ANTIMALARIAL DRUG DISCOVERY: EFFICACY MODELS FOR COMPOUND SCREENING

David A. Fidock, Philip J. Rosenthal‡, Simon L. Croft§, Reto Brun‖ and Solomon Nwaka¶*

Increased efforts in antimalarial drug discovery are urgently needed. The goal must be to develop safe and affordable new drugs to counter the spread of malaria parasites that are resistant to existing agents. Drug efficacy, pharmacology and toxicity are important parameters in the selection of compounds for development, yet little attempt has been made to review and standardize antimalarial drug-efficacy screens. Here, we suggest different *in vitro* and *in vivo* screens for antimalarial drug discovery and recommend a streamlined process for evaluating new compounds on the path from drug discovery to development.

**Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, USA.*
‡Department of Medicine, Box 0811, University of California, San Francisco, California 94143, USA.
§Department of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London, WC1E 7HT, UK.
‖Department of Medical Parasitology and Infection Biology, Parasite Chemotherapy, Swiss Tropical Institute, CH-4002 Basel, Switzerland.
¶Medicines for Malaria Venture, PO Box 1826, 1215 Geneva 15, Switzerland.
Correspondence to D.A.F.
e-mail: dfidock@aecom.yu.edu
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Malaria remains one of the most important diseases of the developing world, killing 1–3 million people and causing disease in 300–500 million people annually. Most severe malaria is caused by the blood-borne APICOMPLEXAN parasite *Plasmodium falciparum* and occurs in children in sub-Saharan Africa. The two most widely used antimalarial drugs, chloroquine (CQ) and sulphadoxine-pyrimethamine (SP, commonly available as Fansidar; Roche), are failing at an accelerating rate in most malaria-endemic regions (FIG. 1), with consequent increases in malaria-related morbidity and mortality[1]. Clinical manifestations can include fever, chills, prostration and anaemia. Severe disease can include delirium, metabolic acidosis, cerebral malaria and multi-organ system failure, and coma and death may ensue. Blood-stage infection also generates sexual-stage parasites (GAMETOCYTES) that are infectious for mosquitoes, leading to fertilization and genetic recombination in the mosquito midgut. This is followed by production of haploid SPOROZOITE forms that invade the salivary glands and are subsequently transmitted back to humans.

To combat malaria, new drugs are desperately needed, but traditional mechanisms for drug development have provided few drugs to treat diseases of the developing world. In this challenging situation, there are some reasons for optimism. First, the determination of the genome sequence of *P. falciparum* offers a multitude of potential drug targets. Second, advances in malaria genetics offer improved means of characterizing potential targets. Third, the recent increased participation of pharmaceutical companies in the antimalarial drug discovery and development process offers hope for the development of new, affordable drugs. Indeed, an unprecedented number of malaria discovery and development projects are now underway (TABLE 1), involving many organizations including the Medicines for Malaria Venture (MMV, BOX 1). However, there is a lack of standardized systems for antimalarial drug-EFFICACY screens. This review discusses *in vitro* and *in vivo* efficacy screens to facilitate standardized evaluation of new compounds as they move along the path towards antimalarial drug development (FIG. 2).

Drug-resistant *P. falciparum* malaria

For several decades, the gold standard for the treatment of malaria was CQ, a 4-aminoquinoline that was previously characterized by its efficacy, low toxicity and affordability (less than US $0.2 for a three-day adult treatment course)[2]. CQ acts by binding to haem moieties produced from proteolytically processed haemoglobin inside infected erythrocytes, thereby interfering with haem detoxification[3,4]. Massive worldwide use of CQ, beginning in the late 1940s, was followed a decade later by the first reports of CQ-resistant strains of

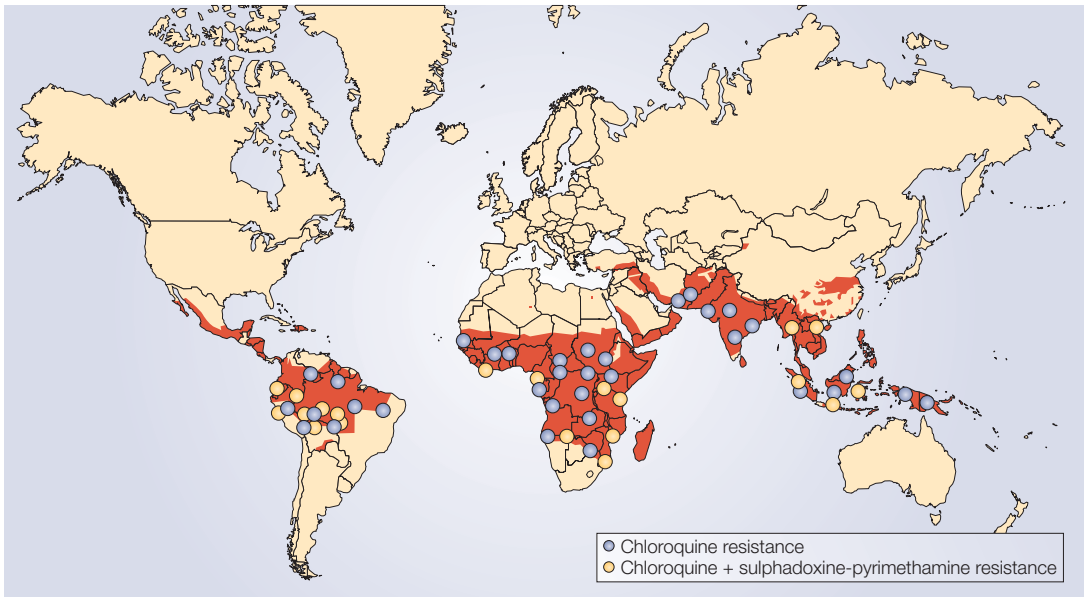

Figure 1 | **The global distribution of malaria, showing areas where *Plasmodium falciparum* resistance to the most commonly used antimalarial drugs, chloroquine and sulphadoxine-pyrimethamine, has been documented.** Resistance is now widely disseminated throughout malaria-endemic regions (coloured in red). Data are from the World Health Organization and are adapted from REF. 11 © Macmillan Magazine Ltd (2002).

APICOMPLEXA
Lower eukaryotic, obligate intracellular parasites characterized by the presence of apical organelles involved in host cell invasion. Includes parasitic protozoa responsible for malaria (*Plasmodium*), toxoplasmosis (*Toxoplasma*), babesiosis (*Babesia*) and coccidiosis (*Eimeria*).

GAMETOCYTES
The sexual haploid stages produced in the blood that are infectious for the mosquito vector. Once inside the mosquito midgut, gametocytes transform into male or female gametes that can undergo fusion, genetic recombination and meiosis.

SPOROZOITES
The haploid parasite forms that reside in the mosquito salivary glands and that are infectious for the human host, where they rapidly invade hepatocytes and transform into liver stage parasites.

EFFICACY
A quantitative index of drug action, in this case related to suppression of malarial infection either *in vitro* or *in vivo*.

ANTIFOLATES
Drugs that target the folate biosynthesis pathway. Antimalarial antifolate drugs target dihydrofolate reductase or dihydropteroate synthase.

INTERMITTENT PREVENTIVE TREATMENT
Entails the administration of full therapeutic doses of a drug at defined intervals. Envisaged to confer a degree of sustained prophylactic protection in the most vulnerable populations, that is, young children and pregnant women.

CHEMOPROPHYLAXIS
Drug treatment designed to prevent future occurrences of disease.

P. falciparum[5]. Today, CQ resistance has spread to the vast majority of malaria-endemic areas, rendering this drug increasingly ineffective (FIG. 1). However, in spite of the prevalence of CQ-resistant *P. falciparum*, this drug continues to be widely used. This is particularly problematic in sub-Saharan Africa, where resource limitations are profound and where highly immune populations often seem to respond — at least partially — to CQ therapy, and therefore somewhat mask the spread of resistance. CQ resistance almost certainly contributes to the recent finding that malaria-associated mortality is on the increase in Africa[6]. SP, a combination ANTIFOLATE drug, is the only other widely used inexpensive antimalarial, but resistance is also leading to unacceptable levels of therapeutic failure in many areas in Asia, South America and now Africa[7]. Despite some optimism about new drug development for the future, as noted above, the malaria-endemic regions of the world are faced with an unprecedented situation in which the only affordable treatment options are rapidly losing therapeutic efficacy.

The urgent need for new antimalarials

New antimalarial drugs must meet the requirements of rapid efficacy, minimal toxicity and low cost. Immediate prospects for drugs to replace CQ and SP include amodiaquine (a CQ-like quinoline) and chlorproguanil-dapsone (LapDap, another antifolate combination that inhibits the same enzymes as SP). These replacements will probably provide a few years of efficacy, particularly in Africa, but they already suffer from some cross-resistance with CQ and SP, which increases the likelihood that full-blown resistance to these drugs will emerge rapidly[8–10]. High on the list of mid-term replacements are artemisinin derivatives. However, these drugs have very short half-lives, which necessitates their use in combination with a longer-acting drug (see below). Clearly, additional new drugs are needed. If we are to avoid an ever-increasing toll of malaria on tropical areas, it is imperative to rapidly put into action strategic plans for the discovery and development of novel antimalarial compounds that are not encumbered by pre-existing mechanisms of drug resistance.

The desired profile for new drugs

Ideally, new drugs for uncomplicated *P. falciparum* malaria should be efficacious against drug-resistant strains, provide cure within a reasonable time (ideally three days or less) to ensure good compliance, be safe, be suitable for small children and pregnant women, have appropriate formulations for oral use and, above all, be affordable[11,12]. Drug development necessarily requires trade-offs among desired drug features, but for the treatment of malaria in the developing world the provision of affordable, orally active treatments that are safe for children is, for practical purposes, mandatory. Cost drives the choice of drugs in most developing countries, especially Africa, where most people must survive on less than US $15 per month.

Additional desirable uses include INTERMITTENT PREVENTIVE TREATMENT in pregnancy and childhood, treatment in refugee camps and other emergency situations, treatment of severe malaria, and the treatment of malaria caused by *P. vivax* (a rarely lethal, but nevertheless debilitating and widespread, agent of malaria). Of less importance to public health, but potentially offering profitability, new drugs should ideally also provide protection against malaria when used as CHEMOPROPHYLAXIS by advantaged non-immune populations travelling to endemic areas.

