

New insight-guided approaches to detect, cure, prevent and eliminate malaria

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Abstract New challenges posed by the development of resistance against artemisinin-based combination therapies (ACTs) as well as previous first-line therapies, and the continuing absence of vaccine, have given impetus to research in all areas of malaria control. This review portrays the ongoing progress in several directions of malaria research. The variants of RTS,S and apical membrane antigen 1 (AMA1) are being developed and test adapted as multicomponent and multistage malaria control vaccines, while many other vaccine candidates and methodologies to produce antigens are under experimentation. To track and prevent the spread of artemisinin resistance from Southeast Asia to other parts of the world, rolling circle-enhanced enzyme activity detection (REEAD), a time- and cost-effective malaria diagnosis in field conditions, and a DNA marker associated with artemisinin resistance have become available. Novel mosquito repellents and mosquito trapping and killing techniques much more effective than the prevalent ones are undergoing field testing. Mosquito lines stably infected with their symbiotic wild-type or genetically engineered bacteria that kill sympatric malaria parasites are being constructed and field tested for stopping malaria transmission. A complementary approach being pursued is the addition of ivermectin-like drug molecules to ACTs to cure malaria and kill mosquitoes. Experiments are in progress to

eradicate malaria mosquito by making it genetically male sterile. High-throughput screening procedures are being developed and used to discover molecules that possess long in vivo half life and are active against liver and blood stages for the fast cure of malaria symptoms caused by simple or relapsing and drug-sensitive and drug-resistant types of varied malaria parasites, can stop gametocytogenesis and sporogony and could be given in one dose. Target-based antimalarial drug designing has begun. Some of the putative next-generation antimalarials that possess in their scaffold structure several of the desired properties of malaria cure and control are exemplified by OZ439, NITD609, ELQ300 and tafenoquine that are already undergoing clinical trials, and decoquinate, usnic acid, torin-2, ferroquine, WEHI-916, MMV396749 and benzothioephene-type N-myristoyltransferase (NMT) inhibitors, which are candidates for future clinical usage. Among these, NITD609, ELQ300, decoquinate, usnic acid, torin-2 and NMT inhibitors not only cure simple malaria and are prophylactic against simple malaria, but they also cure relapsing malaria.

Keywords Artemisinin resistance marker · Hypnozoiticidal · REEAD diagnostic · Mosquito killers · Mosquito repellents · RTS · S+AMA1 vaccine · Schizonticidal · Transmission blockers

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Introduction

Malaria continues to persist in 97 countries inhabited by 3.4 billion people. It is reported that in the year 2013, 219 million persons became sick with malaria disease, and of them, 627,000 died including 483,000 children (WHO; World Malaria Report 2012, 2013a). Malaria mortality was more in the countries of Africa than in Asia or South America. There were 60.7 million new infections of malaria in India in 2013 (Murray et al. 2014). In India, ≤ 0.2 million have been dying of

malaria each year, largely in the states of Chattisgarh, Orissa and Jharkhand (Dhingra et al. 2010; Murray et al. 2014). Climate change is allowing malaria to reach new densely populated areas, such as in the highlands (Siraj et al. 2014). Malaria is caused by the parasites of five species of the genus *Plasmodium* of the phylum Apicomplexa. Malaria disease caused by *Plasmodium knowlesi*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium vivax* is milder than that caused by *Plasmodium falciparum* which is often lethal. *P. vivax* and *P. falciparum* are respectively the principal agents of morbidity and mortality causing malaria (Garcia et al. 2008). Besides causing disease in vertebrates, *Plasmodium* parasites infect mosquitoes of the genus *Anopheles*. Thirty to 40 species of *Anopheles* are sensitive to *Plasmodium*. The main vectors of malaria transmission to humans are *Anopheles gambiae* and *Anopheles stephensi* (Hay et al. 2010). Females of these mosquito species acquire malarial parasite infection by biting and taking a blood meal from infected humans. About a week or more than a week later, infected mosquitoes transmit infection to humans while taking their next blood meal. The life cycle of *Plasmodium* is complex (Fig. 1). It comprises of a gametophytic (male and female gamete forming, haploid) phase followed by a sporophytic (arising from zygote/embryo via mitotic divisions, diploid) phase in a female mosquito and a sporophytic phase each in liver and red blood cells and a gametophytic phase in blood in human host (Rosenberg 2008).

As shown in Fig. 1, *Plasmodium*'s asexual liver stage in human host (exo-erythrocytic schizogony) begins with inoculation of its eight to ten sporozoites, during the meal bite by an infected female mosquito. The sporozoites injected into the bloodstream travel to the liver and invade hepatocytes. In the invaded hepatocyte, the sporozoite differentiates and grows, within 6–8 days into a schizont (mother cell), by dividing mitotically to produce 1×10^4 – 3×10^4 merozoites (daughter cells). The liver merozoites, released into the bloodstream upon rupturing of liver schizont(s), invade erythrocytes, thus, begins *Plasmodium*'s human blood stage life cycle (erythrocytic schizogony). Invading merozoite grows into ring stage trophozoite of large size, at the expense of rich nutrition available in the host erythrocyte. Further progression of trophozoite into schizont involves several rounds of mitotic divisions to produce up to 36 merozoites. Erythrocytic schizonts rupture to release merozoites into the bloodstream. The erythrocytic schizogony is completed in about 48 h. Erythrocytic merozoites invade fresh erythrocytes and merozoites get multiplied. This process leads to the presence of 10^8 to 10^{12} circulating merozoites in the bloodstream. At this stage of 12–14 days from infection, the inoculation period ends and symptoms of parasitaemia get manifested by the infected human. After parasitaemia has set in, merozoites of some erythrocytic schizonts invade erythrocytes and differentiate into male gametocytes. Similarly, merozoites of relatively more schizonts invade erythrocytes to produce female gametocytes. Gametocytes circulate in the blood of parasitaemic human.

Gametocytes are the only forms of *Plasmodium* that survive in female mosquito when ingested from malarial human during its blood meal. The *Plasmodium* life cycle starts in mosquito, when the ingested erythrocytes burst and the released gametocytes produce gametes. The male gametocyte undergoes divisions to produce eight male gametes that are exflagellated. The female gametocyte transforms into a female gamete. Fusion of a male gamete and female gamete results in a zygote, which develops into a ookinete. The motile ookinete migrates to the midgut epithelium to initiate an oocyst. Multiple divisions in oocyst result in the production of thousands of sporozoites. Upon release from oocysts, the sporozoites migrate to salivary glands. Sporozoites from salivary glands are injected into a fresh human host during the next blood meal of the *Anopheles* after 8–10 days of the previous meal (Hall et al. 2005; Eckhoff 2011). The life cycles of *P. vivax* and *P. ovale*, which cause relapsing malaria, somewhat differ at the liver stage from that of *P. falciparum*. The parasites in the hepatocytes invaded by the *P. vivax* and *P. ovale* sporozoites often enter into hibernation for a period up to more than a year. Resumption of liver stage in the hibernated hypnozoites becomes the cause of relapsed malaria. Several aspects of malaria, including parasite biology, clinical features in patients, diagnosis of the infected, protection from the disease and treatment of the diseased, have been broadly reviewed recently by White et al. (2014). Epidemiology and research priorities of malaria and strategies to eliminate malaria have been discussed in some detail in the reviews of Feachem et al. (2010), Enayati and Hemingway (2010), Alonso et al. (2011), Cohen et al. (2012), Burrows et al. (2013), Cotter et al. (2013), Whittaker et al. (2014) and White (2014). The reviews reveal that in the history of malaria, evolution of artemisinin resistance in plasmodia in recent years is a landmark. This development has led to revision of research agenda on diagnosis and treatment of malaria and, therefore, significant changes in plans for malaria control and eradication.

Eradication of malaria from a region requires detection and effective chemotherapeutic treatment of all parasitaemic persons and prevention by blocking of transmission from infected to other persons by a variety of means (Fig. 2). The objective of this review is to discuss new insights into human-*Plasmodium*-mosquito interaction and its control with reference to eradication of malaria, in the background of the origin of artemisinin resistance in malarial parasites.

Origin of artemisinin-resistant malaria

Table 1 gives a list of 15 antimalarials of seven chemical classes that have been used to treat malarial disease, singly or in combination, at different times in different geographical areas (Biamonte et al. 2013). Since 1996, no new class of antimalarial has been added to clinical practice against malaria. On the

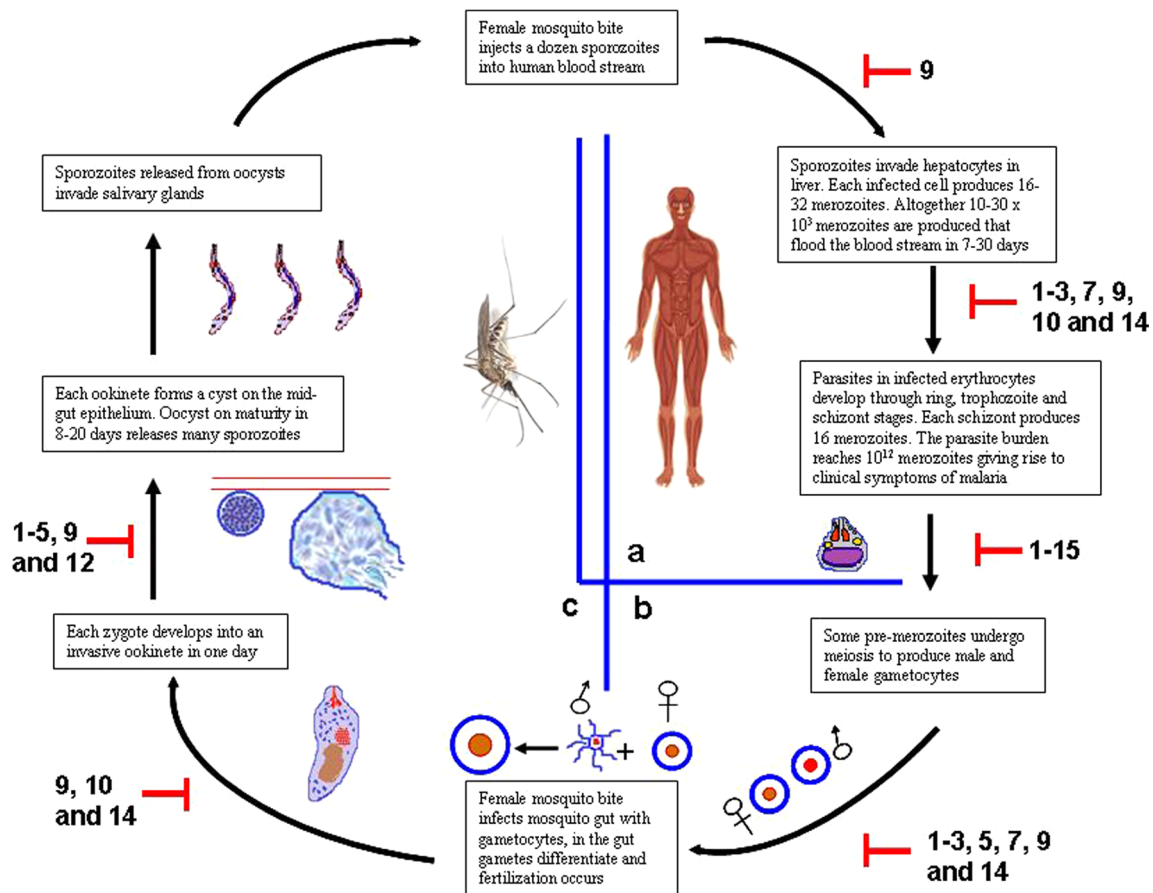


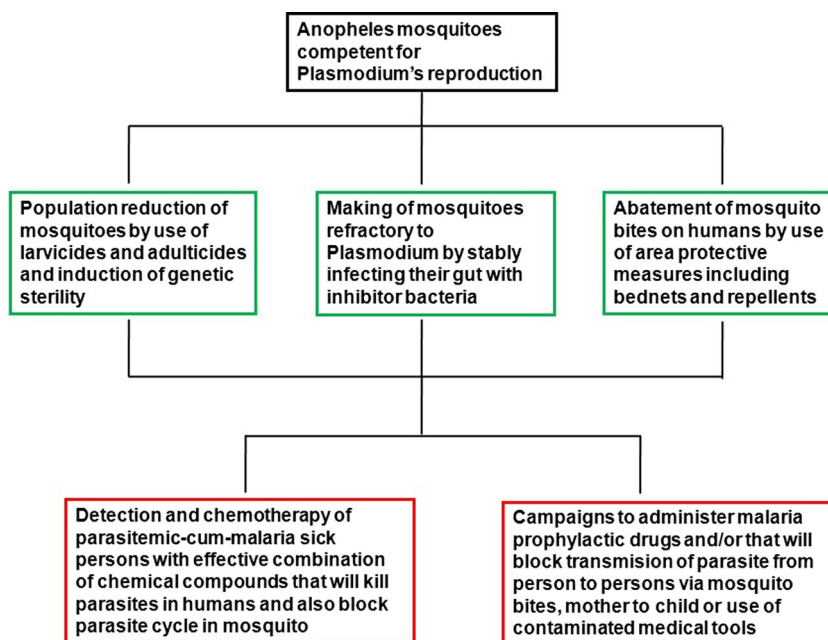
Fig. 1 Life cycle of the protozoan parasite *Plasmodium* whose five species induce malaria in the infected humans: *P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax* and *P. knowlesi*. *P. falciparum* is the cause of maximum mortality (nine out of ten malaria deaths), and *P. vivax* causes maximum morbidity. The genome sequence of *P. falciparum* is known (Gardner et al. 2002). Malaria parasites are transmitted person to person by *Anopheles* mosquitoes. There are 430 species of *Anopheles* of which up to 40 species serve as malaria vectors. Among them, *A. gambiae* and *A. stephensi* are prominent vectors in Africa and India, respectively. Genome sequence of *A. gambiae* is known (Holt et al. 2002). Both mosquitoes have African origin (Liu et al. 2014). The symptoms of malaria in humans are headache, back pain, muscle ache, fatigue, sweats, fever, chills, vomiting and enlarged spleen. The symptoms appear 7 to 40 days after infection. The parasite life cycle is divisible into three parts: *a*, liver and red blood cell stages in the human host wherein parasite multiplies asexually; *b*, sexual stage in human blood-stream where some pre-merozoites undergo gametocytogenesis; and *c*, sexual stages in female mosquito after it has acquired the gametocytes as a part of blood meal from infected human. The antimalarial compounds that interfere with the progression of different stages are shown as numbers

against horizontal bars. The antimalarials 1–15 are as follows: 1, dihydroartemisinin; 2, artesunate; 3, artemether; 4, lumefantrine; 5, piperaquine; 6, sulfadoxine; 7, pyrimethamine; 8, chloroquine; 9, primaquine; 10, atovaquone; 11, proguanil; 12, mefloquine; 13, amodiaquine; 14, pyronadrine; and 15, quinine. The antimalarials shown to act in the *b* and *c* parts of *Plasmodium* life cycle negatively control the transmission of infection and those that act in the *a* part control the progression of malaria and some of them also control sexual gametogenesis and thereby malarial transmission. The blood meal obtained by a female mosquito by biting to an infected person under treatment also contains the drugs which continue to act on the sexual stages undergoing in the mosquito. A developmental stage of parasite in the liver called hypnozoite is not shown in this figure. Hypnozoites are *P. vivax* or *P. ovale* parasites that hibernate in hepatocytes for many months and become the cause of relapsed malaria. The only clinical antimalarial that kills them is primaquine. Besides the bite of an infected mosquito, malaria transmission can also occur via mother to newborn, blood transfusion or sharing of contaminated needle or syringe and organ transplanting

other hand, malaria disease treatment has suffered gravely, for more than 60 years, from acquirement of genetic drug resistance property by malarial parasites (Sibley and Ringwald 2006). Malarial parasites developed chloroquine resistance as early as 1950. Subsequently, resistance was noted against other drugs such as pyrimethamine, sulfadoxine, atovaquone, amodiaquine and mefloquine. Occurrence of drug resistance mutations was rare and sporadic, but they subsequently spread worldwide. The

main reason for their first appearance and selection has been deduced to be drug underdosing of malaria patients. The dominant nature of certain drug resistance alleles like *crt*, *mdr1*, *mrp1* and *dhfrts* was perhaps responsible for the rapid spread of drug resistance in the areas of their origin and subsequently in other areas where migration introduced the resistance alleles (Johnson et al. 2004; Cooper et al. 2007; Plowe 2009; Petersen et al. 2011; Hrycyna et al. 2013; Nwakanma et al. 2013).

