A REVIEW ON ANTI-MALARIAL DRUG DISCOVERY AND ITS SCREENING METHOD

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ABSTRACT

Malaria has been one of the most prominent and ancient diseases which has been profiled and studied. A lot of effort has been done for the design and discovery of anti-malarial drugs. The anti-malarial drug discovery and its screening are urgently needed. Our aim must be to Design safe and affordable noble drugs to control the spread of malarial parasites that are resistant to existing agents. Therapeutic efficacy and toxicity are important for the design and synthesis of compounds, while a minor effort has been done to review and assess the screening of anti-malarial drug-efficacy. In this review, we suggest different in-vitro and in-vivo screening for anti-malarial drugs, and evaluate different process for new compounds from drug discovery to development.

Key words: anti-malarial drugs, drug-efficacy, in-vitro.

INTRODUCTION

Now a day’s malaria is still one of the most important diseases of the developing country, the 1–3 million people died and causing disease in 300–500 million people annually world wide. In its total impact on humanity, Plasmodium falciparum is one of the world’s most pathogenic microbes, which is caused by the blood-borne Apicomplexan parasite Plasmodium falciparum and occurs in children in most of the developing country mainly in sub-Saharan Africa. Efforts to control malaria are becoming decreasingly successful because of anti-malarial drug resistance in the parasite, insecticide resistance in mosquitoes, and socio-economic deficits and warfare in human populations. The two most widely used anti-malarial drugs, Chloroquine (CQ) and Sulphadoxine-pyrimethamine SP, commonly available
as Fansidar; Roche), are failing at an accelerating rate in most malaria endemic regions with consequent increases in malaria-related morbidity and mortality. Plasmodium falciparum is an small haploid, but gonomically complicated eukaryote, able to change its gene expression to generate a sequence of forms that exploit most efficiently quite different environments: liver and red blood cells in humans; gut, vascular system and salivary glands in the mosquito. Clinical symptoms can include fever, shivering, prostration and anemia. It can lead to severe disease 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 (Fig 1).

Fig. 1  Drug-resistant P. falciparum malaria

To control malaria, new drugs are needed, but traditional mechanisms for drug discovery and development have synthesized few drugs to cure diseases of the developing country in whole world. In this challenging situation, there are some reasons for hoping potent drug. First, the confirmation of the genome sequence of P. falciparum offers a multitude of potential drug targets. Second, modification in malaria genetics improved means characterizing potential
targets. Third, the latest increased participation of pharmaceutical industries for anti-malarial drug discovery and development process design 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. However, there is a lack of standardized systems for anti-malarial 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 anti-malarial drug development (FIG. 1).

Now a days, CQ resistance has well known and more common to the vast majority of malaria-endemic areas, For many decades, the standard drug for the treatment of malaria was Chloroquine(CQ), a 4-aminoquinoline, that have more efficacy, low toxicity and affordability. The action of Chloroquine, bind to haem protein produced from hemoglobin through proteolytic enzyme inside infected erythrocytes, thereby interfering with haem detoxification. This is especially in sub-Saharan Africa, where resource limitations are profound and where highly immune populations often seem to get least response to CQ therapy, and therefore somewhat prevent the spread of resistance. CQ resistance almost certainly contributes to the latest survey that malaria-associated mortality is more in Africa.

SP, a combination of antifolate drug is only the other widely used anti-malarial drug, but this also causes resistance leads to therapeutic failure in many areas in Asia, South America and now Africa. Beside this some researchers have being design new drug development for the future, as mentioned above, the malaria endemic regions of the world are faced with an unexpected situation in which the only affordable treatment options are rapidly losing therapeutic efficacy.

Table 1 Current malaria discovery and development projects and associated organizations

<table>
<thead>
<tr>
<th>Discovery projects</th>
<th>Development projects</th>
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<tr>
<td>Improved quinoline (MMV, GSK, U. Liverpool)</td>
<td>Rectal artesunate (TDR)</td>
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<td>Farnesyl transferase inhibitors (MMV, BMS, U. Washington, Yale))</td>
<td>Manzamine derivatives (MMV, U. Mississippi)</td>
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<tr>
<td>Chlorproguanil dapsone artesunate (MMV, TDR, GSK)</td>
<td>Pyronaridine artesunate (MMV, TDR, Shin Poong)</td>
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<tr>
<td>Cysteine protease inhibitors (MMV, UCSF, GSK)</td>
<td>Amodiaquine artesunate (TDR, EU, DNDi)</td>
</tr>
<tr>
<td>Fatty acid biosynthesis inhibition (MMV, Texas A&amp;M U., AECOM/HHMI, Jacobus)</td>
<td>Mefloquine artesunate (TDR, EU, DNDi)</td>
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The urgent need for new anti-malarial

