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Review

Global Phenotypic Screening for Antimalarials

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Malaria, a devastating infectious disease caused by *Plasmodium spp.,* leads to roughly 655,000 deaths per year, mostly of African children. To compound the problem, drug resistance has emerged to all classical antimalarials and may be emerging for artemisinin-based combination therapies. To address the need for new antimalarials with novel mechanisms, several groups carried out phenotypic screening campaigns to identify compounds inhibiting growth of the blood stages of *Plasmodium falciparum*. In this review, we describe the characterization of these compounds, explore currently ongoing strategies to develop lead molecules, and endorse the concept of a "malaria box" of publicly accessible active compounds.

Introduction

Malaria is a mosquito-borne disease that kills roughly 655,000 people every year, mostly young children in Africa. Malaria affects roughly 215 million patients annually (World Health Organization, 2011), and approximately one third of the world's population is at risk for contracting the disease. The World Health Organization has announced a new campaign for global malaria eradication (Wells et al., 2009).

Mosquito-borne diseases are usually controlled by a combination of vector control, vaccines, and chemotherapy. In the case of malaria, economical vector control strategies, including insecticide-impregnated bed nets and localized spraying, have been deployed with success (Okumu and Moore, 2011). Additionally, progress is being made toward effective vaccines with the RTSS vaccine from GlaxoSmithKline (GSK), giving some protection (Schwenk and Richie, 2011). Nonetheless, chemotherapy remains the dominant component of malaria control. Unfortunately, clinical resistance has emerged for most available drugs (Petersen et al., 2011), and there are recent indications of the emergence of resistance to the artemisinin components of artemisinin-based combination therapies, which are a cornerstone of current antimalarial treatment strategies (Dondorp et al., 2009; Mok et al., 2011; Saralamba et al., 2011; Veiga et al., 2011).

Therefore, new antimalarials are urgently needed. The focus of the discovery process is on new medicines that are structurally distinct from existing drugs, act by novel mechanisms, and avoid being acted upon by drug transporters overexpressed or overactive in multi-drug-resistant malaria. In the late 2000s, three groups, one in academia (St. Jude Children's Research Hospital) (Guiguemde et al., 2010) and two in industry (GSK [Gamo et al., 2010] and Novartis [Plouffe et al., 2008]), identified novel leads using screening campaigns measuring the growth inhibitory potential of compounds acting on *Plasmodium falciparum* co-cultured during its asexual stages in human erythrocytes.

In this review, we discuss the driving force for conducting these screens; the results, including similarities and differences between the compounds identified; and the need for further innovation and work in understanding the underlying cellular and physiologic mechanisms by which the new classes of antimalarials work.

Biology

The rate of discovery of novel antimalarials has been slow since the 1980s, after the pharmaceutical industry deprioritized antimalarial drug discovery. Most work until the mid-2000s addressed two themes: (1) incremental modification of existing chemotypes to overcome drug resistance or improve pharmacologic properties; and (2) assessment of new targets proposed from parasitologic insight. The conditions in the field—widespread drug resistance to most classes of drugs, a small number of structural classes of drugs, and a small number of validated targets—constituted a perfect storm, occluding the development of novel drugs.

Drug Resistance

Many targeted antimalarials seemed to rapidly lose efficacy. For example, atovaquone, an analog of ubiquinone that selectively inhibits the parasitic electron transport chain, selected for drug resistance before its introduction into the market (Canfield et al., 1995). The use of dihydrofolate reductase (DHFR)-targeting drugs revealed that multiple drug resistance mutations could accumulate, conferring high levels of resistance (Sridaran et al., 2010). This has left the impression that, in many cases, the parasite can rapidly select for mutations that confer drug resistance during the blood stages and that many compounds that target specific enzymes will fall victim to this issue. On the other hand, resistance to drugs, such as chloroquine, that do not target enzymes seems to happen much less rapidly (Sanchez et al., 2010). The issue for drug discovery then became how to predict which classes of drugs would exhibit this phenomenon and which would not. As many of the targeted therapies matured in the late 1990s, it became increasingly difficult to manipulate the molecules to overcome resistance. Multiple generations of small molecule inhibitors existed for each validated target. Many unsuccessful attempts were made

to discover new chemotypes directed at standing targets. This lack of success perhaps reflects the decades of focused medicinal chemistry efforts aimed at targets like DHFR and thymidylate synthase and the relatively rigid and uncompromising nature of their active sites.

Validated Targets

Many parasitologists have focused on identifying targets through genetics and understanding metabolism. These efforts led to the identification of a number of protein targets that were presumptively essential. The sequencing of *P. falciparum,* which revealed that 60% of its proteins have no orthologs in humans (Gardner et al., 2002), accelerated this approach. However, genetic essentiality alone does not confer validation, because drugs must be able to abrogate function in the intracellular context and possess balanced efficacy, bioavailability, and toxicology. For example, cysteine proteases, such as falcipains, are critical for many cellular processes, particularly catabolism of hemoglobin and merozoite egress (Rosenthal, 2011), and nonspecific cysteine protease inhibitors can kill the parasite (Joachimiak et al., 2001). However, no falcipain inhibitor has been successfully transitioned to the clinic because of issues associated with pharmacokinetics and toxicology (Ettari et al., 2010). Similar scenarios have played out with other proposed targets such as enoyl-ACP reductase (FabI), although in that case it did lead to a proposition that the treatment outcome would be improved if the target were addressed in liver rather than blood stages (Yu et al., 2008). For these reasons, the number of validated drug targets remains rather limited.

Validated Chemotypes

The third major constraint is the relatively small number of antimalarial chemotypes as starting points for drug discovery programs. In a recent review, we estimated that there are fewer than 30 small molecule scaffolds with bona fide in vivo activity against *P. falciparum* (Smithson et al., 2010). Even among the examples that are active in vivo, only a small number prove themselves in clinical development—with the number of distinct antimalarial chemotypes in the clinic being fewer than 10 (Table 1). As discussed above, essentially all of these have been the subject of extensive medicinal chemistry campaigns spanning decades and are unlikely to yield the types of drugs needed to support the malaria eradication campaign. Thus, emphasis needs to be placed on identifying new scaffolds and developing them into viable clinical candidates.

Screening Strategies

Screening strategies to identify leads fall into three categories: virtual screening, target-based screening, and phenotypic screening. Many screening campaigns have been executed for malaria in the past decade in both the academic and industrial sectors, and here we will summarize and discuss the results of some of these efforts.