Fig. 2 Diagramme of a scheme for prevention of malaria in a malaria burdened geographical region. Implementation of such a programme in different regions can roll back by reducing the burden of malaria such that WHO's goal of eradicating malaria worldwide by 2030 is achieved. Statistical analysis has shown that if transmission of parasites is prevented by the use of bed nets by 75 % of population, malaria will be stopped in the concerned geographical region (Agusto et al. 2013). Once transmission is stopped, the disease is unlikely to reappear (Chiyaka et al. 2013)



In response to the prevalence of resistance to antimalarials commonly used in clinical practice, the artemisinin (ART)-based combination therapy (ACT) was adopted in 1978 as the first line of treatment of malaria (Banek et al. 2014). In ACTs, ART or one of its derivatives—dihydroartemisinin, artesunate, artemether/artether— is combined with antimalarial(s) such as piperazine, mefloquine, amodiaquine, sulfadoxine+pyrimethamine, atovaquone+proguanil, pyronaridine and lumefantrine. ARTs via a spectrum of their inhibitory effects, that are not fully understood (Kumar and Srivastava 2005), block the development of parasite's trophozoites and schizonts in red blood cells and also eliminate, at least partially, the parasite's gametocytogenesis in the blood of the treated humans (see Table 1). Thus, ARTs not only remediate malaria disease by killing the asexual parasites, but they also block malaria transmission to some extent. The ARTs are fast acting. They kill the malarial parasites present in the blood of patients in 2 days. Their in vivo half life is short. The accompanying drug in ACTs overcomes this deficiency of ARTs. The presence of two drugs in ACTs also serves to safeguard against development of resistance against either of the antimalarials. ACTs are being used in an area-specific manner, such that resistance to ARTs' partner drug does not exist in the area of usage of an ACT. The ACT comprising of dihydroartemisinin and piperazine was found to cure 97 % of the *P. falciparum*-caused malaria patients in Asia, Africa and South America; thus, ACTs have saved millions of lives (WHO 2013b).

The presence of resistance to ARTs of ACTs has been consistently observed in western Cambodia over about the last 10 years (Noedl et al. 2008; Dondorp et al. 2009; Phyo et al. 2012; Satimai et al. 2012; Miotto et al. 2013). As evidence of resistance development, it was noted that clearing of parasites

following ACT treatment was taking more than 3 days. Survey in Cambodia showed that in many cases of malaria, clearance of parasites by ACT took up to 20 days. More recently, a similar type of resistance to ARTs has been noted in a wider Southeast Asian region comprising Myanmar, Thailand, Cambodia and Vietnam (WHO 2013a, b).

Measures to contain migration of artemisinin-resistant malaria to outside of Southeast Asia

Evolution of artemisinin resistant in *Plasmodium* parasites has been a grievous setback to the national and international programmes aiming to eradicate malaria. There is imminent danger of migration of ART-resistant malarial parasites to Sri Lanka, Bangladesh, Nepal, India, Pakistan and Africa. There is an urgent need to stop such possibilities. It is urgently required to detect in the affected Southeast Asian region all persons infected with malarial parasites and treat them by administering the ACT antimalarial to which the local parasites are sensitive. Further, transmission requires to be blocked by the control of mosquitoes as well as by administration of primaquine or an equivalent drug as a prophylactic, in mass campaigns, taking care to avoid this treatment of people afflicted with heritable glucose-6-phosphate deficiency. Such people may be treated with new complementary safe experimental drugs such as ELQ300, GNF156, NITD609 and torin-2. However, until very recently such a programme suffered from deficiencies in rapid method(s) to detect parasitaemic persons on the one hand and persons suffering from ART-resistant infection on the other hand. These lacunae have now been remedied. Juul et al. (2012) have given a cost-effective

Table 1 Properties of antimalarial compounds in clinical use: chemical structure, biological activity, drug formulation and effectivity against parasites in mosquito vectors and in malaria patients

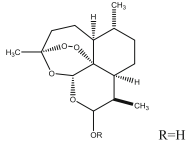
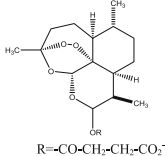
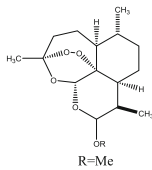
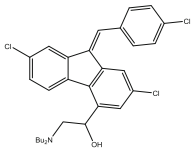
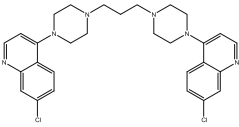
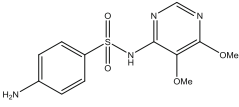
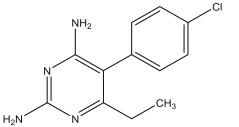
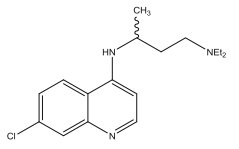
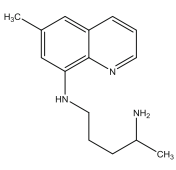
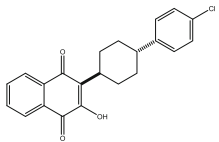
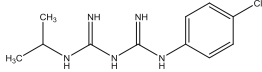
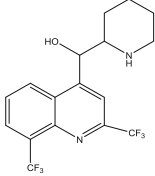
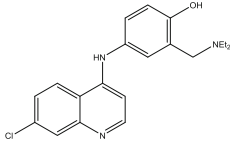
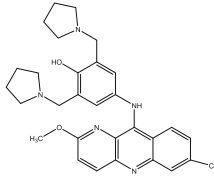
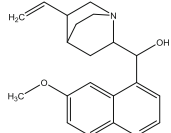
S. no.	Compound name	Class of the compound	Structure of the compound	Whether known to be active against parasite developmental stages in			Remarks	References
				Humans in Liver	Blood	Female mosquitoes		
1	Dihydro-artemisinin	Endoperoxide (artemisinin derivative)		Yes ^a	Yes ^a	Yes ^b	Being an artemisinin compound, it acts by clearing the peroxide bond by Fe (II) present in heme proteins, thus generating free radicals that kill the parasite. Since artemisinins are fast acting, it is used in combination with slow absorbing piperazine (Eurartesine). This artemisinin combination therapy (ACT) comprises of a tablet taken once a day for three days. The effect of treatment lasts for upto nine weeks. Micro-male gametocytes are relatively more sensitive than the female gametocytes.	Duffy and Avery 2013 a, b; Zani et al. 2014; Gargano et al. 2012; Keating 2012; Tarning et al. 2012
2	Artesunate	As above		As above	As above	As above	As an ACT, it is used in combination with pyronaridine. It is also given alone parenterally for severe malaria.	Zhang et al. 2012; Charman et al. 2011; Rottmann et al. 2010
3	Artemether	As above		As above	As above	As above	It is used in combination with lumefantrine (Coartem tablet = artemether 20 mg + lumefantrine 120mg). Four tablets are taken twice a day for four days. This ACT treatment is a little less effective than dihydroartemisinin + piperazine in serving as a prophylactic drug.	Agarwal et al. 2013; Hodel et al. 2013; Masanja et al. 2013
4	Lumefantrine	Amino alcohol		No	Yes	Yes	As for artemether; by itself it is not active against mature gametocytes of parasites formed in humans but is active inhibitor of sporozoite formation in mosquito	As above and Delves et al. 2012 a, b; Lelievre et al. 2012; Slack et al. 2012; Nsoya et al. 2010
5	Piperazine	4-aminoquinoline		No	Yes	NK ^c	As for dihydroartemisinin; highly active against blood stage gametocytes	As above and Duffy and Avery 2013 a, b; Moore et al. 2008
6	Sulfadoxine	Sulfonamide		No	Yes	NK	In combination with pyrimethamine (Fansidar), it is used orally. Fansidar is effective in single dose when malarial parasite is sensitive to it. The drug replaced chloroquine and now it has been replaced by ACTs. It is a preferred treatment for children and pregnant women when malaria is sensitive. It is not gametocytocidal.	Duffy and Avery 2013 a, b; Calderon et al. 2012; Gujjar et al. 2011; Dondorp et al. 2010; Mugittu et al. 2005; Sibley et al. 2001
7	Pyrimethamine	Antifolate		Yes	Yes	Yes	By acting against parasite's enzyme dihydro-folate reductase, it inhibits DNA synthesis in asexual and sexual cell divisions of parasite in humans at liver and blood stages and ookinete and oocyst stages in female mosquitoes. It is a component of Fansidar. Resistance against it is widespread. The drug is not gametocytocidal.	As above and; Duffy and Avery 2013 a, b; Huang et al. 2012; Tanaka and Williamson 2011
8	Chloroquine	4-aminoquinoline		No	Yes	No	It is used as 250 mg oral tablet (Aralen) and is safe in children. It acts by inhibition of hemozoin formation in its digestive vacuole in the process of haemoglobin degradation during parasite's asexual multiplication in erythrocytes. It also inhibits glutathione degradation. Resistance to it results from mutations in parasite's chloroquine transporter (PfcRT) whereby chloroquine is effluxed from the digestive vacuole, its site of action. Chloroquine can cause retinal toxicity. It is also used in the treatment of lupus disease of humans.	Caldarelli et al. 2012; Rottmann et al. 2010; Sullivan et al. 1998
9	Primaquine	8-aminoquinoline		Yes	Yes	Yes	The drug acts slowly therefore it is often used in combination with chloroquine. It is the drug of choice for elimination of hypnozoites that cause <i>Plasmodium vivax</i> and <i>P. ovale</i> malaria relapses. Liver stage schizonts and sexual stage- gametocytes of all malarial parasites are cleared by it. The drug impairs parasite's mitochondrial functions. Low doses (such as 0.4 mg/kg body weight) of the drug clears gametocytes from blood thus showing its strong anti-transmission activity. It causes haemolytic anemia in people with glucose-6-phosphate dehydrogenase deficiency. It's prodrug bulaquine is similarly efficacious.	Eziefula et al. 2014; Graves et al. 2012; Duffy and Avery 2013 a, b; Howes et al. 2013; La Crue et al. 2013 ; Dow et al. 2011; Dondorp et al. 2010; Wells et al. 2010 ; Mazier et al. 2009; Dutta et al. 1989 http://www.who.int/malaria/mpac/sep2012/

Table 1 (continued)

10	Atovaquone	Napthalene		Yes	Yes	Yes	Combined with proguanil (Malarone/Malanil) the drug is used as a prophylactic and in treatment of malaria. The compound targets cytochrome b1 complex of parasite's mitochondria. This drug although active on liver stage parasites does not kill hypnozoites.	Cordel et al. 2013; La Crue et al. 2013 ; Osei-Akoto et al. 2009; Srivastava and Vaidya 1999
11	Proguanil	Antifolate		No	Yes	NK	Being a pro-drug, its antimalarial metabolite active against blood stage parasites is cycloguanil. When used in combination with atovaquone, the parasites from liver stages are also cleared.	As above; and Fernando et al. 2011
12	Mefloquine	Amino alcohol		No	Yes	Yes	It's tablets (Larium) are used both for prevention and treatment of malaria with one dose of 1250 mg for adults, because it has long half life of 2-4 weeks. It acts by blocking heme polymerization by parasites. The drug has serious side effects in the form of depression, psychosis and nightmares. It lacks blood stage gametocytocidal activity.	Lelievre et al. 2012 ; Sullivan et al. 1998; http://www.drugs.com/pro/mefloquin.html
13	Amodiaquine	4-aminoquinoline		No	Yes	Yes	Rapidly absorbed, it kills parasites by interacting with heme iron. It has side effects on patients in the form of hepatitis, myelotoxicity and agranulocytosis. It has anticancer effect against malignant melanoma cells.	Qiao et al. 2013; Kerb et al. 2009
14	Pyronaridine	As above		Yes	Yes	Yes	Two to four Pyramax tablets each containing 180 mg pyronaridine and 60 mg artesunate are prescribed for 3 days as an ACT. The compound acts by inhibiting beta-haematin formation. It is very active against blood stage gametocytes as well as is ookinete inhibitor in mosquito.	Duffy and Avery 2013 a, b; Delves et al. 2012a, b; Croft et al. 2012; Peatey et al. 2012 ; Auparakkitanon et al. 2006 ; Chang et al. 1992
15	Quinine	Amino alcohol		No	Yes	Yes	It's combination with tetracycline, doxycycline or clindamycin is used in first trimester of pregnant malaria patients. It kills parasites by interfering with haemoglobin ingestion by blocking the endocytotic process and haemoglobin degradation by them. The drug is poorly tolerable because of a variety of side effects.	Lelievre et al. 2012; Achan et al. 2011; Dondorp et al. 2010 a;

NK not known

^aThese activities are responsible for the antimalarial treatment efficacy

^bThe activity against the parasite's gametocytes in humans and/or female mosquitoes and against the parasite's ookinete and/or oocyst stages in female mosquitoes allow the arrest of transmission of infection between humans via infected female mosquitoes

field method to detect the presence of even one parasite in a drop of blood or saliva of a person to be tested. Ariey et al. (2014) have revealed a DNA marker to rapidly detect the presence of any artemisinin-resistant malarial parasites. The essential and novel features of these techniques are discussed in subsequent sections.

Procedures for the rapid detection of malaria infection in single drops of blood

Sensitive techniques are required to identify and manage cases of malaria infection in general as well as ART-resistant malaria, in hospital laboratories and field conditions, to screen populations and for surveillance at ports and borders (Moody 2002; Congpuong et al. 2012; Hsiang et al. 2012; White et al. 2014). Three kinds of diagnostic tests are already available. In the conventional optical microscopic techniques, parasites present in erythrocytes are visualised in films of blood smear

made and stained on a glass slide. This technique, developed in the 1900s, does not detect with certainty the *Plasmodium* parasites present in the blood of an infected person in the pre-parasitaemic stages of infection. The microscopic test is difficult to perform, is time consuming and requires considerable skill. The sensitivity of this test is only 50–500 parasites/ μ L of blood (Warhurst and Williams 1996). Despite its limitations, it is the only WHO-standardized and quality-assured test. The immunochromatographic monoclonal antibody-based dipstick rapid diagnostic tests (RDTs) that qualitatively detect the presence of *Plasmodium* antigens in human blood comprise the second kind of malaria test. It was introduced in the 1990s (Beadle et al. 1994; WHO 2011; Global Fund 2011; FIND 2013; White et al. 2014). One of the RDTs detects the presence of histidine-rich protein 2 (HRP2) of *P. falciparum*. HRP2 is a *Plasmodium* protein present in the parasite's cytoplasm and cell membrane of merozoite-invaded erythrocytes. The HRP2-based RDT detects only the *P. falciparum* infections. Its other deficiency is that the test gives a positive result

even after the parasitaemia has cleared. Another RDT detects lactate dehydrogenase (LDH), the final enzyme of the parasite's glycolytic pathway. The LDH-based RDT detects malaria infection of *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. The sensitivity of RDT is $\leq 90\%$ in infections of 100–200 parasites/ μL of blood. The advantage of RDTs is that the results become available in less than 30 min. The third kind of rapid malaria test is the nucleic acid amplification (NAA) polymerase chain reaction (PCR) technique that is able to detect malaria infection in all stages of parasite development in humans with the sensitivity ranging from ≤ 1 to 6 parasites/ μL of blood. The technique was introduced in the 1990s (Barker et al. 1992; Erdman and Kain 2008; Cordray and Richards-Kortum 2012; WHO 2014). The PCR technique amplifies and detects *Plasmodium*-specific DNA or RNA present in the blood sample taken from persons asymptomatic or symptomatic of malaria disease. Several versions of PCR techniques are available: reverse transcriptase-based small or large subunit 18S rRNA molecular amplification (QT-NASBA) that detects *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* infections; nested PCR by the successive use of two sets of *Plasmodium* genome-specific primer; multiplex PCR which allows detection of more than one *Plasmodium* species (mixed infections); and quantitative PCR that quantifies the targeted *Plasmodium* gene(s). The NAAs are more sensitive than RDTs and microscopy and are useful for revealing the areas of oncoming malaria epidemics. Because of the requirement of specialized equipment and trained personnel, the PCR technique is proving difficult to apply in field conditions. The more recently developed technique of Juul et al. (2012) is sensitive and affordable and of practical utility for surveillance of malaria infections in large populations.