The new anti-malarial agent should meet the parameter of more therapeutic efficacy, minimal toxicity and low cost. Instant manner for drugs to replace CQ and SP having amodiaquine (a CQ-like quinoline) and chlorproguanildapsone (LapDap, another antifolate combination that inhibits the same enzymes as SP). These substituent might be provide a few years of efficacy, specially in Africa, but these area already suffer from some cross-resistance with CQ and SP, which enhances the full-blown resistance to these drugs will emerge rapidly.

The mid-term replacements are Artemisinin derivatives. However, these drugs have very short half-life, which is important for their use in combination with a longer-acting drug. That’s why additional new drugs are required. So this plan is necessary for the discovery and development of novel anti-malarial compounds that are not encumbered by pre-existing mechanisms of drug resistance.

The desired profile for new drugs

Basically, new drugs for uncomplicated *P. falciparum* malaria should be efficacious against drug-resistant strains, provide cure within a time (ideally three days or less) to ensure good compliance, be suitable for small children and pregnant women, have appropriate formulations for oral use and above all, be affordable. Drug Design & development necessarily requires among desired drug characteristics, but for the treatment of malaria in the developing world should be affordable, orally active treatments that are safe for children. Cost drives the choice of drugs in most developing countries, especially Africa, where most people must survive on less than about 15 US dollar per month. Additional desirable uses
include Intermittent Preventive Treatment in pregnancy and childhood and other emergency situations, treatment of severe malaria, and the treatment of malaria caused by *P. vivax* (a rarely lethal) of less importance to public health, but potentially beneficial, new drugs should ideally also provide protection against malaria when used as Chemoprophylaxis by advantaged non-immune populations to endemic areas.

**The need for drug combinations**

There is a modern era that drug combinations are important to control the malaria in developing countries. Combinations therapy provides a number of advantages. (i) They need potential efficacy, and might provide synergistic activity. While combination therapy that relies on synergy might not be provide as much protection against the expected selection of resistance. Indeed, the prefer synergistic combination SP acts almost as a single agent in this regard, with rapid selection of resistance, and similar concerns apply to the new atovaquone/proguanil (Malarone; GlaxoSmithKline) combination. (ii) Drug combinations increases the setting of drug resistance, at least one agent would 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. (iii) Probably most important, drug combinations should reduce the selection of anti-malarial drug resistance. 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. It was also recently shown that SP selected for resistance-conferring mutations and subsequent treatment failure, but that SP with Artesunate prevented the selection of SP-resistant parasites in subsequent infections, thereby reducing cost and/or toxicity. Basically, combination regimens will incorporate two agents that are both new 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. It is to combine with Artemisinins — which have no resistance problem but suffer as monotherapy from late recrudescences due to their short half-lives 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, Mefloquine/ Artesunate, Chlorproguanil/Dapsone/ Artesunate or Lumefantrine/ Artemether). The combinations of these are inexpensive and available drugs (for example, Chlorproguanil/Dapsone or Amodiaquine plus Sulphadoxine/ Pyrimethamine), might be appropriate stopgap therapies, especially in Africa.
Target selection and validation

Many anti-malarial drugs that are now in use were not developed on the basis of rational identified targets, but the identification of the anti-malarial 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). (Table2). The identified potential targets that are compared between the parasite and human host offer opportunities for chemotherapy. For example, the dihydrofolate reductase inhibitors Pyrimethamine and Proguanil are important components of anti-malarial drugs, because of their relative selectivity for the parasitic enzyme. The advantage of identified 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 anti-malarial drug design and discovery can be reduced. For examples, anti-malarial drug design and discovery made efforts directed against parasite Cysteine Proteases and Protein Farnesyl Transferases are benefiting from industry projects directed against inhibitors of the human cysteine protease cathepsin K as treatments for osteoporosis and human Farnesyl transferases as treatments for cancer.