Virtual Screening

Virtual screening for malaria is hindered by the paucity of well-validated targets, many of which also lack structural information, although structural genomics efforts exist (Almo et al., 2007; Fan et al., 2008; Vedadi et al., 2007). The limited number of virtual screening studies that have produced viable preclinical candidates for malaria makes it difficult to assess the power of the approach (Nicola et al., 2007; Penna-Coutinho et al., 2011). As new validated targets emerge, it is likely that this approach, together with classical structure-based drug design, will play a more important role.

Target-Based Screening

Target-based screening has dominated drug discovery efforts in the past few decades. As an example, at GSK, lactate dehydrogenase (LDH), FabI, dihydroorotate dehydrogenase (DHODH), and thioredoxin reductase (TrxR) have been screened against the full standard corporate screening library, and, in some cases, also against focused sets of compounds enriched with known inhibitors of orthologous enzymes.

Inhibition of LDH was pursued by many groups (Cameron et al., 2004; Choi et al., 2007a, 2007b; Granchi et al., 2010). A GSK high-throughput screening (HTS) campaign tested 500,000 compounds and identified azole derivatives as selective inhibitors of *Plasmodium* LDH relative to human LDH (Cameron et al., 2004). Although extensive lead optimization produced derivatives with whole cell activity and partial efficacy in malaria animal models, efficacy levels warranting further development could not be achieved, and anti-parasitic activity could not be linked to enzyme inhibition. These results led to the conclusion that LDH is not druggable. TrxR met a similar fate (Buchholz et al., 2010).

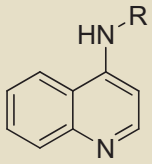
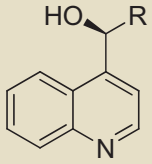
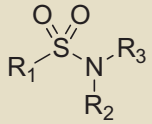
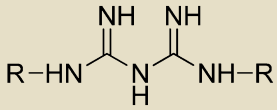
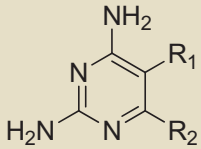
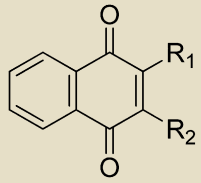
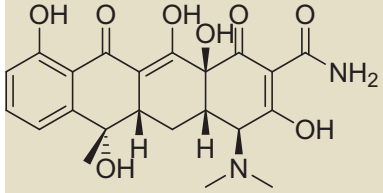
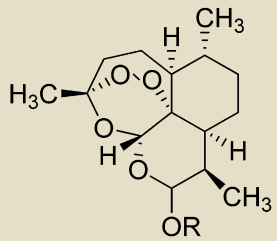
FabI is a validated druggable target for antibacterials (Heerding et al., 2001), and the *Plasmodium* ortholog seemed likely to be a good antimalarial target (Perozzo et al., 2002; Surolia and Surolia, 2001). Three GSK HTS campaigns looking for enzymatic inhibitors of FabI using more than 900,000 compounds identified three chemical families of tractable hits. Optimization of the three chemotypes, directed by co-crystal structures, resulted in highly potent inhibitors of the enzyme with clear structure-activity relationships (SARs). However, whole-cell activity did not correlate with enzymatic potency. Efforts to confirm the target validation finally led to the realization that FabI is dispensable during the intraerythrocytic cycle (Vaughan et al., 2009; Yeh and DeRisi, 2011; Yu et al., 2008). This project was subsequently abandoned.

DHODH was originally chosen because it was hypothesized that the main role of the electron transport chain might be to provide an electron sink for DHODH (Gutteridge et al., 1979). This hypothesis was pharmacologically confirmed later (Baldwin et al., 2005). An enzymatic HTS at GSK using 2,000,000 compounds identified five novel families of inhibitors. They are being held as possible backups for the triazolopyrimidine series currently in preclinical development (Coteron et al., 2011).

Phenotypic Screening

Experience in antibacterial and antifungal drug discovery showed that it is more difficult to add cell permeability without cytotoxicity to an enzymatic inhibitor through medicinal chemistry efforts than to find the target of a selective hit from a growth inhibition screen. A recent study showed that phenotype-based screens identified more first-in-class small molecules between 1999 and 2008 than other screening methods (Swinney and Anthony, 2011). Therefore, phenotypic screening seems a logical approach of choice for lead identification efforts. Additionally, inhibitors identified in phenotypic screening are guaranteed to act against their antimalarial target in its intracellular context, with physiologic concentrations of substrates and effectors. Determining the mode of action for novel hits remains a challenge, but new technologies, including genetics (Diaz et al.,

Table 1. Historical Chemotypes

Scaffold	Chemotype	Target	Examples
	4 aminoquinolines	Hemozoin	Chloroquine, Amodiaquine
	4 Methanolquinolines	Hemozoin	Mefloquine, Quinine
	Sulfonamides	DHPS	Sulfadoxine
	Biguanides	DHFR	Proguanil
	Aminopyrimidines	DHFR	Pyrimethamine
	Naphthoquinones	Cytbc1	Atovaquone
	Tetracyclines	Organellar ribosomes	Doxycycline
	Sesquiterpene lactones	Unclear	Artemisinin, Artesunate

The historical chemotypes and their targets.

2006; Rottmann et al., 2010), cheminformatics (Plouffe et al., 2008), and proteomics (Guiguemde et al., 2010) are proving successful.

Publicly Disclosed Screening Results

Three groups, from academia (St. Jude Children's Research Hospital) (Guiguemde et al., 2010), industry (GSK) (Gamo et al., 2010), and academic/industry consortia (Novartis) (Plouffe et al., 2008) have reported the results of phenotypic HTS campaigns, and the structures of thousands of active compounds have been deposited in public databases (https://www.ebi.ac.uk/chemblntd/). A number of other screens are still ongoing, but this work remains unpublished.

The St. Jude Children's Research Hospital group screened 300,000 compounds against intraerythrocytic *P. falciparum* (*3D7* strain) at a concentration of 7 μM (Guiguemde et al., 2010). The most efficacious 1,300 of these compounds were subjected to concentration-response experiments against multiple strains of *P. falciparum* as well as counter-screening against multiple mammalian cell lines, leading to 560 validated hits with potency at concentrations less than 2 μM against all strains of malaria tested and at least 10-fold selectivity against mammalian cells. A structurally diverse set of 170 compounds was selected from these hits and subjected to extensive profiling designed to illuminate mechanisms of action. Of the validated hits that were fully profiled, 80% appeared to have novel mechanisms of action and no cross-resistance to existing drugs.