Field-applicable rolling circle-enhanced enzyme activity detection diagnostic test of malarial parasites in single drops of blood or saliva

The new technique that detects a *Plasmodium*-specific enzyme in droplets of blood or saliva is considered easy to use in field conditions on a large scale. The technique called rolling circle-enhanced enzyme activity detection (REEAD) system is used in combination with a fluidic lab-on-a-chip microreactor. REEAD is based on the type 1B topoisomerase activity of the enzyme pTOP1 of *Plasmodium* species (Fig. 3). For carrying out the *Plasmodium*-specific REEAD, a DNA sequence or a reaction substrate (called S) for pTOP1 has been designed. On reacting with pTOP1, the sequence folds into a hairpin structure. The DNA sequence (S) has a single-stranded loop and a double-stranded stem. The loop contains a primer annealing sequence and a probing sequence. One of the

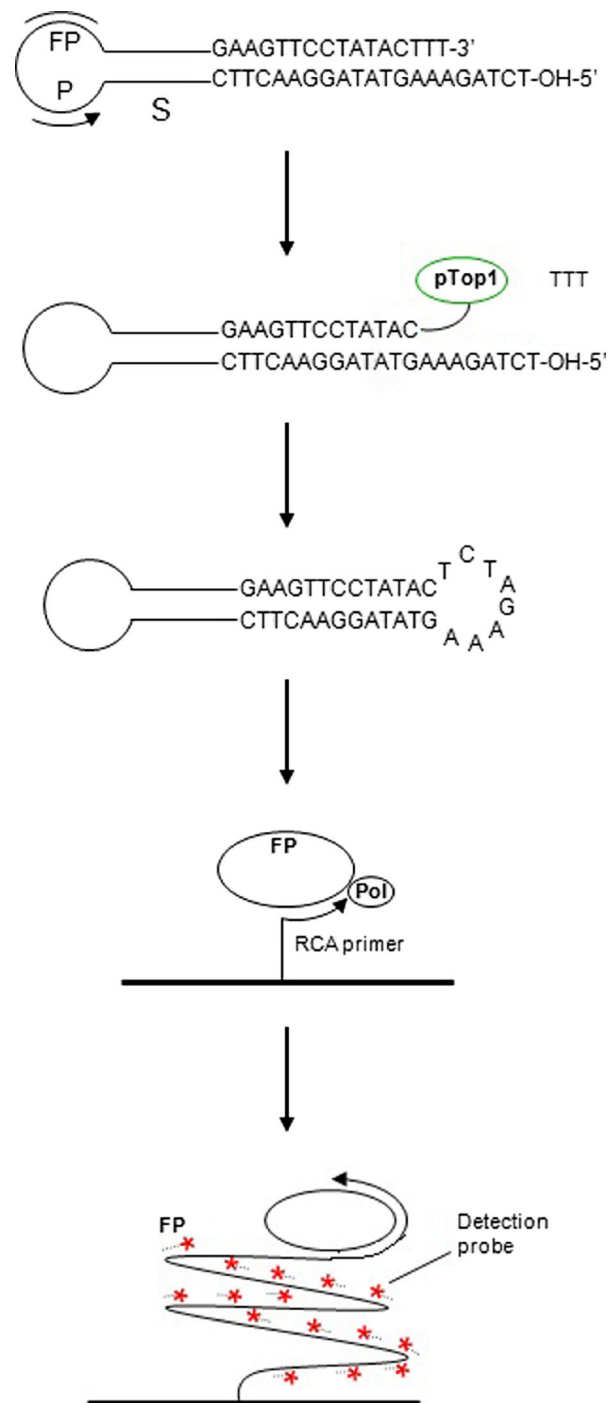


Fig. 3 Design of the rolling circle-enhanced enzyme activity detection (REEAD) system that detects *Plasmodium* in droplets of crude human blood or saliva, for field screening of malaria symptomatics plus asymptomatics in large populations. A drop of blood/saliva and S DNA are added to a lysing buffer. A 5 μL sample of it is deposited on a slide that already has attached to it primers for S DNA. The *Plasmodium* enzyme pTOP1 released from infected blood/saliva reacts with S and via cleavage ligation a single-stranded DNA circle is formed. The P site on it anneals to primer (P) and rolling circle DNA replication occurs producing a long ($\times 10^3$ of original) molecule. Its presence is read by annealing it to a DNA product with rhodamine-labelled complementary (FP) sequence; fluorescent signals are counted microscopically

strands of the stem has a site close to 3'-end that pTOP1 can cleave. Formation of covalent enzyme-DNA intermediate in the stem at the site of cleavage allows religation of the new 3'-end with the protruding 5'-end of the stem's intact strand. Thus, a covalently closed single-stranded circle is formed. The circle serves as template for its extensive ($\geq 10^3$ -fold) amplification by the rolling circle mode, using a small primer (P). The hybridizability of the rolling circle product (RCP) to a short red fluorescent probing nucleotide (FP) sequence allows visual reading of the RCP. The method detects ≤ 1 parasite/ μ L of blood or saliva.

The blood or saliva sample lysed in low salt buffer is added to the DNA substrate and a very small part of it is transferred to a primer-coated slide. The reaction product annealed to the probe is microscopically read for fluorescence. A microfluidic device is used to deposit the reactants on the slide placed on a platform where many reactions can be conducted simultaneously. The REEAD method diagnoses with equal sensitivity the malaria infection caused by the parasites of *P. falciparum*, *P. vivax*, *P. ovale*, *P. knowlesi* and *P. malariae*.

In vitro and ex vivo phenotypic tests of artemisinin-resistant malaria

A significant property of the ART-resistant malaria, of the kind that has emerged in western Cambodia, western Thailand, southern Myanmar and southern Vietnam, is its slower than normal rate of parasite clearance in patients under ACT treatment. The conventional method of measurement of parasite clearance half life is by taking parasite density counts on parasitaemic patients every 6 h, by optical microscopic examination of thick blood films, from the time of start of ART administration to the time disappearance of parasitaemia. However, this kind of diagnosis could require hospitalization of patients for several days. Witkowski et al. (2013) have now provided an in vitro and an ex vivo assay for diagnosing ART-resistant malaria that uses blood sampled from patients before they receive treatment, and hospitalization of patients is not required. In the in vitro ring stage survival assay (RSA_{0-3 h}), the parasites isolated from blood are culture adapted. The parasites in culture are then quantified for the time taken by ART to clear their infection in fresh erythrocytes. In the ex vivo assay, parasites in the blood are cultured directly in the presence and absence of ART. The parasite survival rate suggests whether the patient suffered from ART-sensitive or ART-resistant malaria. The ex vivo test is suitable for large-scale surveillance for ART resistance in affected human populations.

Identification of the principal DNA marker of *P. falciparum* associated with artemisinin-resistant malaria prevalent in Southeast Asia

Artemisinin-resistant malaria in Southeast Asia has been found to be associated with mutations in K13 propeller domain of the PF3D7_134700 gene of *P. falciparum*. Ariey et al. (2014) found that ART resistance experimentally generated in a line of *P. falciparum* and ART resistance that naturally evolved in many *P. falciparum* parasites present in malaria patients in Cambodia carried mutations at the same locus called PF3D7_1343700. They showed that mutations in the kelch K13 propeller domain part of the gene PF3D7_1343700 makes *P. falciparum* resistant to ARTs (Fig. 4).

The wild-type ART-sensitive Tanzanian clone (line) F32 of *P. falciparum* upon in vitro culturing for 125 cycles in the presence of escalating concentrations of artemisinin developed into an ART-resistant line called F32-ART5. Comparison of the whole genome sequences of the parental ART-sensitive F32 line and derived ART-resistant F32-ART5 line detected eight point mutations in seven genes in the F32-ART5 line. Sequencing of 49 parasite lines collected from malaria patients which responded differentially to ART treatment, in the conventional and developed procedures of Witkowski et al., demonstrated polymorphism at the PF3D7_1343700 locus. The ART-resistant Cambodian lines and F32-ART5 carried mutations in the same general region that is kelch K13 propeller region of the PF3D7_1343700 locus. The locus PF3D7_1343700 was wild type in the ART-sensitive Cambodian lines. These observations implied that mutations in the K13 propeller domain of the protein product of PF3D7_1343700 gene rendered *P. falciparum* resistant to artemisinins. It was possible to rule out the association between ART resistance and mutation in five other genes of F32-ART5 and mutations/polymorphism observed at other loci in previous *P. falciparum* population genetic studies in areas of ART resistance (Table 2). Among the $\leq 1,000$ Cambodian clinical parasite isolates that had been genotyped, K13 propeller mutations were found at 17 different sites in PF3D7_1343700 gene. Apparently independent mutations in K13 propeller site were responsible for *P. falciparum* resistance to ART in Cambodia. Involvement of other genes in the determination of ART resistance has not been ruled out. For the present, K13 propeller sequence of PF3D7_1343700 locus can serve as a reliable DNA marker for surveillance of ART resistance in Southeast Asia.

A question has arisen about the origin and selection of 17 independent recessive mutations at a locus in a gene in a small sample of $\leq 1,000$ of *P. falciparum* prevalent in provinces of western Cambodia. What mechanisms could be responsible? There is a possibility that the oxidative stress created by free radicals generated by breakage of the endoperoxide bridge of artemisinin may be responsible. Since the malarial parasite

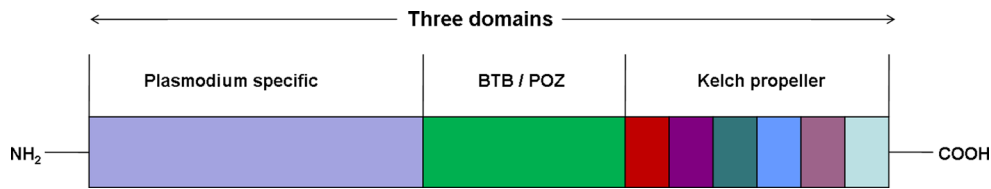


Fig. 4 Diagramme of the structure of PF3D7_143700 protein of *Plasmodium falciparum*. Kelch motives located towards the carboxy terminal are found in a large number of eukaryotic proteins. The KLHL2 human proteins involved in ubiquitin-mediated protein degradation and KEAP1 human protein involved in cellular adaptation to oxidative stress have domains homologous to kelch K13 propeller of *Plasmodium*. It is thought that for its activity, PF3D7_143700 folds into a six-bladed propeller supported by its interaction with C-terminal beta sheet and N-terminal

blade. It is thought that PF3D7_143700 protein is involved in protein degradation-led cytoprotection against oxidative stress imposed by artemisinin. This process is disrupted by mutations in K13 propeller (Ariey et al. 2014; Mitsuishi et al. 2012; Prag and Adams 2003). However, the full mechanism by which the absence of intact PF3D7_143700 gene protects the parasites from the killing action of artemisinin remains to be understood

genome is methylated at cytosines (Gupta et al. 2013; Ponts et al. 2013) and parasite methylome is a known target of the antimalarial drugs, the ART-imposed stress may result in hypomethylation of the parasite genome; a variety of environmental stresses are known to cause genomic hypomethylation in a variety of eukaryotic systems (Kumar et al. 2013). It is also known that demethylated cytosines are mutational hot spots (see Kumar et al. 2013 for review on epigenetics in plants). Although mutations may occur randomly over the genome, the presence of ART may allow selection of resistance mutations against itself in the isolated niches of *P. falciparum* occurrence in Cambodia. Mosquito gut provides the opportunity for the production of homozygotes for K13 propeller mutation from a population of gametocytes segregating for the mutation received by the mosquito via blood

meal, from a patient in whose liver and/or blood cells parasite's schizonts or merozoites acquired the ART-resistant mutation. This idea will get tested in future studies on the ART-exposed *P. falciparum* in vitro and on the extent of genetic polymorphism in *P. falciparum* populations in Southeast Asia.

Advancements towards the prevention of malaria transmission from person to person

Interference with transmission is the means to reduce infection reservoir from areas of occurrence of malaria. Blocking transmission involves several complementary approaches (Fig. 2). These include elimination of the malaria vector mosquitoes, prevention of mosquito bites and killing of malaria parasite

Table 2 Evidence about the association of point mutations in the propeller domain of K13 gene/PF3D7_1343700 locus with artemisinin (ART) resistance, observed in in vitro and in vivo populations, of the malarial parasite *Plasmodium falciparum*. In the malaria patients

undergoing artemisinin combination therapy (ACT), K13 mutant parasites get cleared from the red blood cells much more slowly than the rapidly cleared wild-type parasites

Genetic polymorphism observed in	Genes/loci in which variation was observed to be	
	Associated with ART resistance	Not accountable for ART resistance
In vitro parasite line selected against high concentrations of artemisinin in culture medium	PF3D7_0110400 and PF3D7_1343700 ^a	PF3D7_0213400, PF3D7_1115700, PF3D7_1302100, PF3D7_1459600 and PF3D7_1464500
Natural populations of parasite of western Cambodia and adjoining areas where ART-resistant malaria has been documented, under deployment of ACT as the first line of malaria treatment	PF3D7_1343700 ^{a, b}	PfABC transporter, PfATPase6, Pfert, PfCTP, Pfdhps, Pfdhfr, PfGTP-cyclohydrolase 1, Pfmdr1, Pfmrp1, Pfubcth, PF3D7_145960; MALIO-688956, MAL13-1718319, MAL13-1719976; MAL14-718269, SNPs denoted 1 and 2 and PF10_0355
Natural population in Gambia		PF3D7_1343700 ^c
References	Ariey et al. (2014)	Ariey et al. (2014), Miotto et al. (2013), Takala-Harrison et al. (2013), Amambua-Ngwa et al. (2012), Cheeseman et al. (2012), Cui et al. (2012), Pillai et al. (2012), Van Tyne et al. (2011), Yuan et al. (2011), Plowe (2009), Sidhu et al. (2006)

^a Non-synonymous point mutations in the kelch propeller domain of the K13 gene

^b Seventeen different K13 propeller mutations, responsible for slow clearing of malaria treated with ART, have been identified in 886 parasite isolates from Cambodia

^c Synonymous mutation in the K13 propeller domain

during its development in the vector mosquito. The subject areas of mosquitocides, mosquito repellence and mosquito management in general have been respectively reviewed recently by Prato et al. (2012), Maia and Moore (2011), Dickens and Bohbot (2013) and Beier et al. (2008). New results in each of the three approaches that offer promise of their successful contribution in the control of malaria are discussed below.