Table 1 | **Current malaria discovery and development projects and associated organizations**

Discovery projects	Development projects
Improved quinoline (MMV, GSK, U. Liverpool)	Rectal artesunate (TDR)
Farnesyl transferase inhibitors (MMV, BMS, U. Washington, Yale)	Chlorproguanil dapsone artesunate (MMV, TDR, GSK)
Manzamine derivatives (MMV, U. Mississippi)	Pyronaridine artesunate (MMV, TDR, Shin Poong)
Cysteine protease inhibitors (MMV, UCSF, GSK)	Amodiaquine artesunate (TDR, EU, DNDi)
Fatty acid biosynthesis inhibition (MMV, Texas A&M U., AECOM/HHMI, Jacobus)	Mefloquine artesunate (TDR, EU, DNDi)
Pyridones, peptide deformylase 1, FabI (MMV, GSK)	Artemisone (MMV, Bayer, U. Hong Kong)
New dicationic molecules (MMV, U. North Carolina, Immtech, STI)	Synthetic peroxide (MMV, Ranbaxy, U. Nebraska, STI, Monash U.)
Dihydrofolate reductase inhibition (MMV, BIOTEC Thailand)	Intravenous artesunate (MMV, WRAIR)
Novel tetracyclines (MMV, Paratek, UCSF)	8-aminoquinolines (MMV, U. Mississippi)
Choline uptake inhibition (U. Montpellier, Sanofi)	DB289 (MMV, Immtech, U. North Carolina)
Glyceraldehyde-3-phosphate dehydrogenase inhibition (MMV, STI, Roche)	Azithromycin chloroquine (Pfizer)
Synthetic endoperoxides (Johns Hopkins U., U. Laval, CDRI)	Fosmidomycin/clindamycin (Jomaa Pharma)
Chalcones (National U. Singapore, Lica Pharmaceuticals)	Short chain chloroquine (U. Tulane) Tafenoquine (GSK, WRAIR) Paediatric coartem (MMV, Novartis) Third-generation antifolates (Jacobus)

AECOM, Albert Einstein College of Medicine; BMS, Bristol-Myers Squibb; CDRI, Central Drug Research Institute, India; DNDi, Drugs for Neglected Diseases initiative; EU, European Union; GSK, GlaxoSmithKline; HHMI, Howard Hughes Medical Institute; MMV, Medicines for Malaria Venture; STI, Swiss Tropical Institute; TDR, UDNP/WorldBank/WHO Special Programme for Research and Training in Tropical Diseases; UCSF, U. California San Francisco; WRAIR, Walter Reed Army Institute of Research.

The need for drug combinations

There is a growing consensus that drug combinations are essential to the optimal control of malaria in developing countries[13]. Combinations potentially offer a number of important advantages over monotherapies. First, they should provide improved efficacy. Appropriately chosen combinations must be at least additive in POTENCY, and might provide synergistic activity. However, combination regimens that rely on synergy might not offer as much protection against the selection of resistance as expected, as resistance to either component of the combination could lead to a marked loss of efficacy. Indeed, the widely used synergistic combination SP acts almost as a single agent in this regard, with rapid selection of resistance[14], and similar concerns apply to the new atovaquone/proguanil (Malarone; GlaxoSmithKline) combination[15]. Second, drug combinations increase the likelihood that, in the setting of drug resistance, at least one agent will be clinically active. In East Africa, where resistance to both amodiaquine and SP is quite prevalent, the combination of these inexpensive agents still provides good antimalarial efficacy[16–18]. Third, and probably most important, drug combinations should reduce the selection of antimalarial drug resistance. Resistance has consistently been seen first in areas of relatively low ENDEMICITY, presumably due to the greater likelihood of high PARASITAEMIAS and symptoms leading to treatment in relatively non-immune individuals[19]. In Thailand, the use of an artesunate and mefloquine combination has been accompanied by excellent efficacy and a decrease in the prevalence of mefloquine resistance in infectious isolates[20]. It was also recently shown that SP selected for resistance-conferring mutations and subsequent treatment failure, but that SP combined with artesunate prevented the selection of SP-resistant parasites in subsequent infections[14]. Combinations might offer additional advantages if the separate agents are active against different parasite stages and if they provide the opportunity to decrease dosages of individual agents, thereby reducing cost and/or toxicity.

Ideally, combination regimens will incorporate two agents that are both new (so that parasites resistant to either agent are not already circulating), offer potent efficacy and preferably have similar PHARMACOKINETIC profiles (to limit the exposure of single agents to resistance pressures). Unfortunately, these are challenging requirements that are not met by any combination available at present. One current, widely advocated strategy is to combine artemisinins — which have no resistance problem but suffer as monotherapy from late RECRUDESCENCES due to their short half-lives[21] — with longer-acting agents. The hope is that the potent action of artemisinins will prevent significant selection of parasites resistant to the longer-acting component (for example, amodiaquine/artesunate[22], mefloquine/artesunate[20], chlorproguanil/dapsone/ artesunate[10] or lumefantrine/ artemether[23]). However, artemisinins are natural products that are difficult to synthesize and cannot be sold at cost for less than US $1–2 in curative combination regimens, a prohibitive price in most malaria-endemic regions. Indeed, even if widespread implementation of new artemisinin combination regimens is possible, additional new antimalarial drugs will be needed. Other regimens, offering combinations of inexpensive and available drugs (for example,

POTENCY
An expression of the activity of a compound, in terms of the concentration required to produce a desired effect (for example, 50% inhibition of parasite growth).

ENDEMICITY
An expression of the seasonality and degree of transmission in a malaria-afflicted region.

PARASITAEMIA
A quantitative measure of the percentage of erythrocytes that are parasitized.

PHARMACOKINETICS
The study of absorption, distribution, metabolism and elimination of drugs in a higher organism.

RECRUDESCENCE
The reappearance of parasites or symptoms, in the case of parasitological or clinical recrudescence, in the days following drug treatment. This is a result of incomplete clearance of the infection.

Box 1 | **Medicines for Malaria Venture (MMV)**

MMV, established in 1999 in Geneva, was the first public–private partnership of its kind to tackle a major global disease. This expert, not-for-profit organization brings together public, private and philanthropic partners to fund and manage the discovery, development and delivery of affordable new medicines for the treatment and prevention of malaria in disease-endemic countries. MMV aims to develop one new antimalarial drug every five years.

MMV solicits, selects and manages discovery and development research at different institutions. Projects are selected with the help of an Expert Scientific Advisory Committee on a competitive basis and are reviewed regularly. MMV optimizes the likelihood and cost effectiveness of developing new antimalarials on the basis of its global purview of research projects, ongoing dialogues with scientists from both public and private sectors, and the application of best practices in project management.

The product profiles for MMV for uncomplicated malaria include the following: efficacy against drug-resistant strains; cure within three days; low propensity to generate rapid resistance; safe in small children (younger than six months of age); safe in pregnancy; appropriate formulations and packaging; and low cost of goods. A number of further indications are also of interest: intermittent treatment in pregnancy (and in early infancy if possible); treatments suitable for emergency situations, including, for example, single-dose treatment for refugee camps; *Plasmodium vivax* malaria (including radical cure); severe malaria; and prophylaxis.

At present, MMV manages a portfolio of 21 projects at different stages of the drug research and development process. It hopes to register its first drug before 2010.

chlorproguanil/dapsone[10] or amodiaquine plus sulphadoxine/pyrimethamine[16]), might be appropriate stop-gap therapies, especially in Africa, where the need is greatest and resources most limiting.

Target selection and validation

Most antimalarial drugs that are now in use were not developed on the basis of rationally identified targets, but following the serendipitous identification of the antimalarial activity of natural products (for example, quinine and artemisinin), compounds chemically related to natural products (for example, CQ and artesunate), or compounds active against other infectious pathogens (for example, antifolates and tetracyclines). More recently, an improved understanding of the biochemistry of malaria parasites has identified many potential targets for new drugs and helped shed light on the mode(s) of action of older drugs (TABLE 2, FIG. 3).

Targets that are shared between the parasite and human host offer opportunities for chemotherapy if structural differences can be exploited. For example, the dihydrofolate reductase inhibitors pyrimethamine and proguanil are important components of antimalarial drugs, in large part because of their relative selectivity for the parasite enzyme. One advantage of targets that are also present in the host is that, in certain cases, the host target has already been considered as a therapeutic target for other disease indications. As a result, the cost of antimalarial drug discovery can be reduced if initial work, directed toward more profitable targets, has already been undertaken. As examples, antimalarial drug discovery efforts directed against parasite CYSTEINE PROTEASES[24] and PROTEIN FARNESYL TRANSFERASES[25] are benefiting from industry projects directed against inhibitors of the human cysteine protease cathepsin K as treatments for osteoporosis[26] and human farnesyl transferases as treatments for cancer[27].

Alternatively, targets can be selected from enzymes or pathways that are present in the malaria parasite but absent from humans. Here, the added difficulty of evaluating a target 'from scratch' is offset by the high degree of selectivity that should be provided by inhibitors of the parasite target. In some cases, parasite targets might be shared by other microbial organisms for which classes of inhibitors have already been generated and can be readily screened. One example is the use of prokaryotic protein-synthesis inhibitors, including tetracyclines and clindamycin, which were found to have antimalarial activity. These compounds presumably act selectively against malaria parasites because of their action against prokaryote-like plasmodial organelles known as apicoplasts, which seem to have cyanobacterial origins and are related to algal plastids[28]. Additional, recently identified potential selective targets for antimalarial drugs include components of type II fatty acid biosynthesis[29] and mevalonate-independent isoprenoid synthesis[30]. Both pathways are also targets for existing antibacterial compounds, providing initial leads for antimalarial drug discovery[31–33].

A 'reverse' drug discovery approach is to elucidate the nature of previously unidentified targets of existing antimalarial drugs as a basis for new drug discovery or development efforts. One germane example relates to CQ, which acts by interfering with the production of the malarial pigment HAEMOZOIN, allowing the intraparasitic build-up of toxic free haem[4,34]. This has defined inhibition of haemozoin formation as an attractive target for new antimalarial drugs[35].

