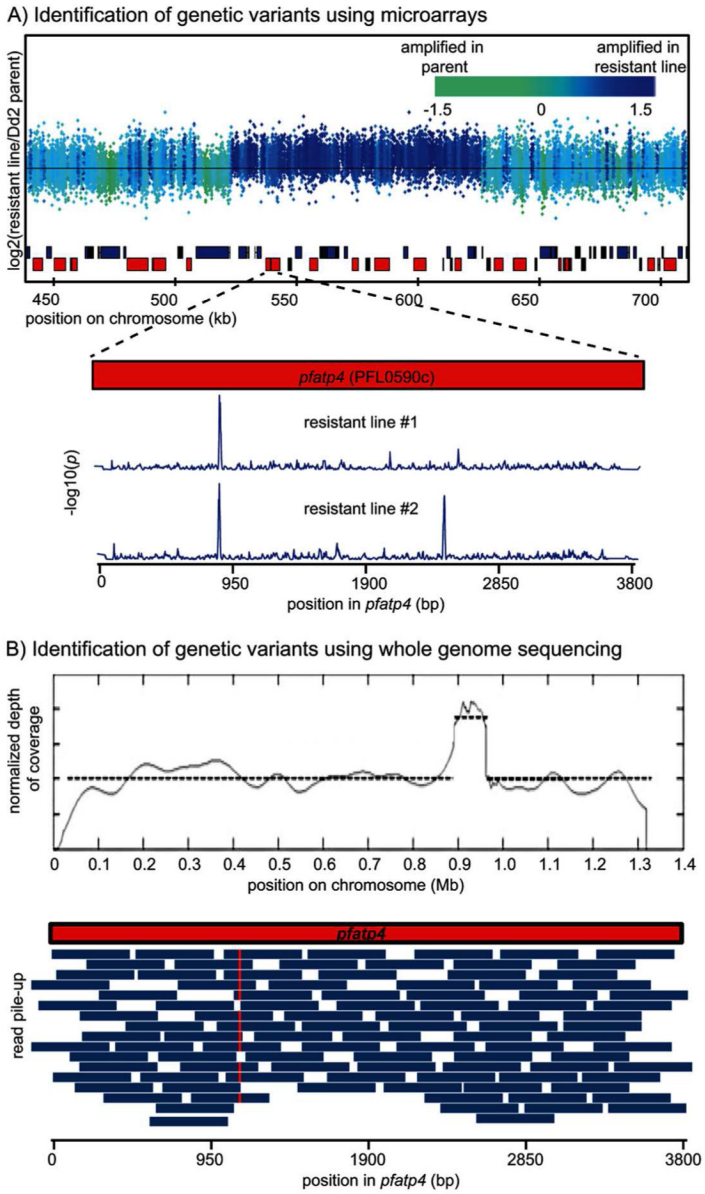

Figure 2. Genome-scanning methods to detect newly emerged changes in the *P. falciparum* genome

A) Tiling microarrays. Plotted values of the log ratio of the hybridization intensities at different probe locations along the genome, colored by fold change, are used to identify locations of copy number variants (CNVs). CNVs often include the putative target gene. Microarrays are used to detect single nucleotide variants (SNVs) by identifying probes that have a loss of hybridization in the resistant line compared to the parent. A decrease in p-value represents a likely SNV, where the probes failed to hybridize in the resistant line.

B) Whole-genome resequencing (WGS). WGS is used to identify CNVs by comparing the average read coverage throughout the genome. Regions in the resistant line that have a higher coverage than the parent contain amplified copies of genes in that region. SNVs are identified by aligning reads to a reference genome and comparing the base call at each position between the resistant line and the parent. The red line in each read represents a base call that differs from the reference strain.