Killing of *Plasmodium* in mosquito

Female *Anopheles* mosquitoes transmit malaria while biting and taking blood meals from humans. When a mosquito bites a malarial human, it obtains along with blood 10^3 to 10^4 sexual stage haploid male and female *Plasmodium* gametocytes. The gametocytes mature into gametes, fertilization occurs and zygotes form in the mosquito midgut. From the zygotes, 10^2 to 10^3 motile ookinetes are formed. These diploid asexual ookinetes form oocysts. Thousands of motile sporozoites released from oocysts into haemocoel accumulate in the salivary glands of the mosquito. Each of the *Plasmodium*'s developmental stages in mosquito is a potential target for controlling malaria transmission. Two approaches are being pursued to make mosquito refractory to *Plasmodium*. One of these aims to genetically engineer mosquito genome by the use of the clustered regularly interspaced short palindromic repeat (CRISPR) Cas9 associated system. It is aimed on the one hand to disrupt or delete the genes in mosquito that are essential for *Plasmodium*'s sporogony and, on the other hand, to add heterologous genes that will antagonise *Plasmodium*'s survival in mosquito (Oye et al. 2014; Esvelt et al. 2014). Upon their release as per regulations, the genetically engineered mosquitoes are expected to spread the *Plasmodium*-resistant trait into the local population of mosquito (Marshall 2009; Reeves et al. 2012). In the second approach, the wild-type or genetically engineered bacteria that secrete compounds lethal to *Plasmodium* are made to co-reside with *Plasmodium* in the gut of the female mosquitoes. Two experimental results of the approach offer hopes for the development of field-applicable technologies to block malaria transmission.

Bian et al. (2013) have isolated a line of *A. stephensi* mosquito in whose gut the gram-negative bacterium *Wolbachia* (strain WA1bB) has resided stably for 34 generations. *Wolbachia* is harmless to mosquitoes and humans. It was already known that *Wolbachia*, not a normal resident of mosquito gut, is lethal to parasite when present together with it. The mosquitoes that are stably carrying *Wolbachia* WA1bB have been observed to be stably refractory to *Plasmodium*. Malarial parasite's reproductive development is completely suppressed in the *Wolbachia*-carrying female mosquitoes. In the new mosquito line, female mosquito egg cytoplasm transmits *Wolbachia* to the progeny upon mating with either infected or uninfected males. An experimental release of

Wolbachia-infected females into a noninfected mosquito population led to all mosquitoes acquiring *Wolbachia* in less than ten generations. Replacement of mosquito population in a malaria-infected area with *Wolbachia*-infected mosquitoes is expected to reduce/stop malaria transmission.

The bacterium *Pantoea agglomerans* is a part of mosquito's microbiome and co-resides with *Plasmodium*. Wang et al. (2012) genetically engineered *P. agglomerans* such that its population in mosquito midgut will kill the sympatric *Plasmodium* population. The genetically modified *P. agglomerans* secreted the peptide scorpine or other such proteins that inhibited *Plasmodium*'s developmental pathway for sporozoite formation. Its presence in the mosquito gut inhibited *P. falciparum* and *Plasmodium berghei* by up to 98 %. In the treated mosquito population, only 16 % carried live parasites. Having no effect on humans and the mosquito life cycle, the engineered *P. agglomerans* is highly promising for field release. It could be spread among mosquitoes by placing in malaria-affected areas many clay jars containing cotton balls soaked in sugar and bacteria to serve as baiting stations.

Malaria prevention by keeping mosquitoes away from the human body

Malaria bites on humans are preventable in several ways. Mosquitocides are sprayed on the walls of homes. Insecticide-treated bed nets are used to protect people while sleeping during nights. Poisonous baits are used to attract and kill mosquitoes in homes and in the open near water bodies (Jawara et al. 2009; Okumu et al. 2010; Smithuis et al. 2013). People are protected by repelling mosquitoes away from them by spraying chemicals that cause hypnosia in mosquitoes, on walls, curtains and other suitable stations in homes (Agusto et al. 2013). Mosquito-repellent-laden bands and patches are used on human clothing and skin; repellents are also added to soaps, shampoos and lotions. There have been developments in each of these strategies to prevent malaria transmission.

Identification of new mosquito repellents

Mosquitoes land on surfaces attracted by certain odours. Their chemoreceptors for odours are borne on sensilla that are attached to the organs antennae, maxillary palp and proboscis (De Moraes et al. 2014; Touhara and Voss 2009). The chemoreceptors are non-selective ion channels, each consisting of two proteins, an odour receptor (OR) and a co-receptor called Or83b (orco) which is sensitive to both agonist and antagonist odours (Dickens and Bohbot 2013). Carbon dioxide, lactic acid and octenol present in breath and sweat attract mosquitoes to humans. Malarial parasite-infected female mosquitoes possess 3-fold more attraction to humans than the uninfected

mosquitoes (Smallegange et al. 2013). The olfactory sensitivity of mosquitoes is extra high at night (Rund et al. 2013). Mosquito repellents act by shutting the receptors that function in tandem with Or83b. The *orco* gets overactivated leading to scrabbling of the sense of smell in mosquito such that humans become invisible to them. N,N-diethyl-meta-toluamide (DEET; Fig. 5a) has been a much used mosquito repellent for the last several decades. DeGannarro et al. (2013) by comparing the effect of DEET on wild-type and mutant *orco* mosquitoes have shown that DEET action is dual. On one hand, it is a strong repellent in the absence CO₂, and on the other hand, it deters biting when present on human skin, irrespective of CO₂. The search for new repellents, in view of the strong unfavourable smell of DEET, epigenetic changes that it causes and development of tolerance towards DEET in mosquitoes (Bernier 2013; Stanezyk et al. 2013), has led to the discovery of safer and more efficacious compounds than DEET.

Bernier (2013) has developed a formulation, containing homopiperazine (Fig. 5b), 1-methylhomopiperazine (Fig. 5c) and several other human skin secretion compounds, which is more effective than DEET. Chen and Leutje (2013) have identified several *orco* antagonists of phenylthiophenecarboxamide class of compounds which have the potential to serve as mosquito repellents (Fig. 5d). Kain et al. (2013) have identified several anthranilates [methyl N,N-dimethyl anthranilate, ethyl anthranilate and butyl anthranilate (Fig. 5e–g)] compounds present in fruits which could be used as pleasant smelling, safe and effective mosquito repellents much superior than DEET. Jones et al. (2011) and Pask et al. (2013) have reported about a group of Vanderbilt University Allosteric Agonists (VUAA) such as VUAA1 (Fig. 5h) and amiloride derivatives which are up to hundred thousand fold more potent in activating *orco* than DEET. Besides, a variety of essential oils rich in geraniol, linalool and/or α -pinene have been shown to be effective mosquito repellents. Some of the fatty oils such as those of castor, *Jatropha* and catnip have also been observed to be effective mosquito repellents. Many such preparations are listed in en.wikipedia.org/wiki/insect_repellent.

Causing reduction in the indoor mosquito population

Placement of aromatic plants such as of *Lantana* species, and likewise those of *Lippia* and *Geranium*, that spontaneously release essential oils into the air, has proven to be an inexpensive means of reducing mosquito population indoors (Mng'ong'o et al. 2011). *Lantana camara* adult plants kept in homes were observed to reduce mosquito population by 50 %. Similarly, cultures of the skin bacterium *Staphylococcus epidermidis* strain DSMZ 11047 can be used to inactivate mosquitoes indoors. The bacterial population produces volatile organic compounds that disrupt the behaviour of mosquitoes (*A. gambiae*). The stations of bacterial culture can also be used

to trap mosquitoes or kill them by combining an insecticide with bacterial culture (European patent application EP2 140764A1/08159618.1, 2010).

Killing of mosquitoes by attracting them to toxic baits

A simple and widely affordable method called attractive toxic sugar bait (ATSB) that controls mosquitoes in the countryside has been found successful when tested at several field locations. In the ATSB procedure, the sweet bait material is produced locally and sprayed on plants growing near the water bodies. The principal component of ATSB is fruit juices from such diverse plants, locally growing in African locations, as of nectarine, guava, honeymelon and cactus. ATSB contains 60–70 % v/v fruit juice, 5–25 % v/v wine and 10–20 % w/v brown sugar. This pulpy material is kept for fermentation for a few days. Finally, 1 % w/v Biostab (a mixture of antibacterial and antifungal substances standardized in Israel that serves as a preservative) and 1 % w/v boric acid (the killing agent) are added. Mosquitoes get attracted to the sprayed plants and intake by them of the sweet mix kills them. The procedure has proven effective in curtailing the local mosquito populations in several different African geographical locations where it was field tested (Muller et al. 2010; Beier et al. 2012; Marshall et al. 2013).

New mosquitocidal formulations

Although costly, spraying of insecticides in homes and the use of insecticide-treated bed nets have contributed immensely in reducing malaria morbidity and mortality. Insecticides of organochlorine, organophosphate, carbamate and pyrethroid classes have been used at various times and locations. Mosquitoes are known to have developed resistance to one or more insecticides of all classes of insecticide compounds at different locations. A single mutation in the upregulated gene for glutathione S-transferase (GSTe2) made a local population of *A. funestus* malaria vector resistant to DDT as well as pyrethroids in Benin, West Africa (Riveron et al. 2014). Mutations allowing overexpression of P450 family genes *CYP6M2* and *CYP6P3* and duplication of the genes *ACE-1* (allele *G119S*) encoding acetylcholine esterase have made *A. gambiae* population of Tiassale in West Africa resistant to all four kinds of insecticides: carbamates, DDT, organophosphates and pyrethroids (Edi et al. 2014).

Pyrethroids are safe, affordable and effective and have long-lasting effects. Therefore, they have been the first choice in malaria control. Since new types of insecticides are yet to arrive, the alternatives to pyrethroids continue to be organophosphates and carbamates. To avoid resistance development, the use of insecticides in rotation is apparent. Whereas one chemical class of insecticide is used for spraying of walls of homes, another

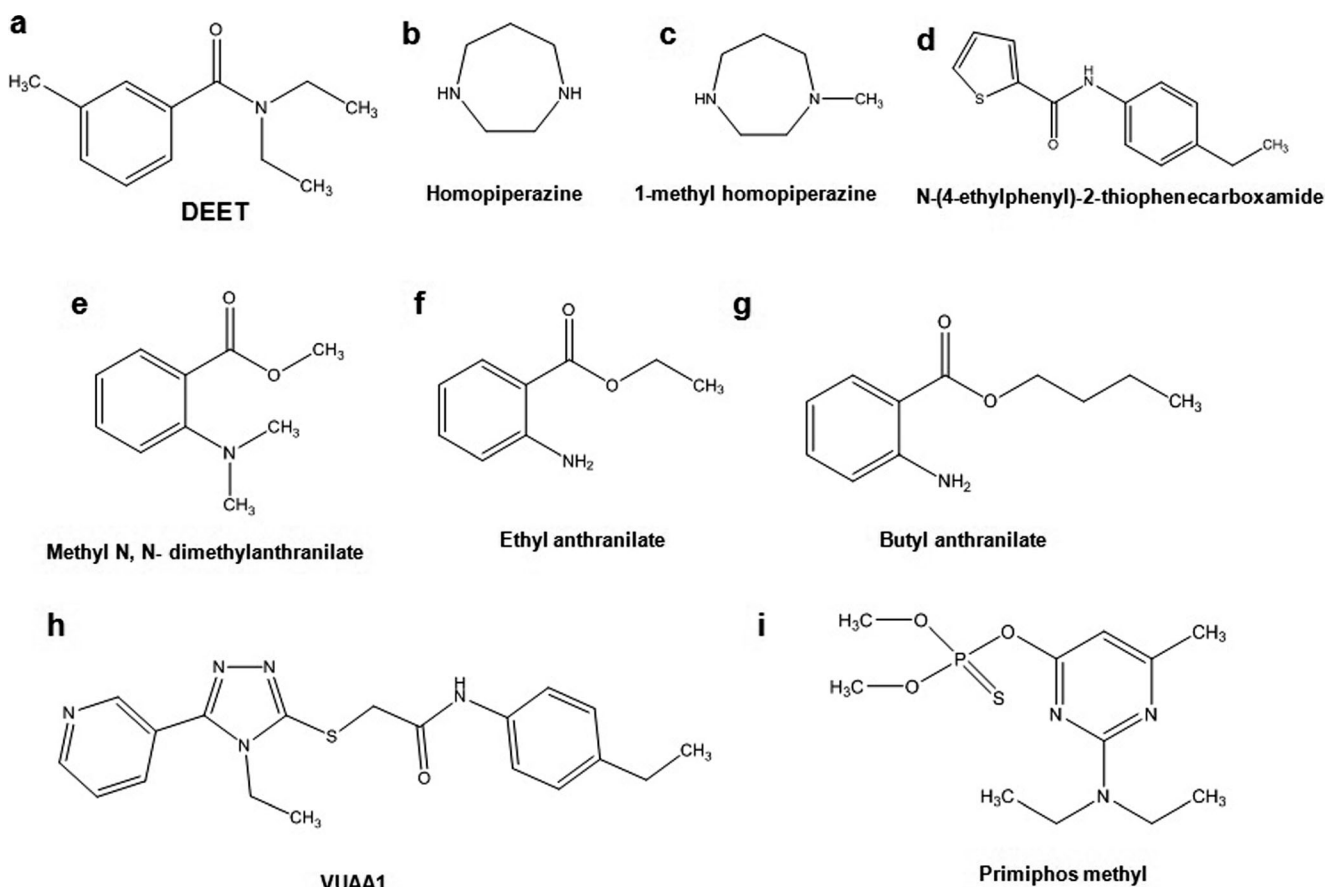


Fig. 5 a–i Chemical structures of the conventional mosquito-repellent DEET and some new mosquito repellents of promise and the insecticide primiphosmethyl

kind of insecticide must be used for preparing insecticide-laden bed nets. To control pyrethroid resistance in the environment of sub-Saharan Africa, Rowland et al. (2013) have recommended a long-lasting encapsulated formulation for use in spraying of walls. It consists of the organophosphate insecticide primiphosmethyl (Fig. 5i). The effect of formulation lasted for about 1 year, whereas the effects of other sprayings lasted for only a few months. Thus far, only the DDT sprays had long-lasting antimosquito effect but DDT is now banned. The new formulation of organophosphate has doubled the lasting effect of DDT which was observed to be 6 months. Several formulations similar to that of Rowland et al. are on the horizon. The use of methoprene on infested soil, mud or puddles and oil film on sedentary water as larvicides and pyrethroid sprays on walls or fogging of environment with malathion in homes as adulticides has proven effective in the control of local mosquito populations (Meister 1992).

Drugs that kill mosquitoes and thereby block sporozoite transmission

The use of the endectocide ivermectin to reduce malaria transmission as well as to kill mosquitoes has been

proposed, and experiments on the effectivity and safety of this tool have been initiated (Chaccour et al. 2013). Ivermectin (Fig. 6) is semisynthesized from a *Streptomyces avermectinius* fermentation precursor. It has been in use for the last 25 years for the control of onchocerciasis and lymphatic filariasis (Omura 2008). Ivermectin is known to agonize glutamate-gated chloride channels of invertebrates which lead to their flaccid paralysis and death. *Anopheles* mosquitoes have proven to be highly sensitive to ivermectin (Tesh and Guzman 1990). Presently, to control onchocerciasis and filariasis, ivermectin is administered to all residents of a village on a single day. In such villages, mosquitoes that bite humans die because they acquire ivermectin with blood meal. Repetition of ivermectin administration in malaria-burdened villages has been identified as a means to kill mosquitoes and thereby reduce transmission. Another approach considered is to add ivermectin to ACT drugs. The new combination containing three drugs, including ivermectin, will kill malaria parasites in the human body and also kill mosquitoes that may bite the treated patients. The transmission of malaria parasite will be checked because the *Plasmodium*-infected mosquitoes will be eliminated.