At GSK, 2,000,000 compounds were screened against intraerythrocytic *P. falciparum* (Gamo et al., 2010). More than 19,000 primary hits inhibited growth by more than 80% at 2 μM. Retesting and counter-screening for LDH inhibitors left 13,500 confirmed hits, referred to as the Tres Cantos Antimalarial Set (TCAMS). When tested at 10 μM, only 1,982 compounds blocked HepG2 growth by more than 50%, leaving roughly 11,500 validated hits. Most of the compounds were also active against multi-drug resistant strains of *P. falciparum*.

At Novartis, a proof-of-concept screen was performed with the Novartis natural products library of about 12,000 pure compounds, yielding 275 hits. After reconfirmation and assessment of cytotoxicity, 17 compounds were selected for further characterization based on their favorable selectivity index (high potency and low cytotoxicity) (Rottmann et al., 2010). After this initial pilot screen, a larger screen of a non-proprietary collection (about 800,000 compounds) was carried out and identified 7,500 primary hits. Dose response studies gave 5,655 hits with 50% inhibitory concentration (IC_{50}) values less than 1.25 μM and selectivity of at least 5-fold against two mammalian cell lines. About 200 distinct chemical classes were further prioritized for hit triage and early hit-to-lead chemistry activity.

Chemistry

Composition and Diversity of the Screening Sets

Generally, the screening compounds were selected to provide diversity and were filtered to remove compounds with predicted poor absorption, distribution, metabolism, and excretion (ADME) properties. The Novartis natural product collection, while diverse, was not prefiltered for ADME properties. The calculated properties were consistent across all three libraries with average molecular weights of 375 Da and an average calculated partition coefficient (ClogP, measuring lipophilicity) of 3.4. The physiochemical characteristics and scaffold composition of the collective hits were analyzed to determine the constraints placed on their activity by the use of whole cell screening.

The distribution of seven relevant physiochemical properties (such as molecular weight, hydrophobicity, and counts of atoms or hydrogen bond donors/acceptors) was statistically analyzed for FDA-approved small molecule drugs, the hits, and screening compounds (commercial HTS, natural products, fragments) using linear discriminant analysis to determine how the property distributions of the hits differed from or were similar to those of the originating compound collections (Figure 1) (Nichols, 2011). Generally, this analysis followed our prior work with screening libraries and can be related to cellular and systemic bioavailability (Shelat and Guy, 2007). The first two ordinates included large contributions from Oprea complexity (Allu and Oprea, 2005), molecular weight, logP, and hydrogen bond acceptors. The antimalarial hit sets did not fully encompass the distributions of properties of most of the approved drugs (shaded area) but were significantly shifted toward higher molecular weight and increased hydrophobicity as well as lower number of hydrogen bond donors, decreased solubility, and smaller polar surface area. The same trend was observed in comparison with the originating HTS libraries. This shift was unexpected for a cellular screen, which more typically yields hits with properties that overlay those of most approved drugs. This finding, from independent compound collections selected to be "drug-like/lead-like", strongly suggests that the parasite has unique requirements for activity, probably associated with the need to cross two different cell membranes to reach the targets and the unusual environment in the erythrocyte.

The analysis of scaffolds (Figure 2) followed our previously reported methods (Shelat and Guy, 2007). Each cluster in the figure represents a set of closely related scaffolds ("scaffold family"); the size and shape of the nodes represent the number of molecules derived from the scaffold and the presence of one or more highly potent hits ($EC_{50} < 100$ nM), respectively. The three largest scaffold families in the aggregate hit set are quinolone, indole, and benzoimidazole. There was a high degree of similarity at the scaffold level between the three hit sets. The lowest overlap was between GSK and Novartis, which shared only 50% of the scaffolds (Tanimoto similarity > 0.8, EPFP_4 fingerprint). St. Jude shared 63% with GSK and 76% with Novartis. Overall, there was a low level of similarity with scaffolds from existing drugs (roughly 25%), which could indicate that the critical targets in *Plasmodium* and humans are significantly different in terms of the molecules that they "like." Another key finding is that while each library contained most active scaffold families, the most active compounds in a scaffold family tended not to be shared between libraries. For example, the inset in Figure 2 shows a scaffold family in greater detail. All three groups sampled from the 1,2,3,4-tetrahydroisoquinolone scaffold and identified potent 1-phenyl substituted analogs (pink diamonds). However the GSK and Novartis groups identified potent, closely related analogs (red and magenta diamonds), yielding a substantially different picture of the structure activity-relationships in this series. In fact, all three groups possessed unique analogs that could have informed hit evolution. Only integration of the data from multiple screens allowed identification of all of the well-validated active compounds.