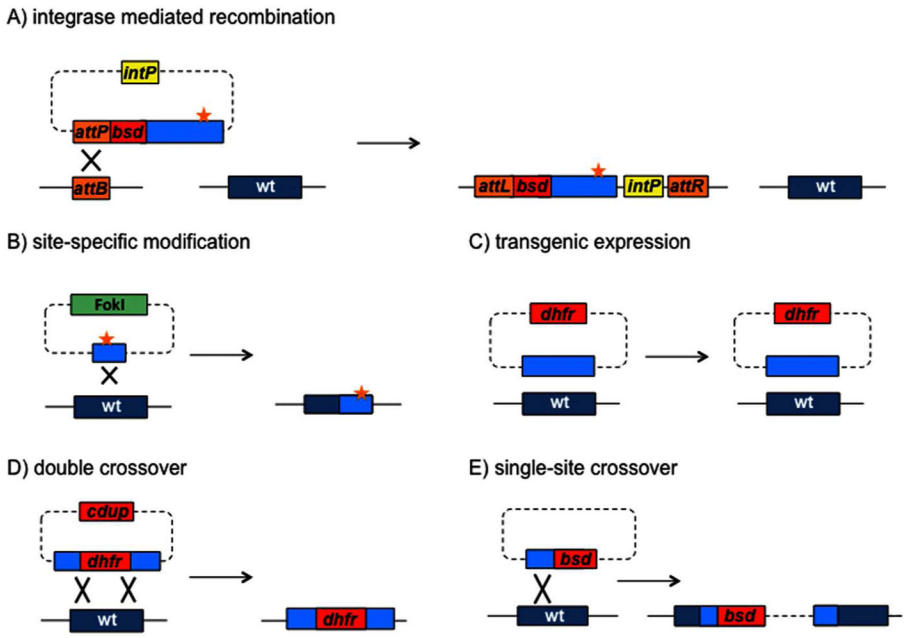

Figure 3. Strategies for linking genotype and phenotype

Dark blue boxes represent wild-type allele of interest, while light blue represents the mutated allele. The orange star represents a single-nucleotide variant (SNV). A) Integrase-mediated recombination uses strains of *P. falciparum* that have been engineered to contain an *attB* locus. Introduction of an episomally replicating plasmid that encodes an integrase and *attP* locus allows for site-specific recombination between the *attP* site on the plasmid and the *attB* chromosomal site.[43] This stably incorporates a plasmid-borne sequence of interest into the chromosome. This can result in introduction of a mutant copy of a locus of interest or a second copy of an allele of interest to test whether additional copies confer resistance. B) Site-specific modification can be achieved by transfecting a plasmid encoding customized zinc finger nucleases.[46] Nuclease pairs bind either side of the target site, where they produce a double stranded break. DNA repair is mediated through homology-directed recombination that can incorporate the donor-provided plasmid-borne sequence into the gene of interest. This is useful for introducing SNVs into genes and can also be used to introduce complete markers or even a full deletion of a locus of interest. C) Transfection with a plasmid that contains a copy of the gene of interest and a selection marker (e.g. *human dihydrofolate reductase (hdhfr)*, which renders parasites resistant to WR99210[67]) allows for stable transgene expression of the gene with the allele of interest.[68] If the phenotype is dominant, the function of the allele can be investigated. D) Allelic exchange uses plasmid containing a positive selectable marker (e.g. a drug resistance gene, such as *hdhfr*) flanked by homology arms that can recombine with the regions flanking the chromosomal gene of interest. Transgenic parasites harboring the resistance gene will escape being killed when drug pressure is applied. Because plasmids can be maintained episomally, a negative selectable marker may be placed on the plasmid (using the *Saccharomyces cerevisiae cytosine deaminase/uracil phosphoribosyl transferase (CDUP)* gene) to force recombination and eliminate the original allele.[69] E) Allelic exchange with a single-site crossover results in integration of a copy of the plasmid within the endogenous gene in the chromosome, resulting in gene disruption or introduction of a mutated allele carrying SNVs of interest. This approach was earlier used to definitely assess drug resistance-associated mutations in several key mediators including *Pfcrt*, *Pfmdr1* and *Pfdhps*.[47, 48, 50, 70, 71]

Table 1

Stage of activity and chemical class of clinically relevant antimalarials.