Protecting humans during their sleep

Malaria vector female mosquitoes mostly bite people at night-time when the latter are asleep. Two inter-country population studies have revealed that malaria transmission can be eliminated altogether by precautionary measures taken at the human population level. Augusto et al. (2013) concluded from their survey that the use of bed nets by 75 % of the population is sufficient for eradication of malaria from any region. Hulden et al. (2013) reached to the conclusion that segmentation of sleeping quarters in households can be a means of stopping malaria transmission. They observed that malaria transmission was negligible where the number of persons sleeping in a bed room was less than four.

Eradication of malaria mosquito by the use of male sterile genetic technique

Experiments are in progress to adapt, in malaria mosquitoes for their control, the conventional and CRISPR-associated genetic engineering techniques to cause heritable male sterility that has been successfully used for eradication of several agricultural pests (Knipling 1959; Whitten and Mahon 2005; Alphey 2014; Esvelt et al. 2014; Oye et al. 2014). In this method, male sterile mosquitoes will be released in cycles until the area becomes free of mosquitoes. The field-released male sterile spermless mosquitoes will mate with females but copulated females will not produce any offspring. Thus, each time male sterile mosquitoes will be released, the size of the mosquito population will get reduced. The technique has been successful in field test conditions for the control of *Aedes aegypti*, the dengue-spreading mosquito (Harris et al. 2012; Lacroix et al. 2012). Thailayil et al. (2011) have developed a male sterile line of malarial parasite transmitting *A. gambiae* mosquito, by RNAi silencing of the germ cell differentiation gene ZERO

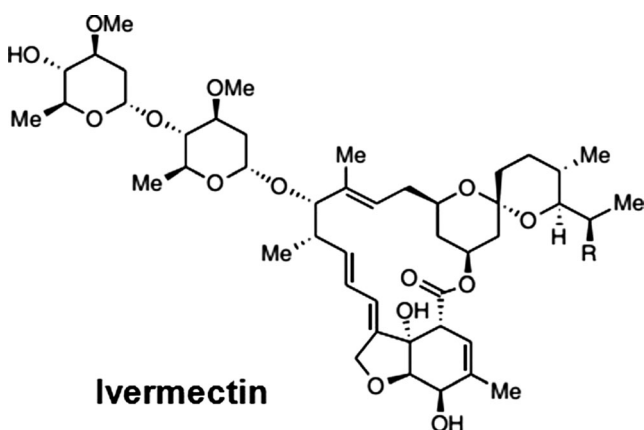


Fig. 6 Chemical structure of ivermectin, the compound suitable to add to artemisinin combination therapy drugs to combat malaria transmission on account of its mosquitocidal activity

POPULATION GROWTH (ZPG). The genetically modified mosquitoes were found to copulate with wild-type females normally. They switched off in the females the receptivity for further copulation. Subsequent responses of the females were also normal. The spermless mosquitoes are expected to be field tested for mosquito control. The use of the GM mosquitoes will require extensive pre-release safety trials.

Vaccine for malaria prevention

Presently, millions of lives are being saved from malaria morbidity and mortality by the use of drugs that either block malaria transmission or treat malaria disease. Vaccine against malaria could be a cost-effective means to eradicate malaria by reducing the transmission of parasite. Several different approaches to develop efficacious vaccine(s) to stop malaria are in progress. The subject has been recently reviewed by Schwartz (2012) and Riley and Stewart (2013). Although 27 malaria vaccines are under clinical trials, no effective vaccine is as yet available (Moorthy et al. 2013; Cowan et al. 2014). The ongoing programmes are using one or more of the proteins present on the surface of sporozoites and/or merozoites of *Plasmodium* as antigens. The genetic polymorphism present in the parasite population of an area and among populations of different areas is proving to be a challenge in vaccine development. The RTS,S has been the first-generation malaria vaccine candidate now undergoing phase 3 trials. Besides, there are a few second-generation promising malaria vaccine candidates on which experiments are continuing. Greater understanding of *Plasmodium* parasite-human host interaction, aided by second-generation genome sequencing and functional genomics of both host and pathogen, will be valuable in improving the vaccines under development and conceptualization of new vaccines.

RTS,S vaccine candidate

The antigen in RTS,S is a hybrid protein, in which R and T domains of the circumsporozoite protein, the major coat protein of the sporozoites of *P. falciparum*, are covalently linked to the hepatitis B virus antigen (HBs). The vaccine is formulated in the form of virus particles in a liposomal adjuvant (A5P1). The vaccine antagonizes the invasion by sporozoites of host liver hepatocytes; thus, the formation of merozoites in the liver is prevented. RTS,S induces high antibody titres against *P. falciparum* circumsporozoite protein (CSP) and a moderate CD4⁺ T cell response. The children vaccinated with RTS,S got protected against uncomplicated as well as severe malarial (Riley and Stewart 2013).

RTS,S is now in the final stages of efficacy trials that started in 1984 and is expected to be released for use in 2015, perhaps

in selected geographical areas of high malaria burden. A year after vaccination with RTS,S, about half of young children subjects (aged 5–17 months at the time of vaccination) and one third of infants (aged 6–12 weeks at the time of vaccination) were found malaria protected. However, protection did not last long. The incidence of malaria among the vaccinated children and infants increased progressively over time. Four years from the time of vaccination, protection against malaria in the vaccinated subjects was zero. Recently summarized results of a phase 3 randomized control trial on 8,923 children and 6,537 infants in Africa have led to the estimate that during 18 months after three dose standard vaccination with RTS,S/ASO 1 malaria vaccine, the vaccinated children and infants respectively averted 83 and 45% of clinical cases of malaria (The RTS,S Clinical Trials Partnership 2014).

It is believed that booster dosing of the safe RTS,S vaccine may provide sustained immunity against malaria (Agnandji et al. 2012; Olutu et al. 2013; Riley and Stewart 2013; Fouquet et al. 2014). GlaxoSmithKline have filed an application with the European Medicine Agency for the licensing of RTS,S vaccine.

Whole parasite vaccine of sporozoites attenuated by ionizing radiation(s)

Seder et al. (2013) have developed the PfSPZ vaccine which gave 100 % protection in the phase 1 safety trial. The vaccine consists of metabolically active but replicatively inactive sporozoites that are attenuated by exposure to ionizing radiations (irrspz). The irrspz sporozoites are isolated from the salivary glands of irradiated mosquitoes which are raised under sterile conditions. To generate sporozoites, mosquitoes are fed with malarial parasite-infected human blood. The purified cryopreserved irrspz are used as intravenously administered vaccine. In the phase 1 trial, six volunteers who had been injected with five doses of vaccine did not develop malaria when they were subjected to bites by malaria-infected female mosquitoes. However, among the nine volunteers who were injected with four doses of vaccine, only four demonstrated complete protection from malaria. In all of the 16 subjects, there was a correlation between vaccine dose and antibody level plus immune response. The RTS,S safe vaccine now requires regulatory standardization. New techniques need to be developed for efficient mass production and genetic attenuation of sporozoites to make the vaccine affordable.

Vaccine of genetically attenuated sporozoites

As an alternative to the irradiation-attenuated sporozoites serving as vaccine, Mikolajczak et al. (2014) have developed a line of *P. falciparum* in which three of the genes involved in the development of malaria disease in humans are deleted.

The deleted genes are *P36* and *P52* whose protein products function in the formation of parasitophorous vacuole in human cells where the parasite invades, grows and multiplies and *SAPI* which specifies a protein that regulates RNA stability and thereby the expression of parasites genome in general. The triple mutant has normal gametocytogenesis, mosquito infectivity and sporozoite production. In the humanized mouse model system that harbors human hepatocytes and human erythrocytes, the sporozoites do not progress to produce merozoites and liver stage does not transition into the blood stage. The *p36⁻ p52⁻ sapI⁻* vaccine now awaits assessment of safety, effective induction of immune responses and efficacy against infectious wild-type sporozoites.

Novel vaccine candidates, synthetic MSP-1 and SEA-1

Two new antigens identified are as follows: SEA-1, a protein required for egress of schizonts from erythrocytes; and MSP-1, a merozoite surface protein. Synthetic antigen consisting only of the N- and C-terminal regions of MSP-1 expressed in the ciliate *Tetrahymena thermophila* as a recombinant protein elicits antibodies in mice which provided protection against lethal malaria (Cowan et al. 2014). rPf SEA-1 vaccinated Tanzanian children did not experience severe malaria. Similarly, Kenyan adolescents and adults possessing antibodies against rPf SEA-1 had lower parasite densities than people who did not produce these antibodies (Raj et al. 2014). The two antigens may be used together with others such as RTS,S and p36p52sap1 vaccines.

AMA1-based vaccines

Apical membrane antigen 1 (AMA1) is a microneme protein of *P. falciparum* present in both sporozoites and merozoites of the parasite. It is essential for the invasion of hepatocytes by sporozoites and of red blood cells by merozoites. Antibodies against AMA1 block the multiplication of parasites in both the liver and blood stages of the parasite's life cycle in human host (Dutta et al. 2013). One of the vaccines based on AMA1 which is undergoing phase 2 trials in Mali is FMP 2.1/A502A. In this vaccine, AMA1 has been resourced from the corresponding gene of the field strain 3D7 of the *P. falciparum* parasite. The recombinant form of AMA1 protein called FMP2.1 has been formulated in the A502A adjuvant system. Children of the age group 1–6 years were administered the vaccine at 0, 1 and 2 months and followed for 1 year. The vaccine proved to be safe and well tolerated; it induced and sustained high levels of antibodies against AMA1 in malaria-exposed children. FMP2.1/A502A, is now undergoing phase 2 trial in Mali. If it is found successful in phase 2, FMP2.1/

A502A may be used in combination with RTS,S vaccine to achieve additive or synergistic effects (Thera et al. 2012).

AMA1 demonstrates considerable variability among *P. falciparum* field isolates. Therefore, there is doubt on its success to stop malaria caused by *P. falciparum* bearing AMA1 alleles genetically diverse from 3D7 type of parasites. Dutta et al. (2013) have developed AMA1-based Quadvax or QV vaccine, a mixed allele vaccine. Here, AMA1 proteins from four strains of *P. falciparum* namely 3D7, FVO, HB3 and W2mef have been combined to serve as antigen. Antibodies formed against Quadvax were inhibitive to a total of 26 parasite strains which perhaps represented global AMA1 diversity. In an early trial, Quadvax provided 100 % protection. Now, to further improve the efficacy of the AMA1 vaccine, to cover all of the polymorphisms in its structure in natural populations of the parasite, the region of AMA1 that interacts with RON2 protein of the parasite, which is perhaps conserved, has been used in vaccine construction (Dutta et al. 2013; Srinivasan et al. 2014). The AMA1 and RON2 complex injected into mice protected the vaccinated mice from lethal malaria (Srinivasan et al. 2014).

AMA1 in its various forms, RTS,S and other vaccines offer promise of affordable vaccines to eradicate malaria. They may be used in combination to harvest their additive or synergistic antibody responses.

Increased genetic variation in parasite, absence of vaccine and resistance towards approved drugs necessitate discovery of new antimalarials

In recent years, studies have compared the genetic structure of *P. falciparum* populations occurring in Africa, America (Oceania) and Asia (Southeast Asia), especially in the context of emergence of ART resistance in Southeast Asia. Van Tyne et al. (2011) investigated polymorphism for 17,000 single nucleotide polymorphic (SNP) markers in 57 culture-adapted parasites from representative countries of the three continents. Manske et al. (2012) genotyped parasites present in 227 blood samples of malaria patients of six countries of Southeast Asia and Africa, for polymorphism at 86,158 SNPs. Takala-Harrison et al. (2013) examined 290 parasite samples from Bangladesh, Thailand and Cambodia for polymorphism at 8,079 SNPs. Miotto et al. (2013) studied 825 parasite samples, from ten locations in several countries in Africa and from Thailand, Vietnam and four locations in Cambodia, for polymorphism at 86,158 SNPs. Ariey et al. (2014) by screening several hundred ($n=886$) blood samples of malaria patients found 17 different alleles of the gene PF3D7_1343700 to be responsible for artemisinin resistance in the studied Cambodian population. The observations from the above-mentioned studies altogether indicate the following about *P. falciparum* malaria: (a) There are inter-continental

genetic differences in the *Plasmodium* populations since the principal component analysis showed that the parasites clustered together continent-wise. Intra-continental genetic variation-wise, the parasites fell in the following order Africa>Asia>America (van Tyne et al. 2011). These observations are consistent with the origin of *P. falciparum* being in Africa and its presence in other continents a result of independent migration of and founding of subpopulations. Very high levels of genetic variability in Africa are related to high levels of malaria transmission there (van Tyne et al. 2011). On account of high transmission, humans infected by sporozoites of different genotypes by a mosquito or more than one mosquito will produce gametocytes of many different genotypes which upon transmission to mosquito will produce gametes that upon recombination will amplify the genetic variability further. (b) In Cambodia and adjoining countries, a high incidence of selection of ART resistance imparting recessive mutations suggests a high level of inbreeding and hypermutability in parasites (Miotto et al. 2013). It is reported that many human communities live there in small villages with little interaction (Takala-Harrison et al. 2013). The hypermutability may be due to the genetic stress imposed by malaria drugs leading to hypomethylation of genomic DNA (discussed in an earlier section). (c) The inter- and intra-continental genetic diversity, mutability imposed by drug pressure and high recombination rate (17 kb/CM; Su et al. 1999) are continuously increasing divergence between parasites at different locations. The growing genetic variability is creating obstacles in the form of new antigenic variation for the formulation of a globally effective vaccine. These concepts are diagrammed in Fig. 7.

It appears that until effective vaccine(s) can be developed, the control of malaria with the use of new antimalarials is an essentiality (Dondorp et al. 2010a, b; Wells 2010).

Search for new antimalarials in the background of artemisinin resistance

In recent years, the ongoing malaria control programme got derailed because of the emergence of resistance in malaria parasites to ART; mosquito vectors of malaria had already developed resistance to insecticides. Artemisinin in ACTs had been performing two functions: it, along with an accompanying compound, cured parasitaemia in infected humans by killing the asexual blood stage parasites and it also killed gametocytes present in the blood (especially male gametocytes) and thereby reduced the occurrence of new infections. Post-ART resistance, the malaria control strategy has been resurrected. In the absence of an efficacious vaccine, it seeks antimalarials that will target sexual (sporogony) stages of the parasite in mosquito and the parasite's asexual schizogonous stages in the liver and blood and sexual blood stage in

humans. These four kinds of antimalarial activities are sought in one compound or in combination of two or three compounds. Most importantly, all of the new generation antimalarials must target strongly the asexual blood stages of parasites resistant or sensitive to the first-generation antimalarials; they must cure malaria and must be safe and selective against parasite. The concepts, approaches and progress being made in the search for the desired kinds of antimalarials have been reviewed by a number of groups including the following: Gamo et al. (2010), Guiguemde et al. (2010), Aguiar et al. (2012), Derbyshire et al. (2012b), Delves et al. (2012b, 2013), Kirkpatrick (2012), Ghosh (2013), Flannery et al. (2013a), Klein (2013) and Sun et al. (2014).