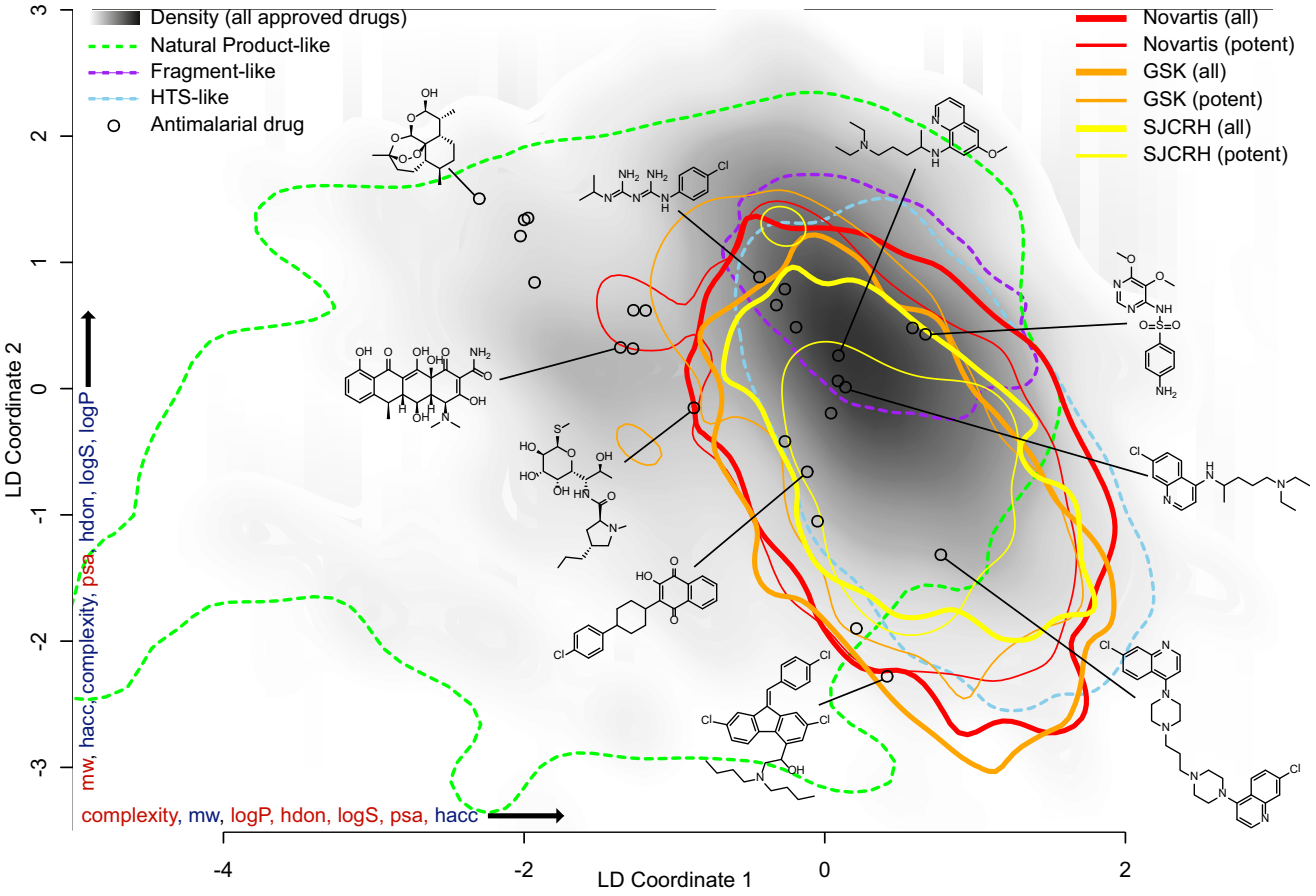


Figure 1. Physiochemical Characteristics of Validated Antimalarial Hits

Linear discriminant analysis of the physiochemical characteristics of validated HTS hits compared with FDA-approved small molecule drugs (oral and parenteral), commercially available HTS compounds, known natural products, and commercially available fragments. Shading represents the density of the distribution of approved drugs within the space. Lines represent the perimeter of the space containing 90% of the described compound collections. The composition of each LDA ordinate is described by the list of the physiochemical properties, sorted in order of decreasing magnitude and colored according to sign (red = negative contribution; blue = positive contribution). Approved antimalarial drugs are depicted by open circles, with structures. The potent subset of each hit set is rescricted to compounds with EC_{50} < 100 nM. Despite all three libraries being designed to overlay the centroid of the drug space, each screen selected compounds that were significantly shifted away from this centroid.

This highlights the need for pre-competitive collaboration in the identification of antimalarial chemotypes and the need to pool as much good quality screening data as possible.

High-Priority Leads

Each group pursued the development of a select series of novel leads identified in the screening efforts. The disclosure of the results from these studies has begun, with some now in literature and some orally disclosed. The lead series that have been actively pursued and publicly disclosed by each group are summarized in Table 2.

The St. Jude group carried out a program of profiling the hit series that involved four major phases (Guiguemde et al., 2010). In the first phase, sets of analogs were constructed for each series to reveal any SARs. Next, these analog sets were tested against a wide panel of strains of *P. falciparum* to empirically establish which series had no cross-resistance with existing antimalarial drugs. At the same time, the compounds were tested against a panel of mammalian cell lines and parasites to establish any appreciable cytotoxicity and selectivity for malaria relative to other protozoa. Third, the series were profiled against a large panel of validated and proposed targets for malaria, using biochemical and biophysical methods to eliminate any that acted by already-known mechanisms. Finally, a select set of compounds was subjected to rapid in vivo testing to establish bioavailability and efficacy using pharmacodynamic models. Following what was learned in these studies, the St. Jude group narrowed its efforts to three validated series: the diaminonaphthoquinones, the dihydroisoquinolones (DHIQs), and the dihydropyridines. Each represented a novel chemotype with a presumptive novel mechanism. In preliminary work, each series had members that were orally bioavailable in mice. All were well tolerated in mice, and each series had at least one example that lowered parasitemia in murine *P. berghei* models. The DHIQs are currently in late lead optimization, and the other two series are just entering the lead optimization stage.

The GSK group carried out an extensive profiling effort to prioritize hits from the TCAMS set (Calderón et al., 2011). The

first approach was mainly computational, looking for novel scaffolds distinct from known antimalarial chemotypes that were hypothesized to be free from cross-resistance. Further analysis focused on those that were chemically tractable, to allow rapid identification and synthesis of derivatives to establish SARs, and that had physicochemical properties compatible with oral bioavailability. The first series to be prioritized from TCAMS using the above criteria was that of the cyclopropyl carboxamides (Sanz et al., 2011). The original hit was both highly potent against multi-drug-resistant strains of *P. falciparum* and free of cytotoxicity against a panel of five mammalian cell lines. The series was judged to be chemically developable, and additional structural analogs were procured or synthesized. Testing of these compounds validated the carboxamides as a novel antimalarial chemotype. In subsequent testing, individual compounds showed oral efficacy in a mouse model of *P. falciparum* infection with potencies similar to that of chloroquine. During the work aimed at selecting resistant mutants for target identification, it was noted that a high level of resistance arose at unusually high frequencies. Consequently, further drug development activities have been halted and await elucidation of the exact molecular target and the mechanism of resistance (Sanz et al., 2011).

The GSK group recently developed a different approach to select chemical series from the TCAMS set with an increased focus on favorable drug-like properties without previous characterization of solubility or pharmacokinetic properties using an in vivo efficacy screening with oral administration in a simplified *P. berghei* mouse model. Several chemical series with potent oral efficacy have been identified and are being characterized.

The Novartis group used a stringent triage process to select leads with favorable physiochemical properties and strong structure activity relationships (SAR) within the screening set, and, often, robust reduction of parasitemia in the *P. berghei* mouse model. The first series reported was a novel benzamide scaffold that was subsequently optimized to afford a compound with good activity against *P. falciparum* (Wu et al., 2009). Another series that progressed was the 1H-imidazol-2-yl-pyrimidine-4,6-diamines (Deng et al., 2010). The next series that was reported by this group was the spiroindolones, including NITD609, which is currently in phase 1 testing and is expected to enter phase 2 trials later this year (Rottmann et al., 2010; Yeung et al., 2010). The current preclinical focus is on the imidazolpiperazine series, including front-runner GNF156, which is anticipated to enter phase 1 trials in early 2012 (Wu et al., 2011). These remarkable outcomes, with two compounds in clinical trials within 5 years of the screen, demonstrate the effectiveness of the phenotypic screening approach.