Antimalarials in Clinical Use							
Compound	**Chemical class**	**L**[a]	**H**[b]	**ABS**[c]	**G**[d]	**Ook**[e]	**Ooc**[f]
Artemisinins (artemether, artesunate, dihydroartemisinin)	Endoperoxide	+	−	+	+	−	+
Lumefantrine	Amino alcohol	−	−	+	−	−	+
Mefloquine	Amino alcohol	−	−	+	+	−	+
Amodiaquine	4-aminoquinoline	+	−	+	+	−	−
Sulfadoxine	Sulfonamide	−	−	+	nd	−	nd
Pyrimethamine	Antifolate	+	−	+	+	+	+
Piperaquine	4-aminoquinoline	−	−	+	[8]	nd	nd
Pyronaridine	4-aminoquinoline	+	−	+	x	+	nd
Quinine	Amino alcohol	−	−	+[72]	+[72]	+	nd
Atovaquone	Naphthalene	+	−	+	+[8]	+	nd
Proguanil	Antifolate	−	−		nd	−	nd
Chloroquine	4-aminoquinoline	−	−	+	−	−	−
Primaquine	8-aminoquinoline	+	+	+[73]	+[73]	+[74]	+[74]
Selected Antimalarials in Development							
Antimalarial	**Chemical class**	**L**	**H**	**ABS**	**G**	**Ook**[b]	**Ooc**[c]
NPC-1161B	8-aminoquinoline	+	nd	+[75]	+	−	+
Methylene blue	Aromatic	+	nd	+[8]	+[8]	+[76]	+[8]
NITD609	Spiroindolone	–[59]	nd	+[23]	+[77]	nd	+[77]
GNF156 (KAF156)	Imidazolopiperazine	+[59]	nd	+[59]	+	nd	nd
OZ439	Endoperoxide	−	nd	+	+	−	+
OZ277	Endoperoxide	−	nd	+	+	−	+
P218	Antifolate	+	nd	+[15]	nd	nd	nd
Tafenoquine	8-aminoquinoline	+[30, 78]	+[79]	+[79]	+[80]	–	+[80]

Abbreviations: L, liver-stage; H, dormant hypnozoite; ABS, asexual blood stage; G, mature gametocyte; Ook, ookinete; Ooc, oocyst; nd, not determined; Pf, *Plasmodium falciparum*; Pv, *Plasmodium vivax*; Pb, *Plasmodium berghei*. IUPAC names for compounds not mentioned in the text are:

Artemether ((1R,4S,5R,8S,9R,10S,12R,13R)-10-methoxy-1,5,9-trimethyl-11,14,15,16-tetraoxatetracyclo[10.3.1.0^{4,13}.0^{8,13}]hexadecane);

Dihydroartemisinin ((3R,5aS,6R,8aS,9R,12S,12aR)-decahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10-ol);

Artesunate (4-oxo-4-{[(3R,5aS,6R,8aS,9R,10S,12R,12aR)-3,6,9-trimethyldecahydro-3,12-epoxypyrano[4,3-j][1,2]benzodioxepin-10-yl]oxy}butanoic acid);

Lumefantrine ((+-)-2,7-Dichloro-9-((Z)-p-chlorobenzylidene)-alpha((dibutylamino)methyl)fluorene-4-methanol);

Mefloquine ((R)-[2,8-bis(trifluoromethyl)quinolin-4-yl]-[(2S)-piperidin-2-yl]methanol));

Aminodiaquine (4-[(7-chloroquinolin-4-yl)amino]-2-(diethylaminomethyl)phenol);

Sulfadoxine (4-amino-N-(5,6-dimethoxypyrimidin-4-yl)benzenesulfonamide); pyrimethamine (5-(4-chlorophenyl)-6-ethylpyrimidine-2,4-diamine);

Quinine ((R)-[(2S,5R)-5-ethenyl-1-azabicyclo[2.2.2]octan-2-yl]-(6-methoxyquinolin-4-yl) methanol);