Campaigns to identify new antimalarials

A variety of in vitro and in vivo tests are available to find out whether or not a given compound has antimalarial activity. Some of the in vitro tests can be practised as throughput screens on 96, 384 or higher number of well plates. In the conventional type in vitro screens, the asexually replicating parasites in culture (Trager and Jensen 1976) synchronized for ring stage are exposed to a specific dose of a test compound

and the effect is monitored variously at the schizont stage, in terms of survival of parasites (by the use of optical microscopy), incorporation of [^3H] hypoxanthine (by measuring the radioactivity), amplification of DNA (by use of PCR), measurement of lactate dehydrogenase or histidine-rich 2 (HRP2) protein [by the use of enzyme-linked immunosorbent assay (ELISA)] or confocal fluorescent image counting following exposure to DNA intercalating dye (Flannery et al. 2013a, b; Nogueira and Estolio do Rosano 2010), in comparison to control. A variant form of the above in vitro assay uses transgenic *P. berghei* that expresses green fluorescent protein (GFP)-linked luciferase (GFP:LUC) in place of normal *P. berghei* or *P. falciparum* parasites. The luciferase activity measured by bioluminescence in the lysates of treated and control parasites tells whether the tested compound is antimalarial (Lin et al. 2013).

On the compounds that are found to be positive in in vitro test(s), the in vivo therapeutic efficacy tests are performed, either in mouse-*P. berghei* or in rhesus monkey-*Plasmodium cynomolgi* model systems, to determine half maximal inhibitory (IC_{50}) concentration. Such a test can also be done on *P. falciparum*-infected immune-deficient mice (Jimenez-Diaz et al. 2009). In these assays, parasite growth is measured by optical microscopy or other sensitive methods such as PCR

Fig. 7 Scheme delineating genetic mechanisms for increase of genetic diversity and emergence of antimalarial resistance in malaria causing populations of *Plasmodium*, in geographical locations favouring outbreeding as in Africa or inbreeding as in parts of Cambodia

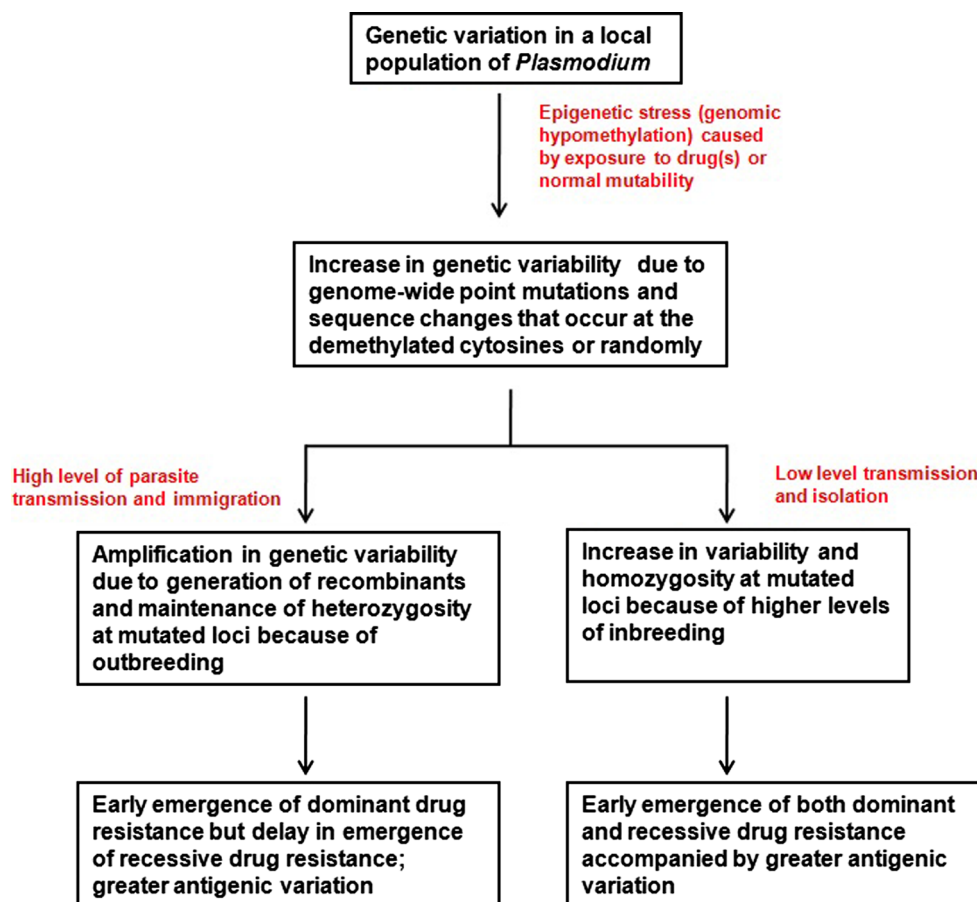
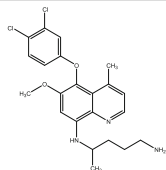
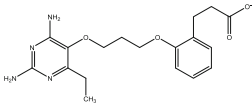
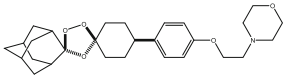


Table 3 Properties of the antimalarial compounds undergoing different stages of development for clinical usage

S. no.	Compound name and class (in parenthesis)	Compound's structure	Whether inhibits parasites in				Remarks	Reference(s)	
			Human liver in the formation / growth of merozoites	Human blood in the formation of hypozoites	Human blood in the formation of merozoites	Their gametogenesis and / or fertilization in human blood and mosquito gut respectively			Female mosquitoes in the development of oocysts and / or oocytes
1	Tafenoquine (8-aminoquinoline)		Yes	Yes	Yes	Yes	Yes	It is a primaquine analogue that is suitable for single dose treatment (unlike 14 days treatment of primaquine) and possesses activity against hypozoites. It has like primaquine liability towards human CYP2D and G6PD polymorphisms.	Duffy and Avery 2013b; La Crue et al. 2013; Llanos-Cuentas et al. (2013); Wells et al. 2010; Ploemen et al. (2009); Crockett and Kain. (2007); Coleman et al. (1999)
2	ELQ-300 4(1H)-quinolone-3-diarylether		Yes	Yes	Yes	Yes	Yes	An endochin like quinoline, it is active against chloroquine and atovaquone resistant parasites at all stages of parasite multiplication and resists resistance development. It blocks pyrimidine biosynthesis in parasite by inhibiting cytochrome b1 activity of mitochondria. Formulation of this stable drug in combination with another antimalarial for an oral drug required.	Duffy and Avery 2013b; Nilsen et al. (2013)
3	NITD 609 (Spiroindolone)		Yes	Yes	Yes	Yes	Yes	Orally bioavailable, it targets parasites by inhibiting their cation channel sodium efflux ATPase4. It is more potent than artesunate and chloroquine. It is suitable for formulation of a single dose treatment.	Smith et al. 2014; Spillman et al. 2013; Van-Pelt Koops et al. (2012); Meister et al. (2011); Rottman et al. (2010); Yeung et al. (2010)
4	GNF156 (Imidazole-piperazine)		Yes	Yes	Yes	Yes	Yes	It is orally bioavailable and safe but mode of action is not known, which is unique.	Smith et al. 2014; Nagle et al. (2012); Meister et al. (2011); Chatterjee (2011); Wu et al. (2011)
5	Albitiazolium (Bisthiazolium)		Yes	Yes	Yes	Yes	Yes	Suitable for severe malaria, it accumulates in very high concentrations and is administered via intravenous or intramuscular injections because of low oral bioavailability. The drug acts by inhibiting choline import which is involved in production of the membrane lipid phosphatidyl choline in parasites. The compound resists resistance developments.	Caldarelli et al. (2013 and 2010); Wein et al. (2012); Le Roch et al. 2008; Vial et al. (2004); Wengelnik et al. (2002)
6	DSM 265 (Trizolopyrimidine)		Yes	NK ^a	Yes	Yes	Yes	It is orally bioavailable and safe drug of long therapeutic half-life. Parasite's enzyme dihydroorotate dehydrogenase, involved in pyrimidine synthesis, is inhibited by it.	Coteron et al. (2011)
7	Methylene blue (Aromatic)		Yes	NK	Yes	Yes	Yes	In combination with dihydroartemisinin, its formulation called Prove blue provides protection against cerebral malaria. The drug acts by inhibiting parasite's monoamine oxidase and glutathione reductase and blocks heme polymerization within the food vacuole.	Dormoi et al. 2012, 2013; Delves et al. 2012; Lelievre et al. 2012; Adjalley et al. 2011

Table 3 (continued)

8	NPC-1161B (8-aminoquinoline)		Yes	Yes	Yes	Yes	Yes	It is a primaquine type drug and generally shares with it the properties of tafenoquine. It is very active against liver stage and inhibits sporogony.	Marcisin et al. (2014); Zeeman et al. 2013; Delves et al. 2012 b; Ganesan et al. (2008); Tekwani and Walker (2006)
9	P218 (Antifolate)		Yes	NK	Yes	NK	NK	It is orally bioavailable drug active against both sensitive and pyrimethamine resistant malaria. It targets dihydrofolate reductase of parasite, like cycloguanil and pyrimethamine.	Yuthavong et al. (2012, 2006)
10	OZ439 (Endoperoxide)		No	NK	Yes	Yes	Yes	Artemisinin resistant parasites are sensitive to it. It inhibits gametocyte / gamete formation and sporogony. Orally bioavailable, it has long half-life and is suitable for development of a single dose formulation.	Moehrl et al. (2013); Wang et al. 2013; Delves et al. 2012 b; Charman et al. (2011)

NK not known

(Flannery et al. 2013a, b). The in vivo drug luminescence (IVDL) assay makes use of *GFP:LUC* transgenic *P. berghei* infections in rodents. In these 4 day suppressive drug tests, the in vivo presence of parasites is quantified by luciferase activity in samples of animal tail blood (Lin et al. 2013). The human-*P. falciparum* system is used to perform ex vivo assays. Parasites taken from a patient are brought to ring stage and exposed to a known amount of the test compound and the effect is monitored in terms of parasites growing into schizonts (Witkowski et al. 2013).

Using one or more of the screening procedures outlined above, several campaigns, to find molecules inhibitive to the asexual blood stage (ABS) of malarial parasites, have been accomplished and a few are in progress. Some new campaigns may arise from new methods for synthesizing compounds of diverse structures (Heidebrecht et al. 2012), such as by the use of reactions of diazo compounds (Karageorgis et al. 2014). The St. Jude Children's Research Hospital (Guiguemde et al. 2010), Genome of Institute of the Novartis Research Foundation (Plouffe et al. 2008) and GlaxoSmithKline Tres Cantos (Gamo et al. 2010) have altogether screened more than four million compounds. The Medicines for Malaria Venture (MMV) continues to test the chemical libraries assembled by sundry biotechnological and pharmaceutical companies. Thus far, more than 25,000 compounds have been found to be active against ABS of malarial parasites. This voluminous portfolio of antimalarials has been open to identify the ones whose targets in parasites are different from those against which resistance has arisen and those that are active against all of the four developmental stages of parasite life cycle in man and mosquito. To share with the researchers the compounds of some promise, an open source Malaria Box of 200 drug-like and 200 probe-like compounds has been formed. The participants of this campaign are expected to conduct

studies on each compound's metabolic properties; effects on different stages of *Plasmodium* life cycle for the identification of cellular and molecular targets; cellular, tissue and organ toxicity; in vivo pharmacokinetics and activity against variants of malaria causing *Plasmodium* species. etc.; and share the results (Spangenberg et al. 2013).

It will be seen from Table 1 which lists the properties of antimalarials in clinical practice and Table 3 which lists antimalarial molecules undergoing clinical trials that majority of the identified antimalarials target hemoglobin degradation or heme detoxification pathways [amodiaquine, piperazine, pyronaridine, quinine, endoperoxides (including OZ-439), NPC-1161B and tafenoquine], folate pathway (pyrimethamine, P218) or mitochondrial pathways (primaquine, atovaquone, proguanil, DSM265, ELQ300). Only albitiazolium, GNF156, methylene blue and NITD 609 target pathways distinct from the above-mentioned. All of the drugs in Tables 1 and 3 got selected because of their pronounced parasitocidal activity on intraerythrocytic asexual stage of parasite replication. Table 4 gives preliminary properties of 26 antimalarial molecules that have proven to be promising in preclinical evaluation experiments. Majority of the molecules in Table 4 have targets distinct from those of the molecules (ACT-21365, alpha-pyrone, azithromycin, bivalent tetrazolium, cladosporin, 4-CF3 phenyl, Genz668764, hydroxy ethylamine, indolisquinolone, ketotifen, ML238, methyl benzylamide, N-myristoyltransferase (NMT) inhibitors, NSC-158011, nutilin-3, phenyl propanoid conjugated iridoid, strictosamide, torin-2) in Tables 1 and 3. The ongoing work on the characterization of the biological effects of the new molecules of variant scaffolds, in search of new clinical series with a wider range of activities against parasite stages in infected individuals and mosquitoes, is bound to enlarge the chemical series represented in Tables 1, 3 and 4.

Table 4 Properties of some leading natural and synthetic compounds found active against in vivo malaria model system(s) and in vitro-grown malarial parasites but non-toxic to human cell lines, which are selectable for preclinical testing

S. no.	Compound name and class (in parenthesis)	Structure	Whether inhibits parasites in				Remarks	Reference(s)	
			human liver in the formation/growth of	human blood in the formation of merozoites	their gametogenesis and/or fertilization in human blood and mosquito gut, respectively	female mosquitoes in the development of oocysts and/or oocysts			
1	Decoquinatone (4-hydroxy quinoline)		Yes	Yes	Yes	Yes	NK	A veterinary drug which also inhibits malarial parasite's liver stages and sexual and asexual blood stages, by acting against their mitochondrial bc1 complex. Because of its insolubility in water, trial on an oral nanoparticle formulation provided complete protection as well as treatment against malaria.	Wang et al. (2014); daCruz et al. (2012)
2	ACT-21365 (Piperazine)		No	No	Yes	Yes	NK	Orally bioavailable and inhibitory to multi-drug resistant protein (MDR-1), it kills both drug sensitive and resistant parasites.	Brunner et al. (2013); 2012)
3	Ketotifen (Cycloheptathiophene)		Yes	NK	Yes	Yes	Yes	This antihistamine cures malaria and blocks transmission by inhibiting ATP-binding cassette (ABC) transporters of parasite.	Eastman et al. 2013
4	Torin 2 (Tricyclic benzonaphthyridinone)		Yes	Yes	Yes	Yes	Yes	Orally bioavailable, it inhibits asexual liver and blood stages and blocks oocyst formation in mosquito. Torins block replenishment of membrane proteins of parasitophorous vacuole of liver stage parasites and trafficking of proteins in general. Drug's action is 1000 times selective on parasite.	Sun et al. 2014; Hanson et al. 2013
5	Nutilin-3 (cis-imidazole)		Yes	Yes	Yes	NK	NK	It reduces liver burden by inhibiting the binding of MDM2 with p53. Thus retention of p53 in the hepatocytes kills the parasites by inducing apoptosis.	Kaushansky et al. (2013); Caldarelli et al. 2010
6	NMT-inhibitor (Benzothiophene)		Yes	Yes	Yes	Yes	Yes	Inhibitor of N-myristoyl transferase that myristates many parasite proteins and thereby acts as parasitocidal. It possibly acts on all stages of parasite division.	Wright et al. 2014
7	Sulphide 3-artesanilide (Trioxane sulfide)		Yes	NK	Yes	Yes	Yes	In combination with mefloquine, it is twice as effective as artemether + mefloquine; it is suitable for single dose therapy.	Slack et al. (2012)
8	C-10 trioxane (Artemisinin-like)		Yes	NK	Yes	Yes	Yes	As above; it is a derivative of artemisinin	Alexander et al. (2012); Posner et al. (2007)
9	4-CF3 phenyl (Aminoquinoline)		Yes	NK	Yes	Yes	Yes	It acts by inhibiting α/β heterodimers of microtubules essential for parasite's cell division.	Mara et al. (2013)

Table 4 (continued)