Target Identification

Target identification and validation are generally perceived as critical components of drug discovery. However, this has not always been the case. Especially for antimicrobials, many drugs currently in use were discovered using the phenotypic paradigm (Keiser et al., 2010). A 2011 study reported that, in the preceding decade (Swinney and Anthony, 2011), 28 first-in-class drugs were discovered using phenotypic screens versus 17 with target-based screens. Using phenotypic methods to identify hits, empirical medicinal chemistry to optimize them, and in vivo pharmacokinetic, toxicokinetic, and pharmacodynamic models to understand their function, a drug discovery program can remove target identification from the critical path needed to move new drugs to the clinic (Figure 3). However, having a target for a small molecule series is practical and useful because it allows the use of both structural and biochemical methods (Stoll et al., 2011; Wilson and Lill, 2011) and can direct the focus of toxicologic studies (Garcia-Serna and Mestres, 2010; Garcia-Serna and Mestres, 2011). Additionally, in malaria drug development, knowing a target for the drug facilitates the study of the acquisition of resistance. Finally, identifying the target opens the possibility of identifying backup molecules hitting the same target and thus better prioritizing efforts. Thus, while not mission-critical, target identification remains an important part of malaria drug discovery.

Many groups have turned to genome scanning when stable resistant mutants can be generated (McNamara and Winzeler, 2011). There are now several examples of successful target identification efforts that relied on the induction of drug resistance and the subsequent characterization of the mutations that conferred the resistant phenotype (Dharia et al., 2010; Istvan et al., 2011; Nam et al., 2011). Using this approach, the putative Ca^{2+}-dependent ATPase, encoded by the gene *PfATP4,* was identified as a candidate target for the spiroindolones (Rottmann et al., 2010). Using a similar approach with the imidazolopiperazine scaffold, drug resistance against this chemotype was found to be mediated by mutations in a gene of unknown function that was named *PfCarl* (cycloamine resistance locus, or Pf0970w) (Meister et al., 2011). An unanswered question in the field is how many new mechanisms of action have been encompassed by the current cellular/phenotypic hits. Based on our experiences in characterizing the hit sets, we believe that only a small set of new mechanisms may have been identified and that this possibility provides a strong impetus for a concerted effort toward systematic target identification globally using these hits (Garcia-Bustos and Gamo, 2011).

The St. Jude group used several strategies to identify potential targets of its hits. Reverse chemical genetic techniques were used to profile the set of antimalarial hits (172) against 66 cellular proteins, including a panel of validated drug targets by direct enzyme inhibition assays, and against a panel of predicted druggable targets by binding assays (thermal melt) (Guiguemde et al., 2010). They also looked for differential sensitivity to the compounds in specific parasite strains, either mutants with genetic linkage to drug resistance (V1S cell line, quadruple DHFR mutant) or transgenic parasites harboring the putative target (PfDHOD) (Painter et al., 2007). The combination of these two target identification strategies allowed identification of known targets for roughly 20% of the screening hits. The only targets giving functional novel inhibitors were the classic ones that have been well validated.

At GSK, rescreening the TCAMS hits against a strain of *P. falciparum* overexpressing DHODH identified roughly 400 inactive compounds, indicating they probably targeted electron transport. Proguanil, a known antifolate, has been previously observed to fully restore the inhibitory activity of cytochrome b1 inhibitors but not DHODH inhibitors against this strain (Ke et al., 2011). This approach identified both cytochrome b (14 novel chemotypes) and DHODH inhibitors (12 novel chemotypes). Antifolates can be identified by supplementing the growth