In addition to benefiting from the entire *P. falciparum* genome sequence[36], investigators have access to a sophisticated database, PlasmoDB (see Further Information), which facilitates genome searches and analysis[37]. The genomes of a number of other plasmodial species have also recently been released and are accessible through this database. These genome sequences can dramatically accelerate the early steps of drug discovery by enabling the rapid identification of putative plasmodial targets that are homologous to validated target proteins from other systems. This, however, does not obviate the need for high-quality biological studies to validate drug targets.

Older approaches to target validation include the demonstration that an inhibitor has potent antimalarial activity. This approach is limited, however, by the fact that it is often difficult to determine whether an inhibitor of a particular plasmodial target is exerting its antimalarial activity specifically by the predicted mechanism of action. This problem is partially solved by the repeated demonstration of antiparasitic activity of different inhibitors of a particular target, by the identification of very potent (generally low-nanomolar) activity and, when possible, by the identification of biologically relevant defects caused by inhibitors (for example, the development of swollen FOOD VACUOLES in parasites treated with cysteine protease inhibitors[24]).

A fourth, unambiguous way of formally attributing the cellular effects of an inhibitor to the putative target is to measure its effects on a transgenic parasite in which the molecular target is resistant. If the inhibitor loses its

CYSTEINE PROTEASES
A class of parasite enzymes involved in crucial processes during parasite development.

PROTEIN FARNESYL TRANSFERASES
These mediate attachment of the prenyl groups farnesyl and geranylgeranyl to specific eukaryotic cell proteins.

HAEMOZOIN
An ordered crystalline assembly of β-haematin moieties produced following haemoglobin digestion in the parasite's food vacuole.

FOOD VACUOLE
An acidic organelle of erythrocytic parasites in which haemoglobin degradation takes place.

ORTHOLOGUE
A gene (or protein) with similar function to a gene (or protein) in a related species.

HAEMATOCRIT
The proportion of the volume of a sample of blood that is represented by red blood cells.

efficacy, this demonstrates that the mutated molecule is indeed the target. This was demonstrated for the *P. falciparum* dihydrofolate reductase gene, which in its mutated form conferred resistance to pyrimethamine in transgenic *P. falciparum*[38]. In a related complementation strategy, transgenic expression of human dihydrofolate reductase in *P. falciparum* conferred complete protection against WR99210, proving that this compound inhibited the parasite ORTHOLOGUE[39].

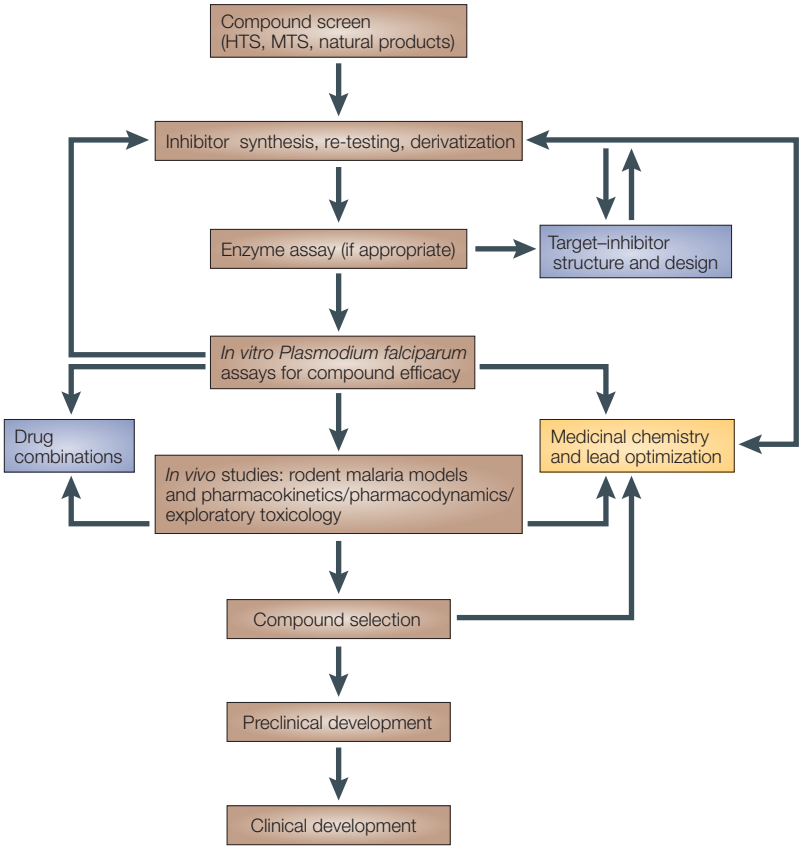

Figure 2 | **Example of a critical path for antimalarial drug discovery.** A discovery programme will typically include compound screening *in vitro* against *Plasmodium falciparum* and *in vivo* against rodent plasmodia. Cut-off values will vary depending on the family of compounds and programmatic decisions, and could be in the order of <1–5 μM for *in vitro* screens and <5–25 mg per kg for *in vivo* screens. Compounds might come from high-throughput or medium- throughput screens (HTS and MTS, respectively), natural product screens or more focused screens for antimalarial activity of known chemical families. Availability of purified, active target enzyme, when possible, enables biochemical screens to be implemented early in the critical path. *In vitro* assays include IC_{50} determinations against drug-resistant and drug-sensitive *P. falciparum* strains, and can be expanded to assessing the rapidity of action, determining the most susceptible stages, and screening for drug-resistant mutants. *In vivo* assays include the primary four-day test for suppression of parasitaemia in rodents. *In vitro* and *in vivo* studies are directed towards compound selection. Medicinal chemistry and lead optimization constitute an essential and iterative component of this part of the critical path. Secondary *in vivo* tests, not a requisite component of a critical path but useful for detailed compound evaluation, include dose-ranging, onset of activity and recrudescence, prophylaxis, and screening for drug resistance (FIG. 4). Detailed protocols for *in vitro* and *in vivo* evaluations of compound efficacy can be found in Further Information, Antimalarial drug discovery: efficacy models for compound screening. Activities off the critical path that can significantly strengthen the programme include parasite versus mammalian cell selectivity screens, biochemical assays, structural analysis and structure-based drug design, and screens of potential drug combinations. Additional screens can include transgenic rodent malaria models to assay the *P. falciparum* (or *P. vivax*) target in an *in vivo* setting, transgenic *P. falciparum* lines that overexpress the target or express the mammalian orthologue (to screen for compound specificity against the desired malarial enzyme), and screens to assess the frequency and biochemical impact of acquiring resistance (either in *Plasmodium* parasites or in bacterial or yeast model systems).

Newer technologies have greatly improved our ability to validate potential drug targets. In particular, methodologies have been developed to transfect *P. falciparum* with plasmids expressing either positive or negative selectable markers, and to thereby alter, replace or knock out genes of interest[38,40–42]. Another promising new avenue made possible by transfection is to express genes encoding drug targets from *P. falciparum* by allelic replacement into the rodent malaria parasite *P. berghei*, for which efficient transfection technology has been developed[43,44]. This enables evaluation of compound efficacy against the correct enzymatic target in an *in vivo* setting. An exciting extension of this approach is the introduction of a gene encoding a target in *P. vivax* — for which *in vitro* culture is unavailable and *in vivo* assays require monkeys — by allelic replacement into *P. falciparum* and *P. berghei*, thereby generating *in vitro* and *in vivo* screens against this understudied human pathogen.

Additional means of validating drug targets are made possible by new genomic and proteomic technologies. The former offer the opportunity to survey transcription across the plasmodial life cycle[45–47], and might provide insight into the transcriptional impact of target inhibition as well as highlight pathways of interest, particularly if functionally related genes share common transcriptional profiles[46,47]. Proteomic approaches, which require the accurate separation of thousands of proteins, are advancing rapidly for *P. falciparum*[48,49] and permit more direct investigations of the biochemical impact of established drugs and potential antimalarials. In a related 'functional proteomics' approach, the inhibition of proteins that have not been biochemically characterized can be surveyed using libraries of inhibitors and competitive binding assays[50]. Proteomic studies should help investigators to identify the mechanisms of action of older drugs, confirm suspected mechanisms for new compounds and suggest novel chemotherapeutic approaches.

In vitro screens of potential antimalarials

In vitro screens for compound activity, which constitute a key component of a critical path for an antimalarial drug discovery programme (FIG. 2), are based on the ability to culture *P. falciparum in vitro* in human erythrocytes. Typically, parasites are propagated in leukocyte-free erythrocytes at 2–5% HAEMATOCRIT at 37°C under reduced oxygen (typically 3–5% O_2, 5% CO_2, 90–92% N_2) in tissue culture (RPMI 1640) media containing either human serum or Albumax (a lipid-rich bovine serum albumin). Multiple drug-resistant and drug-sensitive isolates from around the world have now been culture-adapted and can be obtained from the Malaria Research and Reference Reagent Resource Center (see Further Information).

Details for one standardized protocol for culturing *P. falciparum* and assaying susceptibility to antimalarial compounds are available online (see Further Information, Antimalarial drug discovery: efficacy models for compound screening, which also contains a listing of commonly used *P. falciparum* lines). This protocol describes the measurement of the uptake of

Table 2 | **Targets for antimalarial chemotherapy**

Target location	Pathway/mechanism	Target molecule	Examples of therapies		References
			Existing therapies	New compounds	
Cytosol	Folate metabolism	Dihydrofolate reductase	Pyrimethamine, proguanil	Chlorproguanil	82,83
		Dihydropteroate synthase	Sulphadoxine, dapsone		
	Glycolysis	Thymidylate synthase		5-fluoroorotate	84
		Lactate dehydrogenase		Gossypol derivatives	85
		Peptide deformylase		Actinonin	86
	Protein synthesis	Heat-shock protein 90		Geldanamycin	87
	Glutathione metabolism	Glutathione reductase		Enzyme inhibitors	88
	Signal transduction	Protein kinases		Oxindole derivatives	89
	Unknown	Ca^{2+}-ATPase	Artemisinins		90
Parasite membrane	Phospholipid synthesis	Choline transporter		G25	71
	Membrane transport	Unique channels	Quinolines	Dinucleoside dimers	91
		Hexose transporter		Hexose derivatives	92
Food vacuole	Haem polymerization	Haemozoin	Chloroquine	New quinolines	93,94
	Haemoglobin hydrolysis	Plasmepsins		Protease inhibitors	95,96
		Falcipains		Protease inhibitors	97,98
	Free-radical generation	Unknown	Artemisinins	New peroxides	99,100
Mitochondrion	Electron transport	Cytochrome *c* oxidoreductase	Atovaquone		101
Apicoplast	Protein synthesis	Apicoplast ribosome	Tetracyclines, clindamycin		102
	DNA synthesis	DNA gyrase	Quinolones		
	Transcription	RNA polymerase	Rifampin		
	Type II fatty acid bio-synthesis	FabH		Thiolactomycin	29
		FabI/PfENR		Triclosan	32,33,103
	Isoprenoid synthesis	DOXP reductoisomerase		Fosmidomycin	30
	Protein farnesylation	Farnesyl transferase		Peptidomimetics	25,104
Extracellular	Erythrocyte invasion	Subtilisin serine proteases		Protease inhibitors	97,105

DOXP, 1-deoxy-D-zylulose 5-phosphate; PfENR, *Plasmodium falciparum* enoyl-ACP reductase.