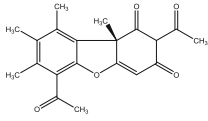
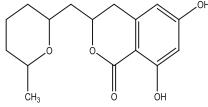
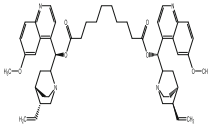
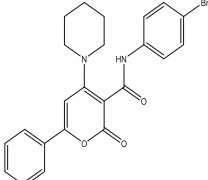
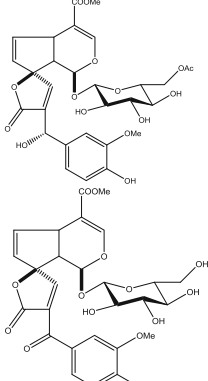

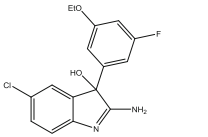
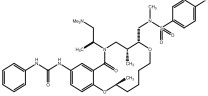
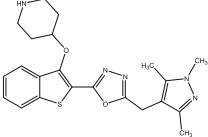
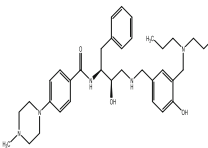
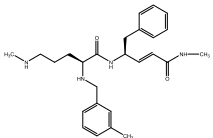
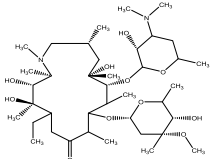
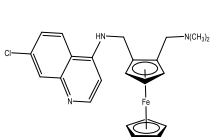
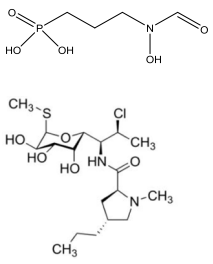
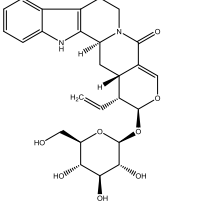
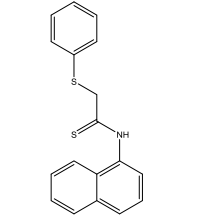
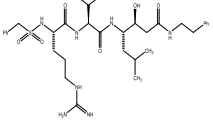
10	Usnic acid (Dibenzofuran)		Yes	Yes	Yes	Yes	Yes	It acts by binding to FAS-II enzyme of type II fatty acid biosynthetic pathway and thereby may be selectively disrupting parasite's mitochondrial membrane.	Launger et al. (2013)
11	Cladosporin (Fungal metabolite)		Yes	NK	Yes	NK	NK	It specifically inhibits parasites cytosolic lysyl tRNA synthetase and is 100 times selective over the human enzyme	Hoepner et al. 2012
12	Quininedimer (Quinine)		NK	NK	Yes	Yes	NK	Malarial parasite resistant to chloroquine, amodiaquine and quinine are all sensitive to quinine dimer class of compounds on account of their anti-hemozoin activity. They also block efflux of quinoline drugs and therefore they could be used in combination with the quinoline antimalarials.	Hrycyana et al. (2013)
13	α -Pyrone		NK	NK	Yes	NK	NK	Parasitocidal by acting against R10-2 kinase (PFD0975W)	Parveen et al. (2013)
14	Phenyl propanoid conjugated iridoid		NK	NK	Yes	NK	NK	Isolated from <i>Marinda morindoides</i>	Tamura et al. (2010)
15	Bivalent tetrazolium		NK	NK	Yes	NK	NK	Orally bioavailable, it acts by disrupting the entry of merozoites into erythrocytes	Vlahakis et al. (2011)
16	Genz-668764 (Aminoindole)		NK	NK	Yes	NK	NK	Orally bioavailable, it is active against both drug sensitive and resistant malarial parasites	Barker et al. (2011)
17	ML238 (Macrocyclic)		NK	NK	Yes	NK	NK	It's mode of action is novel and is therefore active against both drug sensitive and resistant malarial parasites; it is orally bioavailable and safe.	Weiwier et al. (2012)
18	Indolisquinolone		NK	NK	Yes	NK	NK	It's activity corresponds to that of natural dihydrosambarensine alkaloid of <i>Strychnos</i>	Horrocks et al. (2012)
19	Hydroxy-ethyl- amine		NK	NK	Yes	NK	NK	It acts by blocking aspartate proteases like plasmepsin of chloroquine sensitive and resistant parasites.	Ciana et al. (2013)

Table 4 (continued)

20	N'-m-methyl benzylamide		NK	NK	Yes	NK	NK	It acts by inhibiting parasite's calpain protein that mediates merozoite's entry into erythrocytes	Choi et al. (2013)
21	Azithromycin (Macrolide antibiotic)		NK	NK	Yes	NK	NK	Together with chloroquin, fixed doses of azithromycin (AZCQ) are given in intermittent preventive therapy in pregnancy (Iptp). It is also used against sexually transmitted infection of gonorrhea and chlamydial infections. It is a translational inhibitor.	Zhao et al. 2014; Chandra et al. 2012
22	Ferroquine (Ferrocenic chloroquin)		NK	NK	Yes	NK	NK	Orally given, it is a organometallic ferrocene- based chloroquine analog. Active against both chloroquine sensitive and resistant parasites, it kills via generation of hydroxyl radicals in the digestive vacuole of parasite, stopping reinvasion by merozoites. A partner drug is required to completely negate resistance development.	Wang et al. 2014 ; Dubar et al. 2011 and 2013; Salas et al. 2013 ; Supan et al. 2012 ; Biot et al. 2011
23	Fosmidomycin (phosphonic acid antibiotic, upper) and clindamycin, lower (lincosamide antibiotic)		NK	NK	Yes	No	Yes	Both act via action on apicoplasts of the parasite. Fosmidomycin blocks the isoprenoid synthesis.	Weisner et al. 2013; Weisner et al. 2002; Yeh and De Risi 2011; Lell and Kremsner 2002;
24	PR29CT1 (Herbal medicinal)		NK	NK	Yes	NK	NK	80% ethanolic extract of the stem bark of <i>Nauclea pobeguunii</i> containing 56% indole alkaloid strictosamide, the precursor of camptothecin is effective.	Mesia et al. 2012
25	NSC-158011		NK	NK	Yes	Yes	NK	Parasitocidal as well as gametocytocidal because the function of phosphoethanolamine N methyl transferase is blocked.	Bobenchick et al. 2013
26	WEHL-916 (Amide)		NK	NK	Yes	NK	NK	Parasite is killed at the ring-trophozoite transition stage by blocking the plasmepsinV activity. WEHL-916 blocks the enzyme plasmepsinV essential for survival at all stages of parasite in mosquito and man.	Sleebcs et al. 2014

NK not known

New liver stage malaria drugs

The liver stage develops from invasion of hepatocytes by a small number of sporozoites released into the bloodstream via a bite from an infected mosquito. The sporozoites in hepatocytes mature and undergo one cycle of replication to form liver stage schizonts. Clinical symptoms develop when liver schizonts release merozoites into erythrocytes. Since only a small number of hepatocytes initially get infected, the parasite load is low despite long residence time; therefore, liver stage is

asymptomatic, but it is a critical drug target for interrupting further progression of life cycle events of parasite in infected human and for lowering the chances of resistance development. The infecting sporozoites of *P. vivax* and *P. ovale* are able to enter a dormant phase in hepatocytes. The dormant parasites or hypnozoites that survive for many months are the cause of recurring malaria. Among the approved ABS drugs (Table 1), the combination of atovaquone and proguanil clears the parasites (of *P. vivax* or *P. falciparum*) from the liver and that of primaquine and chloroquine clears both liver stage

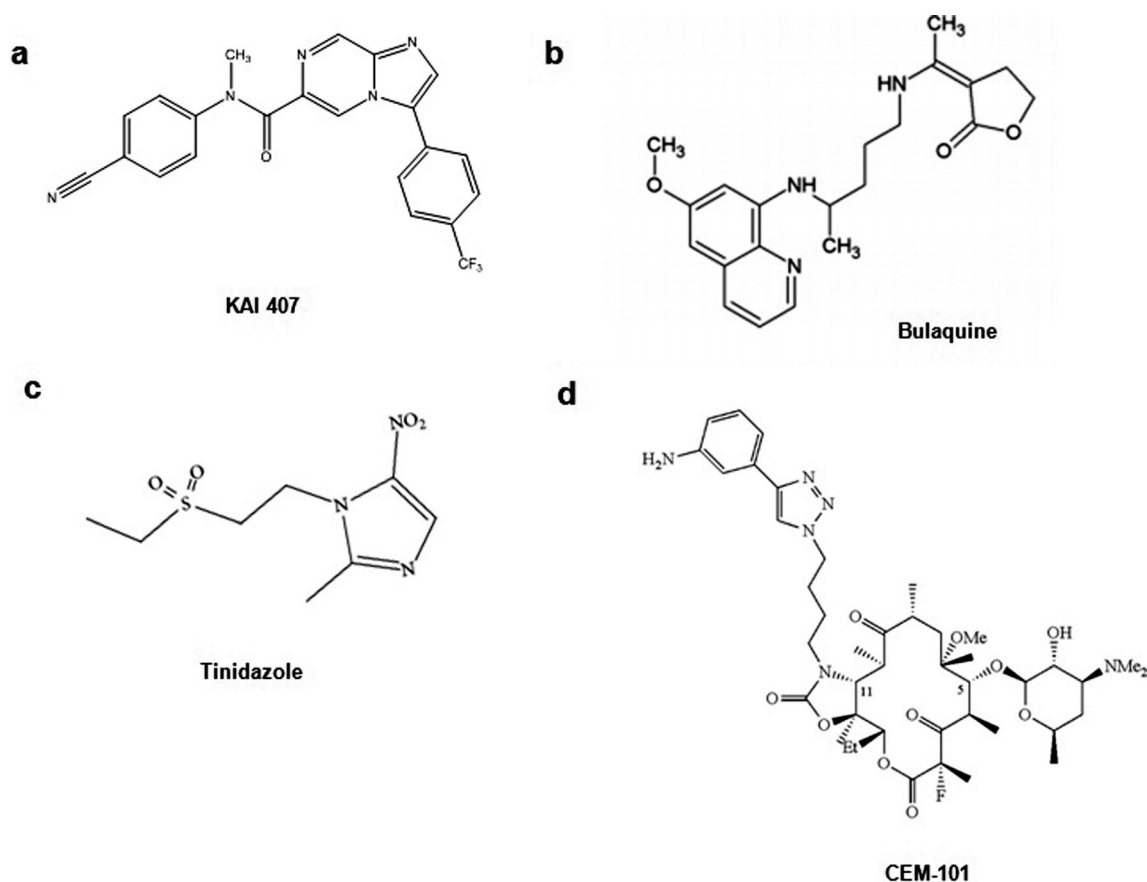


Fig. 8 a–d Chemical structures of some of the important antimalarial compounds that have been found to be active against the liver stage malaria on account of their schizonticidal-cum-hypnozoiticidal activity

schizonts and hypnozoites. However, primaquine has drawbacks: it is slow acting (chloroquine the companion drug is fast acting and therefore clears parasitaemia) and harms people with glucose-6-phosphate dehydrogenase (G6PD) deficiency by causing severe life-threatening adverse reaction. The mechanism by which primaquine and other antimalarials of 8-aminoquinoline family cause haemolytic anemia in G6PD-deficient people is not fully understood (Uthman et al. 2014). G6PD as a part of the pentose phosphate pathway produces the coenzyme nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is involved in the production of glutathione which protects cells against reactive oxygen damage. Reduced levels of glutathione make G6PD-deficient erythrocytes vulnerable to oxidative damage and liable to haemolysis (Cappellini and Fiorelli 2008). G6PD is coded by a 18.5 kb gene comprised of 13 exons on the long arm of the X chromosome. About 400 alleles that reduce the activity and stability of G6PD gene product are known. More than 400 million people worldwide, largely of origin in tropical Africa, tropical and subtropical Asia and Mediterranean regions, carry the deficiency. All the mutation-carrying males and some females who carry the defective gene in heterozygous condition (due to normal X-inactivation) are G6PD deficient. There is an overlap in the geographic distribution of G6PD

deficiency and the spread of malaria (Howes et al. 2013; von Seidlein et al. 2013). Further research needs to provide new families of antimalarials that are safe for G6PD-deficient people and can replace 8-quinolines and rapid low-cost, high-quality field test for G6PD deficiency suitable for screening large populations.

To meet the dearth of liver stage malaria drugs, some of the recent antimalarial drug discovery campaigns have emphasized on liver stage. The success of these campaigns is largely attributable to the use of novel liver stage throughput culture assays that have complemented the hitherto available tedious animal model systems. The liver stage in vitro drug screens have several general features. Cultured primary hepatocytes or cells of hepatoma lines of human, rodent or primate origin are infected with sporozoites of different species of *Plasmodium*. The sporozoites dissected out of the salivary glands of mosquitoes may be fresh or cryopreserved. The infected liver cells are exposed to a known quantity of the test compound, and the treated and control cultures are allowed to grow to produce liver schizonts. At the end of incubation period, parasite cells are observed by optical microscopy, and alternately parasite growth is monitored by bioluminescence imaging, RT-PCR, fluorescence-activated cell sorting or measurement of enzyme activities (Plouffe et al. 2008; Derbyshire et al. 2011; Meister et al. 2011; Delves et al.

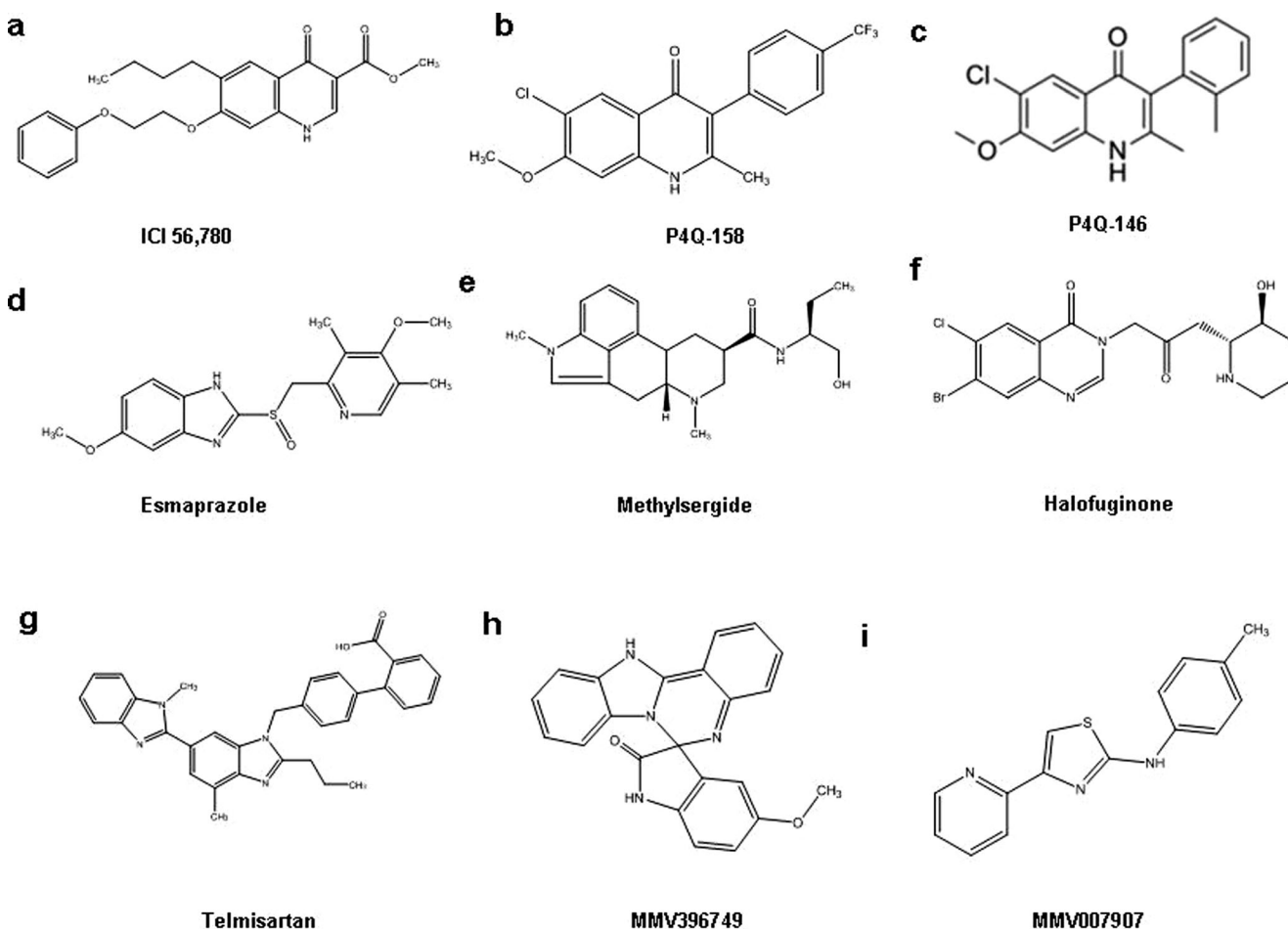


Fig. 9 a–i Chemical structures of some of the important antimalarial compounds that have been found to be active against the liver stage of malaria on account of their schizonticidal activity