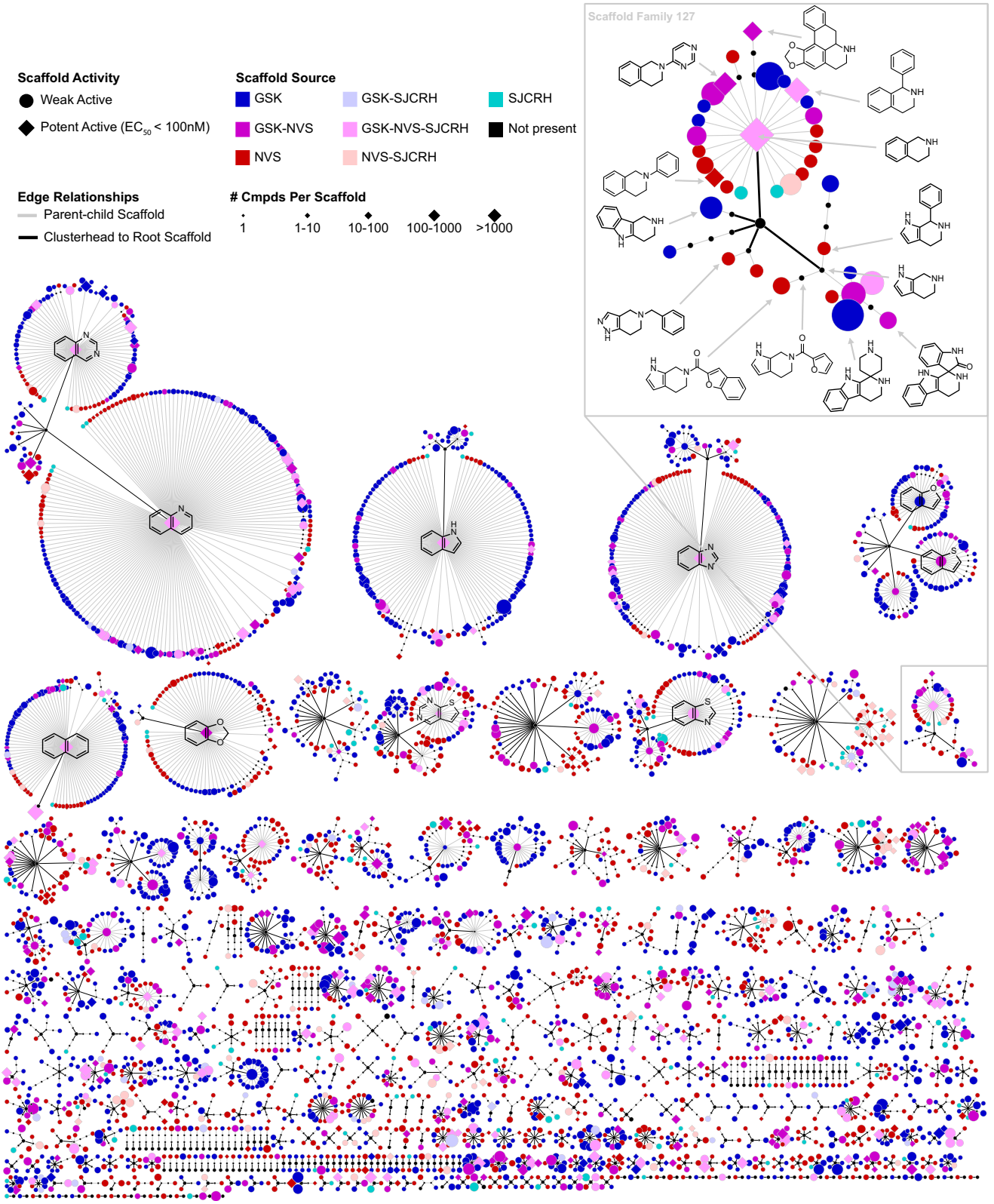


Figure 2. Scaffolds Observed in Validated Antimalarial Hits

A network graph showing the distribution of scaffolds within the hit sets in comparison with one another. Scaffolds were defined from molecules using the Schuffenhauer fragmentation algorithm (Schuffenhauer et al., 2007a, 2007b), but requiring at least two rings unless the molecule was acyclic or possessed only one ring. Scaffold nodes were arranged into parent-child hierarchies based on substructure relationships. The resulting root scaffolds were clustered using

medium with folic acid and, similarly, inhibitors of the isoprenoid biosynthesis pathway by adding geranylgeranyol or isopentenyl pyrophosphate (Yeh and DeRisi, 2011), which shifts the IC_{50} of inhibitors of those pathways.

At Novartis, the full set of confirmed antimalarial hits was screened with biochemical assays against nine putative drug targets. None of these screenings yielded a direct correlation between potency against a target and potency in the whole cell assay. Thus, no chemotype could be unambiguously assigned as an inhibitor of any of the nine targets screened (Crowther et al., 2011). The Novartis hit set was also screened with six other targets; the kinases PfCK2 PfCDPK 1 and 5, and PfGSK3; and the redox enzymes TrxR and glutathione reductase. That study yielded a similar outcome with no clear evidence that the antimalarial activity of those compounds could be attributed to the inhibition of any of the nine molecular targets.

Recently, an in vitro liver-stage assay (Silvie et al., 2003) was used to screen the Novartis hit set to identify compounds with both erythrocytic and hepatic antimalarial activity. The results of that screen showed that the overlap between blood and liver stages was fairly small, with only 229 compounds having an IC_{50} less than 1 μM in the liver-stage assay (Meister et al., 2011). Interestingly, whereas one spiroindolone, NITD609, was found to be completely inactive in this assay, the imidazolopiperazines showed potent activity that translated into robust causal prophylactic activity in the *P. berghei* malaria mouse model. Going forward, such approaches could be used to prioritize medicinal chemistry activities.

Conclusions and Future Directions

The recent publication of thousands of new antimalarial molecules provides a path forward in the field of drug discovery. Although these results are promising, especially with one compound already in clinical trials, it is important to bear in mind that for a new antimalarial, the risk of failure in phase 2 trials is estimated at 50% (Pink et al., 2005). Therefore, there remains a clear need to take full advantage of all of the potential afforded by the existing antimalarial hits to understand what new mechanisms of action and mediators of resistance can be identified and to use these learnings to guide new drug development. This will require strategic adjustments in the malaria drug discovery community, including addressing technical gaps in knowledge and improving global efforts by mobilizing the entire community with improved public disclosure and sharing of data.

A major technical gap is the lack of early identification of potential drug partners for combination therapy and the evaluation of their potential for synergy or antagonism with current antimalarials. The World Health Organization has mandated that all future chemotherapy for malaria will use drug combinations (Anonymous, 2001). Since future drugs will always be used in combination, they should be free of antagonism with potential partner drugs. It is labor-intensive to test large numbers of compounds. Additionally, pharmacokinetic incompatibilities, linked to CYP450 induction or inhibition or mismatched half-lives, are common. A method to estimate half-lives with the throughput to handle the tens of thousands of compounds in the published antimalarial sets would be extremely valuable in prioritizing compounds for further development.

Another technical gap is the lack of simple and powerful assays to determine whether drugs are cytocidal or cytostatic in effect as well as the rate of the effect. Fast-acting cytocidal drugs are desired because they not only alleviate the symptoms of malaria, but they can also delay the onset of drug resistance. Current methods involve long treatment of cultures for fixed periods of time, followed by removal of the drug and determination of effect at a later time. Such methods are labor-intensive, although significant improvements have been reported. The only widely accepted method for determining cytocidality is the clonal dilution assay, which requires weeks of continuous culture and is extremely labor-intensive (Rathod et al., 1997). Recently, a medium-throughput PCR protocol for determining cytocidality was described (Bahamontes-Rosa et al., 2011), but it is unsuitable for handling large numbers of compounds. In addition to determining the speed of parasite killing, assays that could measure the potency of compounds against individual stages of the intraerythrocytic parasite life cycle would be very useful, because a lead compound should be active against all stages. Finally, high-throughput assays against other stages, such as gametocytes and liver stages, are needed as well. Such assays are currently under development in several laboratories (Adjalley et al., 2011; Buchholz et al., 2011; Dembele et al., 2011).

Because drug resistance is the main obstacle to antimalarial drug discovery, it will be critical to assess the potential for drug resistance of lead compounds progressing toward becoming a clinical candidate. Medium-throughput assays to determine parasite resistance acquisition frequencies under drug pressure (Gassis and Rathod, 1996; Rathod et al., 1997; White and Pongtavornpinyo, 2003) would be extremely useful.

Another challenge is understanding the mechanism of action for newly validated hits. Currently, biochemical methods are limited to available proteins, and generating drug-resistant parasites for genome scanning is time-consuming. As discussed above, utilization of high-throughput biochemical screens to select inhibitors for potential targets from the various hit sets has given very low hit rates. This is probably more due to poor validation of the interrogated novel targets rather than to the methods themselves (Pink et al., 2005).