^{3}H-hypoxanthine (which is taken up by the parasite for purine salvage and DNA synthesis) to determine the level of *P. falciparum* growth inhibition. In most applications, parasites are cultured in the presence of different concentrations of test compound in media containing reduced concentrations of hypoxanthine, after which ^{3}H-hypoxanthine is added for an additional incubation period before cell harvesting and measurement of radioactive counts. IC_{50} values can be determined by linear regression analyses on the linear segments of the dose–response curves.

Although ^{3}H-hypoxanthine incorporation is the most commonly used method to assay antimalarial activity *in vitro*, it is costly, radioactive and quite complex, and therefore problematic for resource-poor locations or for high-throughput screening (HTS, reviewed in REF. 51). A low-cost alternative for testing small numbers of compounds is to incubate parasites with test compounds (typically for 48 or 72 hours), and then to compare parasitaemias of treated and control parasites by counting GIEMSA-stained parasites by light microscopy. Another established, but less standardized, assay involves the colorimetric detection of lactate dehydrogenase[52]. Flow cytometry has also been used to test candidate antimalarial compounds, and takes advantage of the fact that human erythrocytes lack DNA. In the simplest use of this technology, parasites are fixed after the appropriate period of incubation with test compounds, then either the parasitized cells are stained with hydroethidine (which is metabolized to ethidium[53]) or the parasite nuclei are stained with DAPI (4′,6-diamidino-2-phenylindole). Counts of treated and control cultures are then obtained by flow cytometry. Appropriate gating can also allow one to distinguish different parasite erythrocytic stages. This relatively simple assay provides quite high throughput and has replaced older methods at some centres, but requires expensive equipment.

Compounds that meet an acceptable cut-off for *in vitro* activity (for example, $IC_{50} \leq 1\ \mu M$) can then be tested for activity against a range of geographically distinct *P. falciparum* lines of differing drug-resistance phenotypes (see Further Information, Antimalarial drug discovery: efficacy models for compound screening) to determine whether resistance to existing antimalarial drugs reduces parasite sensitivity to the compounds under evaluation.

Different research groups have incorporated a variety of modifications of the basic *in vitro* screens listed above, which can influence the measurement of drug activity levels, as follows.

Unsynchronized versus synchronized cultures. For preliminary screening of diverse compounds, the less demanding (but less sensitive) method of using unsynchronized cultures is widely used. SYNCHRONIZED CULTURES are used when comparing a series of compounds, establishing rank order of activities and determining potency against different parasite stages[54].

Duration of incubation. Most assays incorporate incubation with test compounds for 48 hours, the duration of one erythrocytic cycle. Incubations can also be extended to 72 hours or longer. This can generate more reproducible IC_{50} values when working with unsynchronized cultures and is important when testing slower-acting compounds such as antibiotics.

IC_{50}
The drug concentration that produces a 50% inhibition of *P. falciparum* growth *in vitro*. This is frequently determined by calculating the concentration that produces a 50% reduction in uptake of ^{3}H-hypoxanthine.

GIEMSA
A nucleic acid stain used to visually distinguish *Plasmodium* parasites from the surrounding cells.

SYNCHRONIZED CULTURES
Cultures of parasites at the same or similar stage of intracellular development.

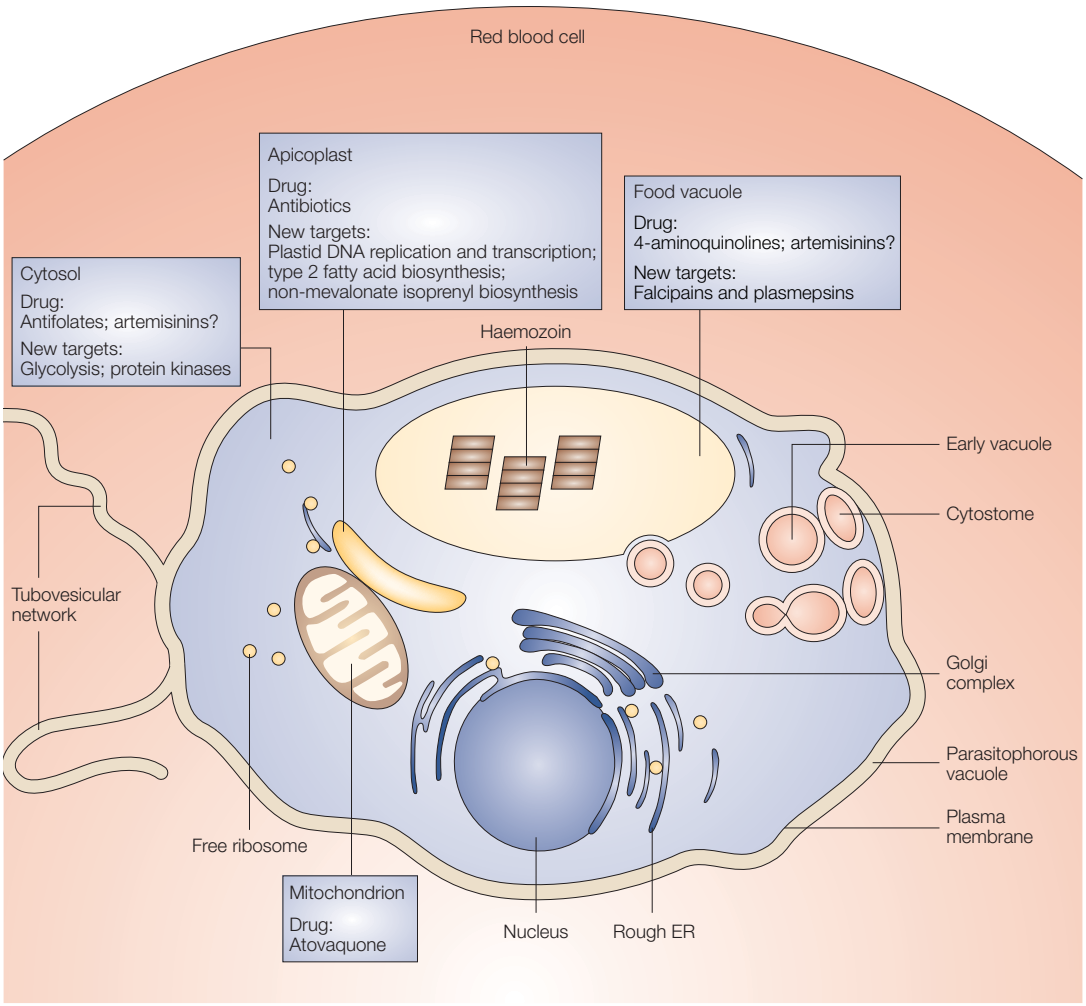

Figure 3 | **Representation of an intra-erythrocytic *Plasmodium falciparum* trophozoite, highlighting key parasite intracellular compartments and the site of action of some of the major classes of antimalarial drugs.** Efforts are ongoing to develop new drugs against an even wider range of subcellular compartments and parasite targets (TABLE 2).

Human serum versus Albumax. In recent years many laboratories have replaced 10% human serum with the serum substitute Albumax. The latter has both clear advantages (for example, reduced batch-to-batch variation) and disadvantages (for example, a higher level of protein binding has been reported with Albumax compared with serum, such that activities of some compounds might differ depending on culture conditions[55]).

Initial percentage of parasitaemia. The number of parasites present at the beginning of the drug assay can have a significant effect on *in vitro* activity (known as the INOCULUM effect)[56].

Numerous variations on these standard assays can be used to gain further insight into compound efficacy. For example, compounds can be added to synchronized cultures at different stages of development to assess which stages are the most susceptible to drug action, and inhibitors can be added for different lengths of time before removal in order to determine the minimum time of exposure needed to achieve parasite killing.

To assess the effects of combining compounds, ISOBOLOGRAM analysis[57,58] can be performed to assess whether two compounds are additive, synergistic or ANTAGONISTIC (see BOX 2, which discusses *in vitro* drug interactions as well as *in vivo* drug combinations). This is conducted using standard dose–response assays over a range of individual drug concentrations, using either a checkerboard technique[59] or fixed-ratio methods[58]. This *in vitro* analysis has been useful in identifying clinical combinations — for example, atovaquone and proguanil[59] — as well as in determining the potential of 'low activity' compounds, such as azithromycin[58].

With the notable exception of the artemisinin family of drugs, almost all antimalarial drugs developed to date are active only against asexual stage parasites and therefore do not prevent transmission of the pathogen[60]. Transmission-blocking activity is nevertheless a desirable property for any new antimalarial drug. To test for this, *P. falciparum* gametocytes can be produced *in vitro*[61] and compounds added to assess the impact on gametocyte development. In addition, transmission-blocking

INOCULUM
A substance or organism that is introduced into surroundings suited to cell growth. In this case this refers to introduction of a defined number of parasitized erythrocytes into *in vitro* culture or a susceptible rodent.

ISOBOLOGRAM
A graphical representation of growth inhibition data for two compounds, plotted as fractional IC_{50} values on an X–Y axis, permitting determination of whether the two compounds are synergistic, additive or antagonistic.

ANTAGONISM
An interaction between agents in which one partially or completely inhibits the effect of the other.