The targets can be opted from enzymes or pathways that are present in malarial parasite but absent from humans. 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. The example is the use of prokaryotic protein synthesis inhibitors, including Tetracyclines and Clindamycin, which were found to have anti-malarial activity. These compounds presumably act selectively against malaria parasites because of their action against prokaryote-like plasmodium organelles known as apicoplasts, which have cyanobacterial origins and these are related to algal plastids. Additional, recently identified potential selective targets for anti-malarial drugs include components of type II fatty acid biosynthesis and Mevalonate-independent Isoprenoid synthesis. These pathways are targets also for existing antibacterial compounds, providing initial leads for anti-malarial drug discovery. A ‘reverse’ drug discovery approach is to elucidate the nature of previous unknown targets of existing anti-malarial drugs as a basis for new drug design and discovery or development efforts. This example relates to CQ, which acts by interfering with the production of the malarial pigment Haemozoin, allowing the intra-parasitic build-up of toxic
free haem. This explains the inhibition of haemoglobin formation as a supportive target for new anti-malarial drugs. These genome sequences can dramatically accelerate the early steps of drug discovery by enabling the rapid identification of putative plasmodium targets that are homologous to validated target proteins from other systems.

Previous parameter to target validation includes the demonstration that an inhibitor has potent anti-malarial activity. This problem is partially solved by the repeated demonstration of anti-parasitic activity of different inhibitors of a particular target, by the identification of potent activity and when possible, by the biologically relevant defects caused by inhibitors (for example, the development of swollen FOOD VACUOLES in parasites treated with cysteine protease inhibitors). This demonstrates that the mutated molecule is essential for target. This was demonstrated for the *P. falciparum* dihydrofolate reductase gene, which in its mutated form conferred resistance to Pyrimethamine in transgenic *P. falciparum*.

*Figure.2 Example of a critical path for anti-malarial 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 $\mu$M for \textit{in vitro} screens and <5–25 mg per kg for \textit{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.

Medicinal chemistry and lead optimization constitute an essential and iterative component of this part of the critical path. Secondary \textit{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 \textit{in vitro} and \textit{in vivo} evaluations of compound efficacy can be found in Further Information, Anti-malarial 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 \textit{P. falciparum} (or \textit{P. vivax}) target in an \textit{in vivo} setting, transgenic \textit{P. falciparum} lines that over express 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 \textit{Plasmodium} parasites or in bacterial or yeast model systems).

Advance technology has greatly enhanced our ability to validate potential drug targets. In particular, methodologies have been developed to transfect \textit{P. falciparum} with plasmids expressing either positive or negative selectable markers, and to thereby alter, replace or knock out genes of interest. Another promising new avenue made possible by transfection is to express genes encoding drug targets from \textit{P. falciparum} by allelic replacement into the rodent malaria parasite \textit{P. berghei}, for which efficient transfection technology has been developed. This enables evaluation of compound efficacy against the correct enzymatic target in an \textit{in vivo} setting. In a related ‘functional proteomics approach, the inhibition of proteins that have not been biochemical Characterized, can be surveyed using libraries of inhibitors and competitive binding assays. The protein based 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 anti-malarial

In vitro study for compound activity, which constitute a key component of a critical path for an anti-malarial drug discovery and development (FIG. 2), are based on the ability to grow the P. falciparum in vitro in human erythrocytes. Typically, parasites are propagated in leukocyte-free erythrocytes at 2–5% Haematocrit at 37°C under low pressure of oxygen (typically 3–5% O₂, 5% CO₂, 90–92% N₂) in tissue culture (RPMI 1640) media should have either human serum or Albumax (a lipid-rich bovine serum albumin). Multiple drug-resistant and drug-sensitive isolates from around the world have been culture-adapted and can be obtained from the Malaria Research and Reference Reagent Resource Center. Brief explanation of one standardized procedure for culturing P. falciparum and assaying susceptibility to anti-malarial compounds are available online (Anti-malarial 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 3H-hypoxanthine (which is taken up by the parasite for purine salvage and DNA synthesis) to determine the level of P. falciparum growth inhibition. In several literature, parasites are cultured in the presence of different concentrations of test compound in media containing reduced concentrations of hypoxanthine, after which 3H-hypoxanthine is added for an additional incubation period before cell harvesting and measurement of radioactive counts. IC50 values can be determined by dose–response curves. Although 3H-hypoxanthine incorporation is the most commonly used method to assay anti-malarial activity in vitro, it is costly and problematic for resource-poor locations or for high-throughput screening (HTS). A low-cost alternative for testing few 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 method involves through colorimetric detection of lactate dehydrogenase. Flow cytometry technique has advantage of the fact that human erythrocytes lack DNA. In the simple 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 also allow one to distinguish different parasite erythrocytic stages.
<table>
<thead>
<tr>
<th>Target location</th>
<th>Pathway/mechanism</th>
<th>Target molecule</th>
<th>Existing therapies</th>
<th>New compounds</th>
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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 \textit{in vitro} activity (for example, IC50 ≤ 1 M) can then be tested for activity against a range of geographically distinct \textit{P. falciparum} lines of differing drug-resistance phenotypes (see Further Information, Anti-malarial drug discovery: efficacy models for compound screening) to determine whether resistance to existing anti-malarial drugs reduces parasite sensitivity to the compounds under evaluation. Different research groups have incorporated a variety of modifications of the basic \textit{in vitro} screens listed above, which can influence the measurement of drug activity levels, as follows.
Unsynchronized vs 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 for comparing a series of compounds, establishing order of activities and determining potency against different parasitic stages.