The identification of essential genes encoding drug targets is still a challenge. Strobel (Strobel and Arnold, 2004) speculated that eukaryotes have approximately 400 essential eukaryotic genes. As has previously been pointed out in analyzing other organisms, using the reasonable assumption that enzymes, receptors, and channels are the most likely targets for small

Tanimoto distance (FCFP_4 fingerprint) to generate 399 scaffold families. Each family was then assigned a node. Parent-child scaffold substructure relationships and cluster membership define graph edges. Scaffold nodes are colored by library membership, sized by number of molecules derived from the scaffold, and shaped according to whether or not at least one molecule from the scaffold is potent (EC_{50} < 100 nM). Structures of the root scaffolds from the ten largest scaffold families are shown. Each group independently identified most scaffold families, although the coverage of each scaffold by analogs present in individual hit sets was highly variable. As a result, often only one library contained the most potent member of a given scaffold. This may have driven the divergence in "high priority" scaffolds among the group.

Table 2. High-Priority Series from Each Group

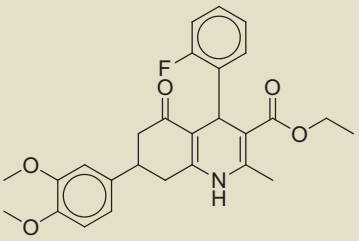
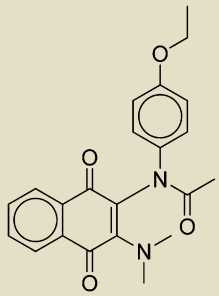
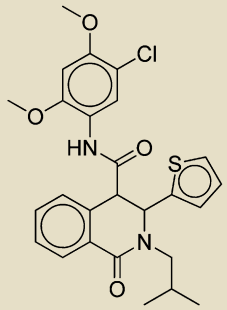
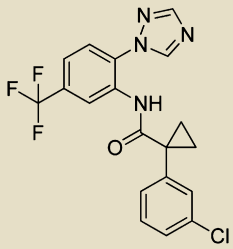
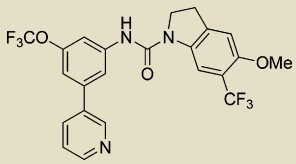
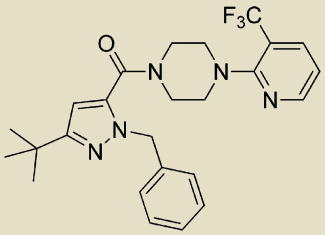
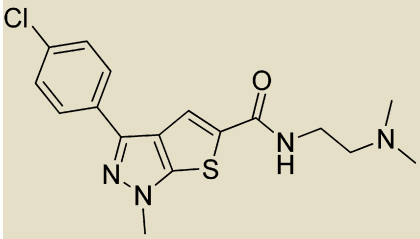
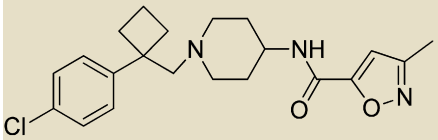
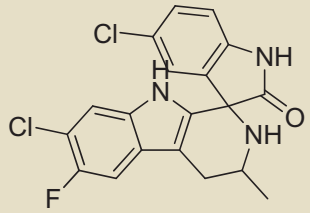
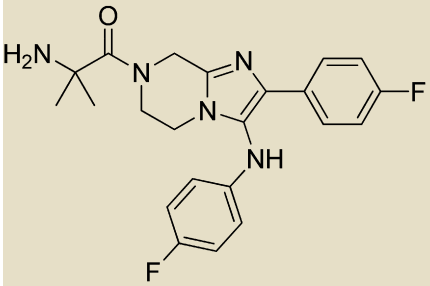
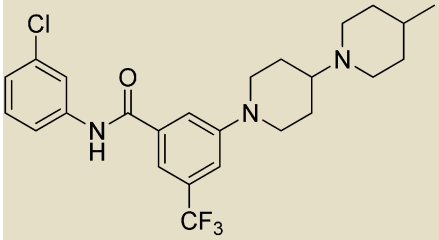
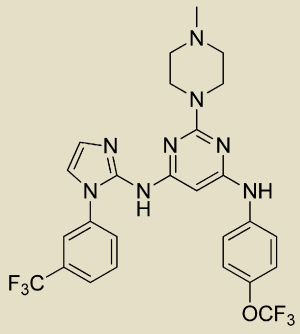
Molecule	Series Example	Institution	Development Stage
	Dihydropyridine SJ000025081	SJCRH	Lead optimization
	Diaminonaphthoqinone SJ000030570	SJCRH	Lead optimization
	Dihydroisoquinoline SJ000101247	SJCRH	Preclinical
	Carboxamide GSK2611622A	GSK	Lead optimization
	Indoline TCMDC-139046	GSK	Lead optimization
	Alkylpyrazole TCMDC-134142	GSK	Lead optimization

Table 2. *Continued*

Molecule	Series Example	Institution	Development Stage
	Thienopyrazole TCMDC-123580	GSK	Lead optimization
	Aminopiperidine TCMDC-124833	GSK	Lead optimization
	Spiroindolone NITD609	Novartis	Phase I
	Imidazolo piperazine	Novartis	Preclinical
	Benzamide	Novartis	Lead optimization
	Pyrimidine-4,6-diamine	Novartis	Lead optimization

In retrospective analysis, it is clear that each group independently detected most of the chemotypes present in this table, but, although uncoordinated, each group focused later efforts on a restricted set of series identified from their screens.

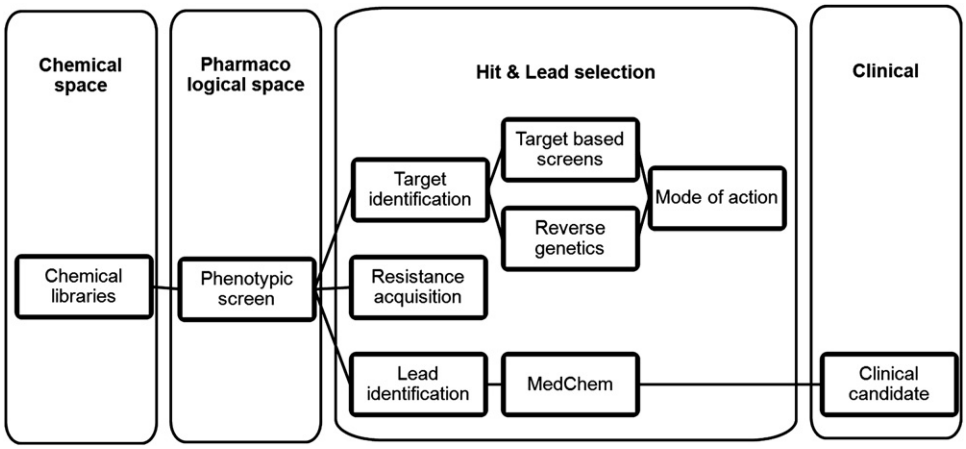


Figure 3. Phenotypic Drug Discovery Strategy for Malaria
The phenotypic strategy utilizes early deployment of cellular and pharmacologic constraints on molecule selection, leading rapidly to compounds with a high probability of activity in vivo. This pushes target identification and validation to later in the process, off the critical path. However, understanding the mechanism of action and acquisition of resistance are related.