Box 2 | **Drug-interaction studies**

In vitro drug interactions: Interactions of two antimalarials are investigated *in vitro* using standard dose–response assays over a range of individual drug concentrations[57,58]. These assays use either a checkerboard technique[59] or fixed-ratio methods of IC_{50} values[58].

Fractional inhibitory concentrations (FIC) are calculated for each drug on the basis of equation 1, in which $IC_{50}\,A_{(B)}$ is the 50% inhibitory concentration of drug A in the presence of drug B:

$$\text{FIC Drug A} = \frac{IC_{50}\,A_{(B)}}{IC_{50}\,A} \qquad (1)$$

Isobologram analysis, based on calculation of the sum of FICs (∑FICs)[57], gives an indication of whether the interaction is antagonistic, additive or synergistic. Although there are no defined breakpoints for antimalarial combinations, a recent study defined antagonism as ∑FICs >2.0 and synergism as ∑FICs <0.5 (REF. 106). Analysis of interactions based on IC_{50} and IC_{90} values can produce different results[106], and variation between studies is expected.

In assessing the clinical potential of a combination, studies should include a comparison of interactions involving the parent drug or the metabolite (for example, artemisinin versus dihydroartemisinin, or amodiaquine versus desethyl-amodiaquine), as well as studies on a panel of *P. falciparum* isolates with known patterns of drug resistance or sensitivity to established drugs[58].

Although limited in predicting *in vivo* events, data can be analysed in relation to potential tissue concentrations[106].

In vivo drug combinations: *In vivo* assays to evaluate drug combinations are more complex than *in vitro* studies because of the need to consider additional parameters, including the route of administration, number of applications and the half-life of the drug. Isobologram analysis can be performed by plotting fractions of the ED_{90} for the single drugs[107,108]; however, it should be noted that accurate ED_{90} values might be difficult to achieve for low-activity compounds (including antibiotics and sulphonamides). Clearer analysis can be obtained using killing curves that demonstrate a shift in ED_{50} or ED_{90} values over a range of doses[44].

assays can be conducted by feeding starved female *Anopheles* mosquitoes a blood meal containing infectious gametocytes via an artificial-membrane feeding apparatus. Mosquitoes can then be maintained for one week, after which the midguts are dissected. The number of midgut oocysts resulting from treated and control parasites are then compared by light microscopy[62].

In vivo screening of antimalarial compounds

Plasmodium species that cause human disease are essentially unable to infect non-primate animal models (with the exception of a complex immunocompromised mouse model that has been developed to sustain *P. falciparum*-parasitized human erythrocytes *in vivo*[63]). So, *in vivo* evaluation of antimalarial compounds typically begins with the use of rodent malaria parasites. Of these, *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei* (see Further Information, Antimalarial drug discovery: efficacy models for compound screening) have been used extensively in drug discovery and early development[64]. Rodent models have been validated through the identification of several antimalarials — for example, mefloquine, halofantrine and more recently artemisinin derivatives[65–68]. In view of their proven use in the prediction of treatment outcomes for human infections, these models remain a standard part of the drug discovery and development pathway. Individual species and strains have been well characterized, including duration of cycle, time of SCHIZOGONY, synchronicity, drug sensitivity and course of infection in genetically defined mouse strains (see below; REFS 69,70).

The most widely used initial test, which uses *P. berghei* or less frequently *P. chabaudi*, is a four-day suppressive test[44], in which the efficacy of four daily doses of compounds is measured by comparison of blood parasitaemia (on day four after infection) and mouse survival time in treated and untreated mice (a detailed protocol is provided in the online document 'Antimalarial drug discovery: efficacy models for compound screening'; see Further Information). Rodent infection is typically initiated by needle passage from an infected to a naive rodent via the intraperitoneal or preferably the intravenous route, often using a small inoculum (typically in the range of 10^6–10^7 infected erythrocytes). Compounds can be administered by several routes, including intraperitoneal, intravenous, subcutaneous or oral. CQ is often used as the reference drug and typically has an ED_{50} value against *P. berghei* (ANKA strain) of 1.5–1.8 mg per kg when administered subcutaneously or orally.

Compounds identified as being active in four-day assays can subsequently be progressed through several secondary tests (FIG. 4), as follows. In the 'dose ranging, full four-day test', compounds are tested at a minimum of four different doses, by subcutaneous and/or oral routes, to determine ED_{50} and ED_{90} values. This test also provides useful information on relative potency and oral BIOAVAILABILITY. In the 'onset/recrudescence' test, mice are administered a single dose (by the subcutaneous or oral route) on day 3 post-infection and followed daily to monitor parasitaemia. Results are expressed as the rapidity of onset of activity (disappearance of parasitaemia), time to onset of recrudescence, increase of parasitaemia and survival in number of days. Compounds can also be tested for prophylactic activity by administering the compound prior to infection, followed by daily examination of smears. Additional screens have been developed to assess cross-resistance and the potential for *in vivo* selection of resistant parasites (see Further Information, Antimalarial drug discovery: efficacy models for compound screening).

When using rodent models, several key variables need to be considered during experimental design and interpretation. Foremost is the choice of rodent malaria species and mouse strains. As alluded to before, rodent plasmodia can differ significantly in their degree of infection, lethality and synchronicity, which can dramatically affect the results. These factors also broaden the range of possible assays for compound evaluation. For example, *P. chabaudi* and *P. vinckei* generate a high parasitaemia and produce synchronous infections, enabling studies on parasite stage specificity. Rodent malaria species can also differ significantly in sensitivity to certain classes of compounds. For example, *P. chabaudi* and *P. vinckei* are more sensitive than *P. berghei* to iron chelators and lipid biosynthesis inhibitors[44,71]. The course of infection can also vary enormously depending on the mouse strain, and models exist that are amenable to studies on chronic infection or SEQUESTRATION[70]. For example, the *P. chabaudi* AS strain in CBA mice produces

SCHIZOGONY
The intracellular process whereby multinucleated *Plasmodium* parasites differentiate to form multiple infectious forms (merozoites), which then egress from the infected cell in order to invade uninfected erythrocytes.

ED_{50}
The drug concentration that produces a 50% reduction in parasitaemia *in vivo*, typically in a rodent malaria model.

BIOAVAILABILITY
The degree to which a drug is available to the body. This is influenced by how much the substance is absorbed and circulated.

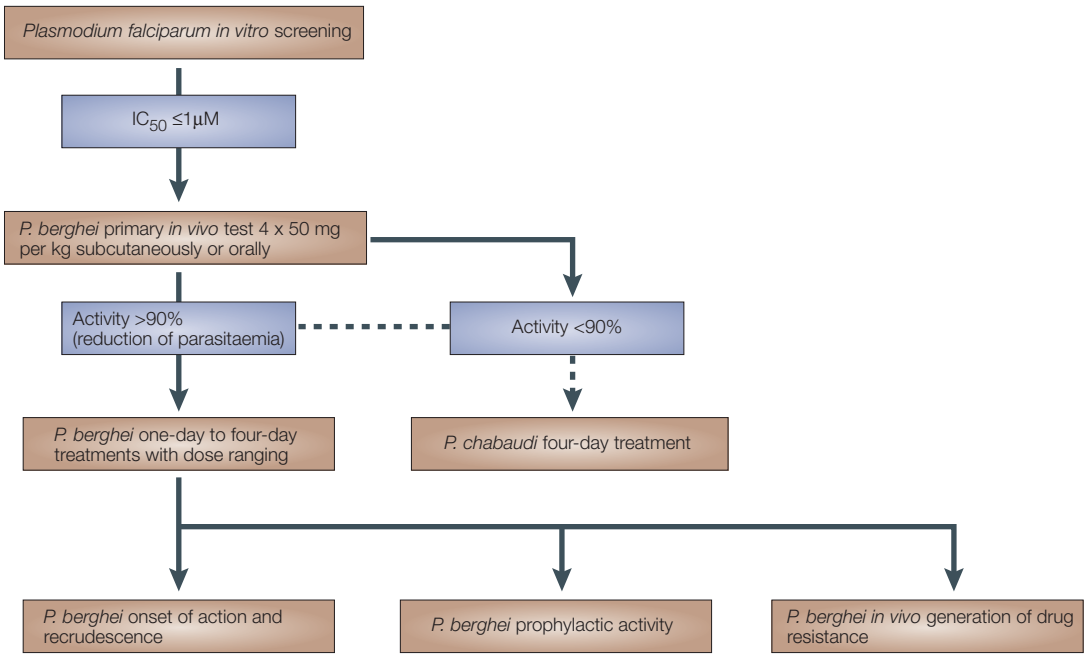

Figure 4 | **Flow chart of one scenario for *in vivo* screening for antimalarial activity in rodent malaria models.** Most tests can be conducted with five mice per group. Protocols for antimalarial efficacy testing *in vivo* and a description of the different rodent malaria models can be found in Further Information, Antimalarial drug discovery: efficacy models for compound screening.

a chronic infection with a defined immune response that can be used in studies of immunomodulators. It is important to note that the drug sensitivity of a given rodent malaria species does not always mirror that of *P. falciparum* and can limit the types of investigations that can be performed. For example, cysteine proteases in rodent plasmodia show subtle active site differences to those in *P. falciparum*, leading to questions about the use of these models in LEAD OPTIMIZATION[72]. Also, the frequently used *P. yoelii* 17X strain is intrinsically partially resistant to CQ and is therefore a poor model for studying acquisition of *P. falciparum* CQ resistance.

Primate models have also had an important role in preclinical development, by providing a final confirmation of the choice of a drug candidate. Infection with certain strains of *P. falciparum* has been well characterized in both *Aotus* and *Saimiri* monkeys[73]. Primate models provide a clearer prediction of human efficacy and pharmacokinetics than rodent models, providing a logical transition to clinical studies[71].

From antimalarial drug discovery to development

The challenge of any drug discovery effort is to identify and develop compounds with properties that are predictive of good efficacy and safety in humans. For malaria, additional hurdles that need to be overcome include the selection of compounds that are reasonably cheap to produce and that are effective against drug-resistant strains. The potential of a candidate compound to be used by the most vulnerable populations (young children and pregnant women) in disease-endemic countries also needs to be assessed as the candidate moves into development. In addition to being effective against uncomplicated *P. falciparum* malaria, compounds that can be easily formulated for severe disease (that is, amenable to parenteral administration) bring an added benefit, as do compounds that work against *P. vivax*[11,12]. Their ultimate success depends on the intrinsic qualities of the molecule, as well as how the development of the drug is planned and implemented.