**Duration of incubation:** Most of the assays do not support 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.

**Human serum versus Albumax:** Now Days many laboratories have replaced 10% human serum with the serum substitute Albumax and have disadvantage (for example, a higher level of protein binding has been reported with Albumax compared with serum).

**Initial percentage of parasitaemias:** 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).

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.

**In vivo screening of anti-malarial compounds**

*Plasmodium* species that causes various problems in human and 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*). So, *in vivo* evaluation of anti-malarial compounds typically begins with the use of rodent malaria parasites. i.e, *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei* have been used extensively in drug discovery and development. Rodent models have been validated through the identification of several antimalarials — for example, mefloquine, halofantrine and more recently artemisinin derivatives.
These models remain a standard part of the drug discovery and development. 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. The most widely used initial test, which uses *P. berghei* or less frequently *P. chabaudi*, is a four-day suppressive test\(^4\). Rodent infection is initiated by needle passage from an infected to a rodent via the intra-peritoneal or preferably the intravenous route. Synthesized drug can be administered by several routes, including intra-peritoneal, intravenous, and 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.

Synthesized drug identified as being active in four-day assays can subsequently be progressed through several secondary tests (FIG. 4), as follows. To determine ED\(_{50}\) and ED\(_{90}\) values. This test also provides useful information on relative potency and oral bioavailability. Synthesized drug can also be tested for prophylactic activity by administering the compound prior to infection, followed by daily examination of smears. Additional screening have been developed to assess cross-resistance and the potential for *in vivo* selection of resistant parasites. Foremost is the choice of rodent malaria species and mouse strains. For example, *P. chabaudi* and *P. vinckei* generate a high parasitaemia and produce synchronous infections, enabling studies on parasite stage specificity. Rodent malarial 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.

**From anti-malarial drug discovery to development**

The design and drug discovery effort is to identify and develop synthesized drug with their properties that are predictive for good efficacy and safety in humans. The potential of a synthesized drug 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 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, Lead Identification and optimization involving cycles of chemistry and biology, and compound selection and preclinical development. This is followed by clinical development. The discovery platform is high risk and needs continuous support to maximize the chances that one or more compound(s) moves into
development. This includes having access to reasonable compound collections, HTS techniques, and medicinal chemistry and pharmaceutical development expertise. 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 anti-malarial 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.

For example, the development of numerous Aminoquinoline and Artemisinin anti-malarials was driven fully by chemistry, but several newer projects are based on identifying the biological relevance of specific targets (Table 2). Promising hits from a screening campaign can be developed into a lead series following a comprehensive assessment of chemical integrity, synthetic robustness, functional behavior, 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, entailing structural modifications with the goal of achieving optimal efficacy and pharmacokinetic/pharmacodynamic properties.

The development of information technology and databases now also enables chemists to identify molecular groups that are likely to be metabolically labile or associated with adverse toxicological properties. This requires that candidate progression criteria be established. The in vitro and in vivo efficacy, chemistry, exploratory pharmacokinetics, ADME and toxicology, in comparison to standard agents. 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.
This R&D pipeline is encouraging, but it must be emphasized that drug development has a high failure rate and 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 anti-malarial drugs and R&D.

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). 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, which is a proven target in several organisms including *Mycobacterium tuberculosis*.

**Future perspectives**

Future efforts is to design new anti-malarial drugs have increased 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.

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**Figure 5** Crystal structure of *Plasmodium falciparum* enoyl-ACP reductase (PfENR) complexed with NAD+ cofactor and the inhibitor triclosan.
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 anti-malarial drugs.

REFERENCES