molecules leads to the logical conclusion that the number of tractable essential targets for malaria probably is around 100 to 200 (Hopkins and Groom, 2002; Landry and Gies, 2008). In an ideal scenario, where the available hit sets serve all possible druggable targets, the probability of detecting inhibitors with an assay using a genetically validated enzyme would be roughly 50%. Because most putative targets in *Plasmodium* lack stringent experimental validation, and the limited work with the hit sets shows that they can contain 10 or more chemical families hitting the same target (tested for DHODH, cytochrome bc1 complex, or others), the true probability is much lower, as discussed above. On the other hand, compounds capable of inhibiting certain targets, such as protein kinases (Doerig and Meijer, 2007), are overrepresented (Gamo et al., 2010), so screening such target classes is almost assured to identify biochemical inhibitors. Nevertheless, identifiying the targets acted upon by the current hit sets is a critical step toward enabling the next generation of drugs.

Finally, there is a need to adjust the research culture within malaria drug discovery to make up for the poor resourcing compared with other diseases. For example, in the United States, the National Institutes of Health expect to spend roughly $5.8 billion on cancer research and $2.1 billion on cardiovascular research, but only $134 million on malaria. We point to two key areas: (1) more rapid public disclosure and sharing of relevant data coming from malaria drug discovery efforts to enable communitywide learning and crowdsourcing (FitzGerald, 2011; Robertson and Mayr, 2011); and (2) a freely accessible collection of physical samples of well validated antimalarial hits and leads with annotated functional data to enable the study of basic malaria biology. Both of these efforts are under way, and although there is a general shift, there needs to be stronger adoption by the community (Bhardwaj et al., 2011; Lessl et al., 2011). Private-public partnerships have laid the groundwork for the discovery of new antimalarial chemical space and will continue to do so (Burrows et al., 2011; Nwaka and Ridley, 2003). In addition, data management interfaces are becoming available in the malaria drug discovery field to allow investigators to query and exchange information. Such examples are Hit Explorer Operating System, Collaborative Drug Discovery, ChEMBL (Gaulton et al., 2012), and PubChem. Public disclosure can stimulate scientific collaborations and, more importantly, can decrease the attrition of drug failures. The failure rate was estimated at 70% at the exploratory early discovery stage based on data from Medicines for Malaria Venture (MMV) (Nwaka and Ridley, 2003).

We believe that the next step to further drive malaria drug discovery will be the establishment of a well-validated collection of novel antimalarial compounds to be used community-wide for the study of mechanisms of action and identification of targets. One of the authors (T.T.D.) coined the term "malaria box" to describe such a collection of compounds. A preliminary malaria box has already been established by MMV, and distribution is beginning. We propose a global consolidation and expansion of the malaria box—to provide higher quality lead materials, stronger chemical diversity, and an open source data exchange component (Figure 4). The malaria box should be a physical depository of high-quality chemical materials of known concentration, purity, and identity. They should not be just "hits," but rather compounds that have been extensively characterized. For example, the collection optimally should be restricted to validated series with known SARs, containing a few analogs each, as well as inactive but structurally related control compounds. An ideal collection would include mostly compounds that are active in vivo. The malaria box should be actively managed to keep its size workable for most biology groups, meaning that it will contain no more than a few hundred diverse compounds.

The same distribution center for the malaria box should maintain full annotation of the compounds, including biological data, ideally confirmed by independent laboratories or orthogonal methods. Utilization of the malaria box by investigators should carry a requirement to make data available to the community through this mechanism. Ideally, each compound would have an associated synthetic route to enable crowd-driven SAR studies.

The malaria box should be available to investigators upon request and as freely accessible as reasonable. The responsible

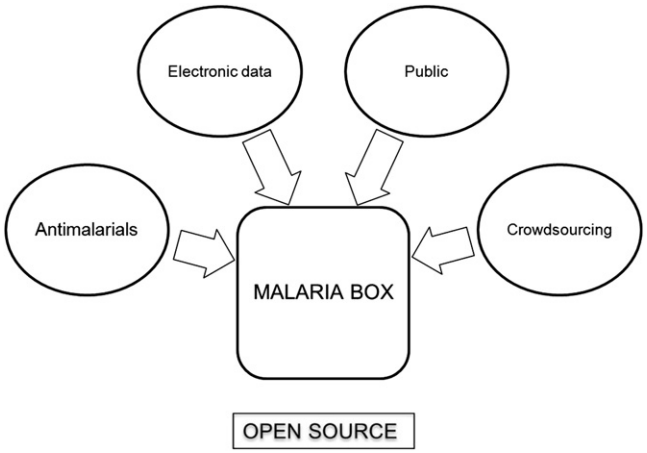


Figure 4. The Malaria Box Concept
The malaria box is a single clearinghouse that would warehouse data, models, and physical compounds and serve as a hub for collaboration. Community members would release constituents to the box, which would then be an open source to facilitate further work on parasitology, chemistry, pharmacology, and model building. To access the contents of the box, collaborators would need to agree to participate in the open source model.icipate in the open source - crowdsourceing contents of the box collaborators woudl k on parasitology, chem

agency holding the collection would not make scientific prejudgments on the work to be done, but rather would distribute reasonable quantities for cellular work. Intellectual property should be deliberately nullified, and there should be a requirement that studies be published if at all possible. While funding such an endeavor may seem daunting, a similar depository exists for malaria cell lines and reagents: the Malaria Research and Reference Reagent Resource Center. GSK has taken a similar approach to enabling kinase biology with its Published Kinase Inhibitor Set. These examples indicate it should be possible to convince all relevant stakeholders to participate and to identify sources to cover costs.

The malaria box will operate at two levels: first, as an open source providing data and chemicals and, second, as the platform where the scientific community would engage in discussions, contribute new data, and self-correct in a way similar to Wikipedia. Ultimately, each scientist around the world would be able to participate in guiding the drug discovery process. We anticipate, in the context of malaria, a disease with low economic reward for the pharmaceutical industry; such a concept will not only accelerate the discovery process but also will increase the success rate.

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