The different drug R&D stages include target selection and validation (as already discussed), LEAD IDENTIFICATION and optimization involving iterative cycles of chemistry and biology, and compound selection and preclinical development. This is followed by clinical development and registration. The discovery platform is high risk and needs continuous support to maximize the chances that one or more compound(s) moves into development. Having an excellent interface with basic research is crucial for moving forward from discovery lead identification, as this transition requires appropriate expertise and resources to identify and progress leads into drug candidates. This includes having access to reasonable compound collections, HTS techniques, and medicinal chemistry and pharmaceutical development expertise. The limiting factors in this transition are target validation and the availability of the necessary resources and expertise for a full discovery programme. An important potential application of chemical libraries and HTS is in using chemical leads to aid target validation, potentially integrating proteomic or genomic approaches. In antimalarial drug discovery terms, a viable drug target is one that has a specific inhibitor that kills the parasite. The inhibitor should possess the right pharmacological and toxicological characteristics to enable successful development. The management aspect of the entire process is equally important for success.

SEQUESTRATION
The ability of *P. falciparum* to sequester in capillary beds as a result of binding of the parasitized erythrocyte to endothelial cell surface receptors. This binding is mediated by parasite-encoded variant antigens presented at the erythrocyte surface.

LEAD OPTIMIZATION
The process of chemically optimizing a compound to improve antimicrobial activity and pharmacological properties.

LEAD IDENTIFICATION
The process of identifying an acceptable lead compound with potent *in vitro* and *in vivo* activity.

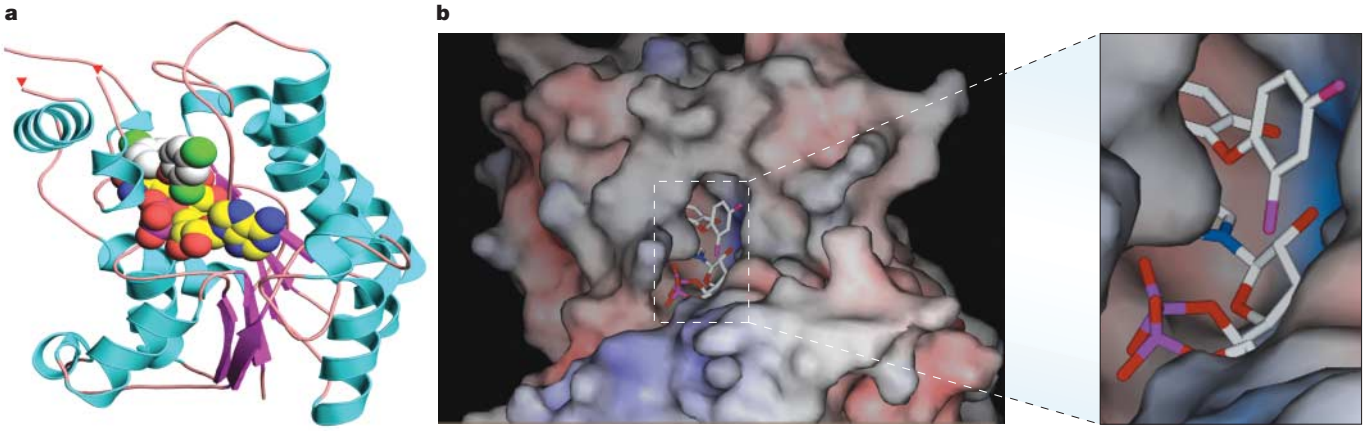

Figure 5 | **Crystal structure of *Plasmodium falciparum* enoyl-ACP reductase (PfENR) complexed with NAD⁺ cofactor and the inhibitor triclosan. a** | The ribbon colours correspond to the PfENR secondary structural elements (pink denotes coils, cyan indicates helices, and β-strands are purple). The chain breaks (red triangles) are due to the low-complexity region in the PfENR substrate-binding loop that was not resolved in the crystal structures. Triclosan and the NAD⁺ cofactor are depicted as space-fill spheres, and coloured by individual elements (nitrogen in blue, oxygen in red, phosphate in purple and chlorine in green). Triclosan and NAD⁺ are distinguishable from each other by the colour of the carbon spheres (triclosan has white carbons, and NAD⁺ has yellow carbons). **b** | Molecular surface coloured according to electrostatic potential of the active site portal of PfENR with bound triclosan. This close-up view is in a similar orientation to **a** and shows triclosan binding, with NAD⁺ lying below. Carbons are represented in white, chlorines in pink, oxygens in red and phosphates in blue. This structure-based analysis of PfENR–inhibitor complexes provides a powerful route by which increasingly potent compounds are being sought to inhibit this key malarial enzyme[33,77], which is a proven target in several organisms including *Mycobacterium tuberculosis*.

Lead identification can be driven by both biology and chemistry, or purely by chemistry through the modification of existing classes of antimalarials to achieve improved potency, safety or ease of synthesis. For example, the development of numerous aminoquinoline and artemisinin antimalarials was driven fully by chemistry, but several newer projects are based on identifying the biological relevance of specific targets (TABLE 2). A project driven by biology and chemistry requires enzyme and/or whole-cell assays to support medium- or high-throughput screens. Promising hits from a screening campaign can be developed into a lead series following a comprehensive assessment of chemical integrity, synthetic robustness, functional behaviour, and structure–activity relationships, as well as bio-physicochemical and absorption, distribution, metabolism and excretion (ADME) properties. Once lead series with some desirable profiles are identified, the compounds can progress to lead optimization[12,74–76], entailing structural modifications with the goal of achieving optimal efficacy and pharmacokinetic/PHARMACODYNAMIC properties. It must be highlighted that structural biology is an important part of modern drug discovery efforts as it helps to facilitate rational drug design. Several antimalarial drug discovery projects, including the dihydrofolate reductase and enoyl ACP reductase inhibition projects, are benefiting from the power of structure-based drug design to improve inhibitor potency[33,77,78] (FIG. 5).

The development of information technology and sophisticated databases now also enables chemists to identify molecular groups that are likely to be metabolically labile or associated with adverse toxicological properties[79,80]. Exploratory toxicity studies clear the way for extensive preclinical toxicology studies subsequent to entry into humans. Lead optimization is labour intensive and perhaps the most important phase in drug discovery, as many potential drugs fail at this stage. Established milestones and deliverables for a lead optimization project ensure proper management of the crucial discovery-to-development transition. This requires that candidate progression criteria be established. These criteria have been customized for antimalarial drugs by MMV (BOX 2) and pertain to *in vitro* and *in vivo* efficacy, chemistry, exploratory pharmacokinetics, ADME and toxicology, in comparison to standard agents[12]. Compounds that are ultimately selected for development also need to be easy to manufacture, stable, readily formulated, bioavailable, have an acceptable half-life and not show any overt toxicity. Development can proceed with parallel process chemistry to assess the cost of goods, compound stability and safety under scale-up, and Good Manufacturing Practice conditions. The compound undergoes preclinical animal safety studies under Good Laboratory Practice conditions, followed by entry into human trials (Phase I–III clinical trials), and then regulatory submission and approval[12,81].

Most of the projects in the current pipeline of antimalarial drug discovery and development have been initiated and managed through partnerships, and MMV has played a leading role in this process (TABLE 1). This R&D pipeline is encouraging, but it must be emphasized that drug development has a high failure rate and already MMV has terminated some projects in its portfolio. Therefore it is essential to continue to identify and fund new viable programmes to ensure sustainability and to prevent future gaps emerging in antimalarial

PHARMACODYNAMICS
The study of the mechanisms of actions of a drug, and the relationship between how much drug is in the body and its effects.

drug R&D. Most of the projects in the development stage are artemisinin combinations that in some cases incorporate improvements on existing drugs aimed at overcoming the current problem of resistance. Some of these development projects will result in new drugs in the short term; however, they will not offer a lasting solution. Longer-term innovative and more challenging discovery projects are required to ensure sustainability and affordability. These projects will increasingly require focused medicinal chemistry and screening (efficacy, pharmacokinetics/ADME and toxicity) strategies for them to be effectively moved into development.

Future perspectives

Efforts to discover and develop new antimalarial drugs have increased dramatically in recent years, both as a result of the recognition of the global importance of fighting malaria, and the dedicated public–private partnership strategy to discover, develop and deliver new drugs. Increased funding from the public sector and philanthropic agencies has fuelled strong academic engagement in drug discovery, and increased partnerships with pharmaceutical companies mean that sets of complementary expertise are becoming available to drive and sustain the development of new drugs for diseases of low commercial return. Yet at the same time malaria mortality is on the increase — due in large part to the increasing ineffectiveness of the two first-line drugs, CQ and SP, and the current lack of affordable alternatives. Current enthusiasm for combining scientific innovation with expertise in the drug discovery and development process offers hope that a concerted effort can allow us to gain the upper hand in treating this disease. Time is a cruel judge, and we cannot afford to miss the current window of opportunity to develop new, affordable and effective antimalarial drugs.

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Competing interests statement

The authors declare that they have no competing financial interests.

Online links

FURTHER INFORMATION

Antimalarial drug discovery: efficacy models for compound screening: http://www.mmv.org/FilesUpld/164.pdf
Malaria Foundation International: http://www.malaria.org.
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Plasmo DB: http://PlasmoDB.org.
World Health Organization malaria page: http://www.who.int/health-topics/malaria.htm.
Access to this interactive links box is free online.

Antimalarial drug discovery: efficacy models for compound screening (supplementary document)

David A. Fidock[*], *Philip J. Rosenthal*[¶], *Simon L. Croft*[‡], *Reto Brun*[§] *and Solomon Nwaka*[#]

[*] **Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York, 10461, USA. E-mail: dfidock@aecom.yu.edu**

[¶] **Department of Medicine, Box 0811, University of California, San Francisco, California, 94143, USA. E-mail: rosnthl@itsa.ucsf.edu.**

[‡] **Department of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London, WC1E 7HT, UK. E-mail: simon.croft@lshtm.ac.uk**

[§] **Department of Medical Parasitology and Infection Biology, Parasite Chemotherapy, Swiss Tropical Institute, CH-4002 Basel, Switzerland. E-mail: reto.brun@unibas.ch.**

[#] **Medicines for Malaria Venture. International Center Cointrin, Block G, 3rd Floor. 20, route de Pré-Bois, PO Box 1826, 1215 Geneva 15, Switzerland. E-mail: nwakas@mmv.org**

1. A Protocol for Antimalarial Efficacy Testing *in vitro*

This protocol for assessing compound efficacy against *Plasmodium falciparum in vitro* uses [^{3}H]-hypoxanthine as a marker for inhibition of parasite growth [1,2]. Many alternative protocols exist, including ones based on microscopic detection of Giemsa-stained slides, assays based on production of parasite lactate dehydrogenase, and the use of flow cytometry [3].