Atovaquone (3-[4-(4-chlorophenyl)cyclohexyl]-4-hydroxynaphthalene-1,2-dione);

Proguanil ((1E)-1-[amino-(4-chloroanilino)methylidene]-2-propan-2-ylguanidine);

Chloroquine (4-N-(7-chloroquinolin-4-yl)-1-N,1-N-diethylpentane-1,4-diamine);

OZ277([(N-(2-amino-2-methylpropyl)-2-cis-dispiro(adamantane-2,3′-[1,2,4]trioxolane-5′,1″-cyclohexan)-4″-yl]acetamide);

Methylene blue ([7-(dimethylamino)phenothiazin-3-ylidene]-dimethylazanium;chloride);

NPC-1161B((-)-(R)-8-[(4-amino-1-methylbutyl)amino]-6-methoxy-4-methyl-5-[3,4-dichloro- phenoxy]quinoline succinate);

[a] Based on *P. yoelii* invasion of HepG2-CD81 hepatocytes (IC50 < 1 uM) as described in [81], unless otherwise noted

[b] Reviewed in [82].

[c] Inhibition of asexual blood stage activity at less than 20 nM as described in [81] unless otherwise indicated.

[d] Inhibition of male mature gamete exflagellation by 50% at 10 uM as described in [81] unless otherwise indicated.

[e] Inhibition of Pb ookinete formation at less than 50% of control at 10 uM [81] unless otherwise indicated.

[f] Inhibition of Pf oocyst development in membrane feeding assay with fewer than 2 oocysts per mosquito[81] unless otherwise indicated.

Table 2

Summary of targets or resistance genes identified after *in vitro* selection and whole-genome analysis.

Compound	Presumed Mechanism of Action	Allelic changes
Atovaquone	Cytochrome bc1	SNVs in *cytochrome bc1*, CNV around the *multidrug resistance associated protein gene*.[31]
Cladosporin	Unknown	CNVs in *lysyl-tRNA synthetase* in three independent clones, SNVs found in *S. cerevisiae lysyl tRNA synthetase*.[56]
Decoquinate	Electron transport	SNV in *mitochondrial cytochrome bc1*.[24]
Fosmidomycin	Inhibits DXR	CNV of region surrounding *pfdxr*.[33]
GNF179	Unknown	SNVs in the *P. falciparum cyclic amine resistance locus*.[24]
Mupirocin	Bacterial protein synthesis	SNV and CNV in *apicoplast isoleucyl tRNA synthase* in three clones.[34]
NITD609 (KAE609)	Unknown	2 unique SNVs in each of 2 clones, CNV + SNV in 3rd clone, all in *pfatp4*,[23] involved in Na+ homeostasis.[55]
NITD731	Presumed protease inhibitor	L333F mutation in *P. falciparum signal peptide peptidase* (PF3D7_1457000).[58]
Piperaquine	Digestive vacuole	SNV in *pfcrt*, CNV of multiple genes around *pfmdr1*.[63]
Tetracyclic benzothiazepine	Unknown	SNVs in *cytochrome bc1*.[57]
Thiaisoleucine	Isoleucine analog	SNV in cytoplasmic *isoleucyl tRNA synthase*.[34]

Notes: CNV, copy number variant; DXR, DOXP reductoisomerase; SNV, single nucleotide variant or small indel. Decoquinate (ethyl 6-decoxy-7-ethoxy-4-oxo-1H-quinoline-3-carboxylate); Fosmidomycin (3-[formyl(hydroxy)amino]propylphosphonic acid)); Mupirocin (9-[(E)-4-[(2S,3R,4R,5S)-3,4-dihydroxy-5-[[(2S,3S)-3-[(2S,3S)-3-hydroxybutan-2-yl]oxiran-2-yl]methyl]oxan-2-yl]-3-methylbut-2-enoyl]oxynonanoic acid); Thiaisoleucine (2-Amino-3-(methylthio)butyric acid).