Parasite strain: Several well-characterized strains (see Supplemental Table 1) are available, either from academic laboratories or from www.malaria.mr4.org (reagents available to registered users). One recommendation would be to test activity against a drug-sensitive line such as 3D7 (West Africa), D6 (Sierra Leone) or D10 (Papua New Guinea), as well as a drug-resistant line such as W2 or Dd2 (both from Indochina), FCB (SE Asia), 7G8 (Brazil) or K1 (Thailand).

Malaria Culture Media: RPMI 1640 medium containing L-glutamine (Catalog number 31800, Invitrogen), 50 mg/liter hypoxanthine, 25mM HEPES, 10 μg/ml gentamicin, 0.225% $NaHCO_3$ and either 10% human serum or 0.5% Albumax I or II (purified lipid-rich bovine serum albumin, Invitrogen). Medium is typically adjusted to a pH of 7.3 to 7.4.

Low Hypoxanthine Media: Same as above except that the hypoxanthine concentration is reduced to 2.5 mg/liter.

Serum (as opposed to Albumax) is important for culturing fresh isolates, and for maintaining properties of cytoadherence and gametocyte production (the latter is required for transmission back to mosquitoes). Some strains also prefer to propagate in serum. Batch-to-batch variation is nonetheless a problem, with occasional batches not supporting robust parasite growth. Accordingly, many laboratory lines have been adapted to propagate in the presence of Albumax, which typically gives more consistent growth between batches (variation was a problem in the past, but now appears to have been resolved). Albumax appears to reduce both the rate at which erythrocytes deteriorate *in vitro*, and pH drift when cultures are exposed to ambient air (i.e. during tissue culture hood manipulations).

Preparation of host erythrocytes: Human erythrocytes for parasite culture are prepared by drawing blood into heparin-treated tubes and washing several times in RPMI 1640 medium to separate the erythrocytes from the plasma and buffy coat. Separation can be achieved by centrifuging the blood at 500 $\times g$ for 5 minutes in a swing-out rotor. Leukocyte-free erythrocytes are typically stored at 50% hematocrit (i.e. 1 volume of malaria culture media for 1 volume of packed erythrocytes, corresponding to approximately 5×10^9 cells/ml).

Parasite Culture Conditions: *P. falciparum* asexual blood stage parasites are propagated at 37°C in malaria culture media at 3-5% hematocrit in a reduced oxygen environment (e.g. a custom mixture of 5% CO_2, 5% O_2 and 90% N_2). Lines can be conveniently cultured in 6-24 well tissue culture plates in a modular chamber (Billups-Rothenberg,Del Mar, CA, www.brincubator.com),with plates containing sterile water on the bottom to increase humidity and minimize desiccation. These chambers can be suffused with the low O_2 gas and maintained at 37°C in an incubator designed to minimize temperature fluctuations. Parasites can also be cultured in flasks that are individually gassed, or alternatively placed in flasks that permit gas exchange through the cap (in which case the incubator needs to be continuously infused with a low O_2 gas mixture). Depending on the line, parasites typically propagate 3-8 fold every 48 hr, thus care must be taken to avoid parasite cultures attaining too high a parasitemia (i.e. percentage of erythrocytes that are parasitized) for healthy growth. Most lines grow optimally at 0.5 – 4% parasitemia. Parasites are most suitable for drug assays when they are 2-5% parasitemia, and mostly ring stages with few or no gametocytes.

Compounds: Compounds can often be dissolved in 100% dimethyl sulfoxide (DMSO) and stored at –80°C (or –20°C). Particle size of insoluble compounds can be reduced by ball-milling or sonication. For the drug assays, serial drug dilutions (either 2× or customized) are made in low hypoxanthine medium (see above) and added to 96-well culture plates at 100 µl per well. Drugs are added to columns 3-12 (test samples), with columns 1 and 2 reserved for wells with low hypoxanthine medium without compound. All drugs are typically tested in duplicate or triplicate for each parasite line. Once completed, plates are placed into their own modular chamber, gassed and placed at 37°C. These plates should be set up no more than a few hours prior to addition of the parasites.

Drug Assay Conditions: Parasites are diluted to a 2× stock consisting of 0.6% to 0.9% parasitemia (depending on the growth rate of the line) and 3.2% hematocrit in low hypoxanthine medium, and 100 µl are added per well already containing 100 µl of low hypoxanthine medium with or without compound (present at different concentrations). Plates are then incubated in a gassed modular chamber at 37°C for 48 hr (some labs prefer 24 hr). After this time, 100 µl of culture supernatant from each well is removed and replaced with 100 µl of low hypoxanthine medium containing a final concentration of 7.5 µCi/ml of [^{3}H]-hypoxanthine (1 mCi/ml stock, Amersham Biosciences). After a further 24 hr, the plates are placed at–80°C for at least 1 hr to freeze the cells. Plates are then thawed and the cells are harvested onto glass fiber filters (Wallac, Turku, Finland). Filters are dried for 30 minutes at 80°C, placed in sample bags (Wallac), and immersed in scintillation fluid (Ecoscint A; National Diagnostic, Atlanta, GA). Radioactive emissions are counted in a 1205 Betaplate reader (Wallac). Mean counts per minute (cpm) are generally in the range 20,000-60,000, with an acceptable minimum of 10,000. Percentage reduction in [^{3}H]-hypoxanthine uptake is equal to 100× ((geometric mean cpm of no drug samples) – (mean cpm of test

samples)) / (geometric mean cpm of no drug samples). Percentage reductions are used to plot percentage inhibition of growth as a function of drug concentrations. IC_{50} values are determined by linear regression analyses on the linear segments of the curves (IC_{90} values can also be determined by curve-fitting and can provide an useful measure of variation between experiments). Assays are typically repeated on two or three separate occasions. Within each experiment, standard deviations are typically less than 10% of the mean. Differences in parasite stages of development can lead to up to two-fold shifts in the IC_{50} values between experiments; however, these differences rarely affect the overall relationships between the parasite lines in terms of their differences in drug response [4-11].

Supplemental Table 1. Standard *Plasmodium falciparum* strains.

Name	Clone	Origin	Resistant to	Multiplication Rate	Reference
Dd2	Yes (from WR'82)	Indochina	CQ, QN, PYR, SDX	5-6	[3]
W2	Yes (from Indochina-3)	Indochina	CQ, QN, PYR, SDX	5-6	[4]
HB3	Yes	Honduras	PYR	4	[5]
3D7	Yes (from NF54)	Apparently West Africa	--	4	[5]
D6	Yes (from Sierra Leone-1)	Sierra Leone	--	4	[4]
D10	Yes	Papua New Guinea	--	4-5	[6]
CAMP	No	Malaysia	PYR	4-5	[7]
FCB	No	Apparently SE Asia	CQ, QN, CYC	7-9	[8]
7G8	Yes	Brazil	CQ, PYR, CYC	4-5	[9]
K1	No	Thailand	CQ, PYR	4-5	[10]

CQ, chloroquine; QN, quinine; PYR, pyrimethamine; SDX, sulfadoxine; CYC, cycloguanil.

Multiplication rate refers to increase in total numbers of viable parasites per 48-hr generation. These rates and the drug phenotypes refer to data from the Fidock laboratory (Albert Einstein College of Medicine, NY) and may not be the same elsewhere. See www.ncbi.nlm.nih.gov/projects/Malaria/epid.html for additional information on these and other *P. falciparum* strains.

2. Protocols for Antimalarial Efficacy Testing *in vivo*

These protocols are meant to facilitate early investigations into the *in vivo* efficacy of candidate antimalarial compounds, using the rodent parasite *P. berghei.* We nevertheless recognize that many different protocols exist, some involving different rodent malaria models (Supplemental Table 2).

1. Primary Biological Assessment of *in vivo* Antimalarial Efficacy using the *P. berghei* Rodent Malaria 4-Day Suppressive Test

Parasite strain: *Plasmodium berghei*, ANKA strain

Standard Drugs: Chloroquine (Sigma)
Artemisinin (Sigma)

Standard Conditions:

Mice: NMRI mice, SPF, females, 25 ± 2 g. Free from *Eperythrozoon coccoides* and *Haemobartonella muris*.

Cages: Standard Macrolon type II cages

Maintenance: Air conditioned with 22°C and 50-70% relative humidity, diet with *p*-aminobenzoic acid content of 45mg/kg, and water *ad libitum*

Test procedure

day 0 Heparinized blood is taken from a donor mouse with approximately 30% parasitemia, (i.e. 30% of the erythrocytes are parasitized), and diluted in physiological saline to 10^8 parasitized erythrocytes per ml. An aliquot of 0.2 ml (=$2x10^7$ parasitized erythrocytes) of this suspension is injected intravenously (i.v.) or intraperitoneally (i.p.) into experimental groups of 5 mice.

2-4 hr post-infection, the experimental groups are treated with a single dose of test compound (for example 30 or 50 mg/kg), normally by the i.p. or subcutaneous (s.c.) route. Other routes of application can also be tested, including i.v. and oral (p.o.). The compounds are prepared at an appropriate concentration, as a solution or suspension containing 7% Tween80/ 3% ethanol (see below). In a more advanced stage of screening, compounds can also be prepared in standard suspending vehicle (SSV, see below).

day 1 to 3 24 hr, 48 hr and 72 hr post-infection, the experimental groups of mice are treated again with the same dose and by the same route as on day 0.

day 4 24 hr after the last treatment (i.e. 96 hr post-infection), blood smears from all

animals are prepared and stained with Giemsa. Parasitemia is determined microscopically by counting 4 fields of approximately 100 erythrocytes per field. For low parasitemias (<1%), up to 4000 erythrocytes have to be counted. Alternatively the parasitemia can also be determined by FACS analysis, which works particularly well for parasitemias >1%. The difference between the mean value of the control group (taken as 100%) and those of the experimental groups is calculated and expressed as percent reduction (= activity) using the following equation:

$$\text{Activity} = 100 - \left\{ \frac{\text{mean parasitemia treated}}{\text{mean parasitemia control}} \times 100 \right\}$$

For slow acting drugs, additional smears should be taken on day 5 and day 6, the parasitemia determined and the activity calculated. Untreated control mice typically will die approximately one week after infection. For treated mice the survival-time (in days) is recorded and the mean survival time is calculated in comparison to untreated and standard drug treated groups. Mice still without parasitaemia on day 30 post-infection are considered cured. Observations concerning adverse effects due to the drug, including weight loss, are recorded.

7% Tween80/ 3% ethanol:
Compounds are dissolved in 70% Tween 80 and 30% ethanol. This solution is further diluted 10-fold with distilled sterile water to result in a stock solution containing 7% Tween 80 and 3% ethanol.

Standard suspending vehicle (SSV):

Na-CMC (carboxy methylcellulose)	5.0 g
Benzyl alcohol	5.0 mL
Tween 80	4.0 mL
0.9% aqueous NaCl solution	1.0 L

SSV should be prepared one day before use. This solution is stable for 3 weeks at 4°C.

2. Secondary Biological Assessment.

Compounds showing good in vivo activity in the primary 4-day suppressive test should be evaluated further in secondary *in vivo* models (Supplemental Box 1). Mice, maintenance, drug preparation and application, and assessment of parasitemia are the same as for the basic 4-day treatment protocol. These secondary *in vivo* methods include:

Dose ranging test: Here, compounds are tested at a minimum of 4 different doses (the "dose ranging, full 4-day test", typically 100, 30, 10 and 3 mg/kg, by both s.c. and p.o. routes of administration). ED_{50} and ED_{90} values are calculated by plotting the log dose against probit activity (for example, using Microcal "Origin"). These reflect the drug concentrations at which 50% and 90% of suppression of parasitemia is achieved. Note that with short half-life drugs it may be necessary to dose multiple times per day. Two other parameters measured in this study are relative potency compared to an appropriate standard drug, and oral bioavailability (assessed by comparison of oral and parenteral administration). However,

bioavailability can be affected by formulation and may vary significantly within the same series of compounds, confounding interpretation of activity. For example, a recent study on artemisinin derivatives showed > ten fold variation in efficacy depending on the formulation [12].

Onset of activity and recrudescence test: For this, a single 100 mg/kg dose is administered on day 3 post-infection by the s.c. route. Control mice receive the suspension vehicle alone as a placebo control. Beginning after 12 hours, 24 hours and extending to day 33, tail blood smears are taken daily and Giemsa stained, followed by an assessment of parasitemia. Results are expressed as the rapidity of onset of activity (disappearance of parasitemia), time point of recrudescence, increase of parasitemia and survival in number of days.

Prophylactic test: Once a therapeutic lead has been established, it is important to establish prophylactic activity. For this, mice are administered 100 mg/kg doses at -72 hr, -48 hr, -24 hr and 0 hr relative to the time of infection. Smears are examined daily to assess suppression of parasitemia, and survival times are measured in days.

For all these assays, CQ can be used as the reference drug. Typically, CQ has an ED_{50} value of 1.5 – 1.8 mg/kg for *P. berghei* ANKA when administered s.c. or p.o. respectively, as determined in the full 4-day suppressive test (data not shown).

3. Tertiary Biological Assessment.

Cross-Resistance: For many antimalarial drug discovery projects, which depend on refining existing antimalarials or synthesizing new antimalarials that act upon the same target as an existing drug, the question of cross-resistance is paramount. Many drug-resistant strains of rodent malaria parasites have been selected over the past four decades by Professor Wallace Peters (Northwick Park Institute for Medical Research, Middlesex, UK). This includes 27, 7 and 14 lines of different drug resistance profiles for *P. berghei*, *P. yoelii* and *P. chabaudi* respectively. Some of these strains are available from MR4 (www.malaria.mr4.org). Cross-resistance studies employ the 4-day suppressive test described above, in order to determine whether the same ED_{50} and ED_{90} values are observed in drug-resistant strains of rodent malaria when compared to *P. berghei* ANKA.

***In vivo* generation of drug resistance:** It may also be helpful to determine the potential of parasites to develop resistance to a new compound *in vivo*, although the clinical relevance of these assays is unknown. Several high dose and incremental increase approaches have been used to select resistant lines. Of these, a widely used 2% relapse method [13] has proven to be useful for most classes of compounds. This entails administering a dose of compound that when given 1 hr before infection, delays the development of 2% parasitaemia until about 7-10 days post-infection. When the 2% level is reached, parasites are again passaged into fresh groups of mice 1 hr after administering the same dose of compound. The time to reach 2% parasitaemia is monitored on a daily basis. The degree of resistance is expressed as the reduction in "delay time to 2%". In rodent models, the rate of selection of resistance varies from 90% within 10 days for atovaquone to 50% after 300 days for artemisinin [13]. The method can then be extended beyond this indicator of selection of resistance to demonstrate the stability of resistance following removal of drug pressure and to select a partner compound(s) that can slow the rate of development of resistance.

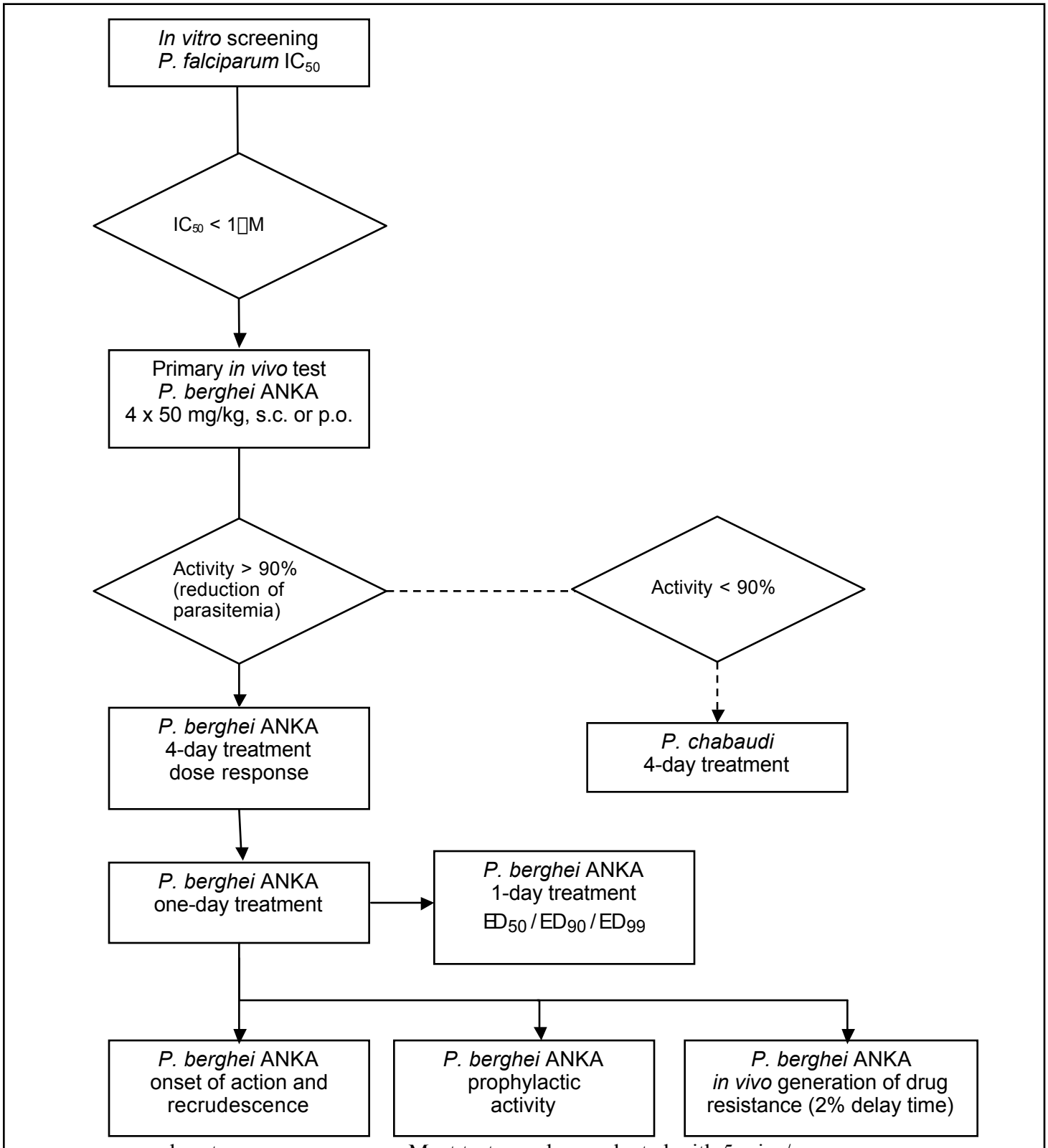
Supplemental Table 2. Characteristics of rodent malaria infections.

	Plasmodium species			
	P. berghei	*P. yoelii*	*P. chabaudi*	*P. vinckei*
First isolated	1948 (Zaire)	1965 (CAR)	1965 (CAR)	1952 (Zaire)
Cycle	asynchronous	asynchronous	synchronous	synchronous
Periodicity	22-25 hours	22-25 hours	24 hours	24 hours
Host cells	reticulocytes	reticulocytes	Mature RBC	Mature RBC
Mz per schizont	12 to 18	12 to 18	6 to 8	6 to 12
Primary use	Drug screening	Liver stage biology and vaccine studies	Mechanisms of drug resistance & antigenic variation	

Mz, merozoites; CAR, Central African Republic; RBC, red blood cells.

See www.ncbi.nlm.nih.gov/projects/Malaria/Epid/Originlist/rodstrain.html (compiled by Dr. David Walliker) for further information.

Supplemental Box 1. Flow chart of one scenario for *in vivo* screening for antimalarial activity.



s.c, sub-cutaneous; p.o., *per os*. Most tests can be conducted with 5 mice/group.

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