2012a, b). In the variant forms of the in vitro drug screens, the normal sporozoites may be replaced by transgenics carrying a fluorescent *GFP:LUC* gene (Derbyshire et al. 2013; La Crue et al. 2013) or a centromere (Voorberg-van der Wel et al. 2013). Both, freshly derived or cryopreserved hepatocytes can be used (Zou et al. 2013). In human hepatocytes infected with *P. vivax* sporozoites or monkey hepatocytes infected with *P. cynomolgi* sporozoites, formation of both liver large multinucleate schizonts and small uninucleate hypnozoites can be quantified (Dembele et al. 2011, 2014). Besides the above, March et al. (2013) have developed a micropatterned coculture MPCC platform. Here, cryopreserved primary hepatocytes derived from individual human donors are cocultured with the support of stroma cells. Liver cells are infected with fresh or cryopreserved *P. falciparum* or *P. vivax* sporozoites. Infected liver cells are overlaid with erythrocytes. Since the MPCC platform can be maintained for 4 to 6 weeks, the drugs can be screened against liver stage schizonts as well as hypnozoites.

In the conventional in vivo test, monkeys are infected with *P. cynomolgi* sporozoites followed by treatment with a compound such as chloroquine that eliminates the blood stage parasites and then the test drug is administered. Infected

monkeys are monitored for several months to observe if malaria reoccurs (Schmidt 1983). This test for anti-hypnozoite drugs is complemented by a test on a rodent model for liver stage. Rodent malaria sporozoites are infected into a mouse shortly before or after the mouse has been treated with the drug under testing. The infection is visualized by estimation of luciferase (if *GFP:LUC* transgenic sporozoites are used) or by measuring the level of blood stage parasitemia or survival (Mwakingwe et al. 2009; Flannery et al. 2013a, b).

The in vitro and in vivo reevaluation campaigns, on molecules established as asexual blood stage antimalarials, possessing sundry biological activities and drugs approved for diverse ailments, to identify molecules having liver stage antimalarial activity have designated many of them as liver stage schizonticidal, and among the latter, some as hypnozoiticidal. In Tables 3 and 4, the proven liver stage hypnozoiticidal and schizonticidal molecules having potential for development into clinical drugs or prophylactics are as follows: albitiazolium, decoquinat, ELQ300, GNF156, NITD609, NMT inhibitor (a benzothioephene), NPC-1161B, nutilin-3, tafenoquine, torin-2 and usnic acid. Most of them have different modes of inhibitory action against parasites.

Besides, Zeeman et al. (2013) have observed a new molecule KAI-407 (imidazopyrazine) as much effective against hypnozoites as are tafenoquine, bulaquine (Dutta et al. 1989) and NPC-1161B (LaMontagne et al. 1982). Tinidazole (an antiprotozoal nitrogroup containing imidazole synthetic derivative; Wells 2010; Wells et al. 2010; Amit et al. 2013; Macareo et al. 2013), inidazolidinone (Wells et al. 2010) and CEM-101 solithromycin (a fluoroketolide antibiotic; Wells et al. 2010; Wittlin et al. 2012) are also found to be active against hypnozoites (Fig. 8). The liver schizonticidal antimalarials not possessing the hypnozoiticidal activity listed in Tables 3 and 4 are as follows: cladosporin, C-10 trioxane, DSM265, 4-CF3 phenyl, ketotifen, methylene blue, OZ439, P218 and sulphide-3-artesanilide. Some additional molecules that offer scope for using their liver schizonticidal activity are MMV007907 (an Atg8-Atg3 protein-protein interaction inhibitor; Hain et al. 2014), ICI 56.780 (a phenoxyethoxy quinoline), P4Q-146 and P4Q-158 (3-phenyl-4(1H)-quinolones) (Hanson et al. 2013), esmeprazole (a proton pump inhibitor that is used to treat gastroesophageal reflux disease), methylsergide (a migraine drug), salinomycin (a breast cancer drug), telmisartan (drug for management of hypertension), halofuginone (synthetic halogenated derivative of the plant *Dichroa febrifuga* quinazolinone alkaloid febrifugene that is a traditional Chinese antimalarial medicine) (Derbyshire et al. 2012a, b) and MMV396749 (a benzimidazole; Burrows et al. 2012) from the Medicines for Malaria Venture (Fig. 9). The bulk of the molecules listed above represents antimalarially active chemical scaffolds offering possibilities for derivation of more active and safer malaria-stopping molecules.

Stopping of transmission and treatment of malaria simultaneously

This approach targets individual, more than one or all of the developmental stages of *Plasmodium* in female mosquito, to thereby stop parasite transmission via drug(s) administered to people for treatment of malaria or for prophylaxis. The aim is to give a drug or drug combination to infected humans so that on the one hand formation of merozoites and gametocytes is interfered in patients and on the other hand parasite's development in mosquito is also blocked. The drugs to be given for prophylaxis will also possess these dual properties, including action against liver stages of the parasite. Several of the early antimalarials did not possess such properties. The concept is that the drug administered to people will get passed on to the female mosquito through its blood meal. Thus, by biting to a treated person, the mosquito gut will receive parasite gametocytes as well as the drug active against subsequent development of gametocytes. The passaged antimalarial will therefore incapacitate parasites in mosquito. Mosquito stages of parasite development are now important targets in programmes

of development of antimalarials, and several effective and safe compounds have been identified. The candidate compounds that are simultaneously used for the treatment of malaria as well as transmission blockers are described below.

Discovery of new transmission blocking drugs

The life cycle of *Plasmodium* (Fig. 1) in the human host begins with the bite by an infected mosquito for a blood meal which releases sporozoites along with some saliva into the bloodstream. The sporozoites invade the hepatocytes and develop into schizonts. The merozoites released from liver schizonts invade erythrocytes where they grow through ring and trophozoite stages into schizonts which release mitotically multiplied blood stage merozoites into the blood for their further asexual replication in erythrocytes. Some of the merozoites instead enter sexual cycle and go through five stages of gametocytogenesis, involving meiosis, to produce stage V mature male and female gametocytes. The gametocytes start to appear a few to 12 days from the appearance of malaria symptoms. They persist even after remission of malaria symptoms. The gametocytes are the only parasite cells that survive transmission from the human host to the mosquito host. A mosquito through its bite to obtain a blood meal ingest about 10^3 – 10^4 gametocytes. These undergo differentiation to produce gametes. In the process, male gametocytes undergo three mitotic divisions to produce exflagellated male gametes. Fusion of male and female gametes forms a zygote which differentiates into ookinete. An infected mosquito may produce 10^2 – 10^3 ookinetes. The motile ookinetes traverse to midgut epithelium where each of them forms an oocyst. Each oocyst upon maturation releases thousands of motile sporozoites which migrate to salivary glands. The mosquito possessing mature sporozoites releases about a dozen of them through its bite to a human, thus completing the mosquito-human-mosquito transmission cycle (Baton and Ranford-Cartwright 2005). There are two phases to block transmission: one in humans that produces mature gametocytes and other in mosquito. In mosquito, the parasite sexual stages, gametogenesis and fertilization and sporogony stages ookinete→oocyst→sporozoites are targets to block transmission. The ACT drugs reduce the transmission only partially because they are active against early stages of gametocytogenesis. At low concentrations, some of them actually increase the gametocyte burden (Peatey et al. 2012). Primaquine is the only fully gametocytocidal among the currently approved antimalarial drugs. There is an urgent need for new transmission blockers because primaquine has G6PD liability, malaria vaccines are awaited and therefore interruption of malaria transmission via drugs is necessary to control, eliminate and eradicate malaria (Alonso et al. 2011). High-throughput in vitro, semi-in vitro and in vivo reliable assays have been recently developed to test the efficacy of candidate molecules against gametocytogenesis,

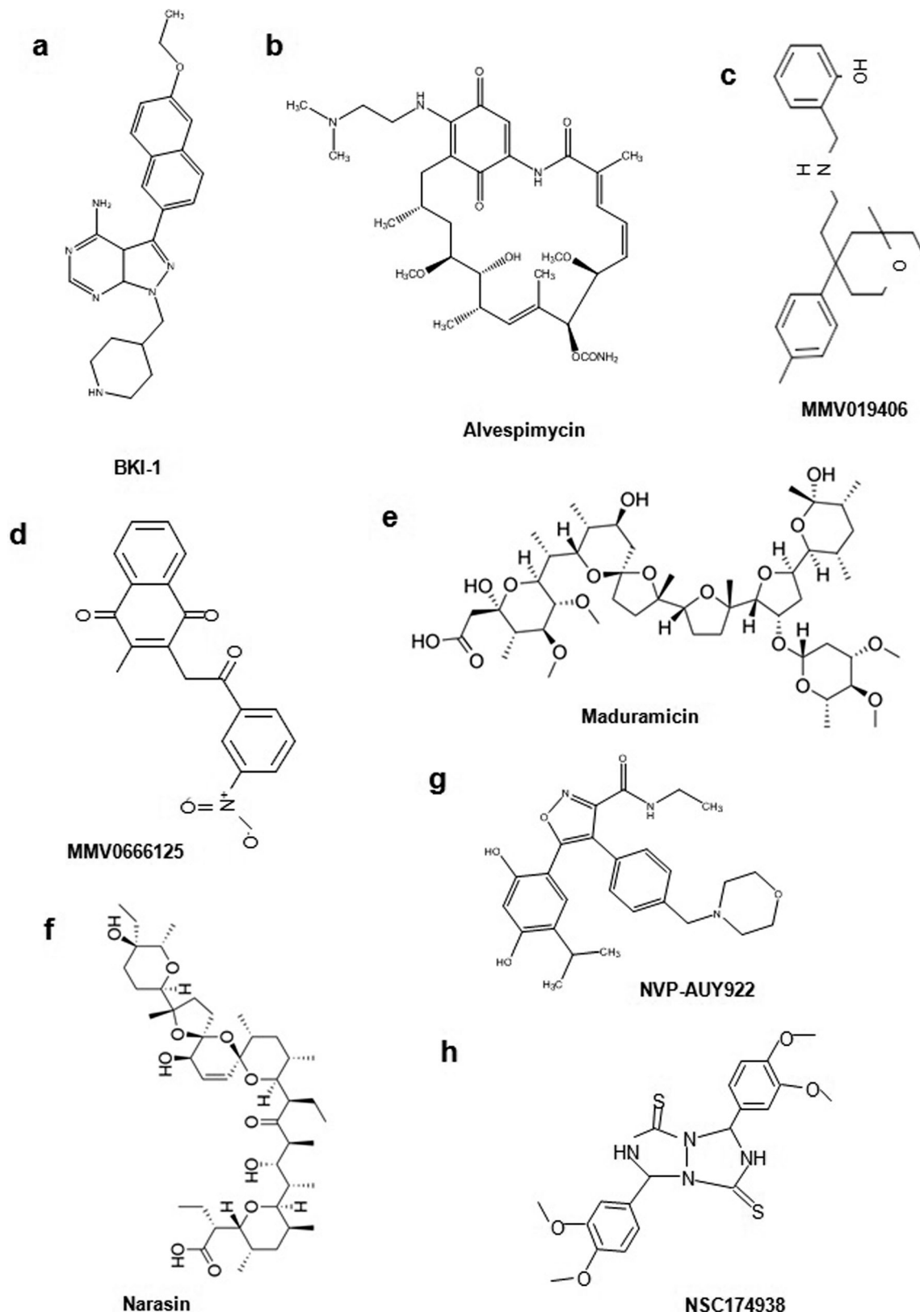


Fig. 10 a–h Chemical structures of some of the important antimalarial compounds that have been found to be sporogonocidal and antagonistic to malaria transmission

gametogenesis and sporogony (Roncales et al. 2012; Ojo et al. 2012).

The available in vitro procedures for testing the efficacy of candidate compounds for killing the mature gametocytes are

typified by that given by Sun et al. (2014). Here, ring stage parasites are cultured conventionally to 0.1 % parasitemia. And at day 9 or 10, the culture is exposed to 50 mM *N*-acetylglucosamine to block further asexual growth. At day 12, the stage III–IV gametocytes are isolated by Percoll density gradient and recultured for 24 h to reach 70 % parasite cells to stages IV–V. The subculture of about 20,000 gametocytes, medium and test compound is incubated for 72 h at 37 °C in 5 % CO₂ atmosphere. Then, AlamarBlue (oxido-reduction indicator) dye is added to the subculture, and after 24 h of further incubation, fluorescence is read such that the fluorescence signal has a linear relationship with surviving gametocyte number. In the procedure of Lelievre et al. (2012), Pf3D7HT-GFP gametocytes in *N*-acetylglucosamine-treated culture are separated by density gradient-cum-magnetic column. About 50,000 gametocytes are subcultured in the presence of test compounds for 48 h before the effect of the test drug is measured by ATP luminescence produced by oxygenation of luciferin catalyzed by luciferase or microscopically by examination of Giemsa-stained thin blood films. The assay of Roncales et al. (2012) uses D-sorbitol treatment to remove asexual parasites and induce gametocytogenesis in cultures. The procedure of Duffy and Avery (2013a, b) deploys confocal fluorescence microscopy on gametocyte-specific protein pfs 16-LUC-GFP and a viability marker MTR (Mitotracker Red CM-H₂X ROS) that allow automated image detection and quantitation of surviving gametocytes. D'Alessandro et al. (2013) in their procedure use calorimetrically estimated lactate dehydrogenase as measure of surviving gametocytes following drug treatment. Kaushal et al. (1980) suggest the use of 1 mM cyclic AMP in static asexual culture to achieve abundant gametocytogenesis.

The exflagellation assay uses *in vitro* cultures rich in mature gametocytes, such as those in Sun et al. (2014) and variant procedures. The test compound is added and exflagellation is triggered by lowering the incubation temperature to about 21 °C. Motile exflagellated cells are counted microscopically in samples withdrawn 20 min later (Sun et al. 2014). In the ookinete development assay, gametocytes are cultured in fresh medium together with the test compound for 24 h at 19 °C. Giemsa-stained ookinetes are counted microscopically (Sun et al. 2014; Delves et al. 2012a, b, 2013).

In the membrane feeding assay (FMA) of sporogony, *Anopheles* mosquitoes starved overnight are fed gametocytes through a parafilm membrane (Churcher et al. 2012). In the case of *P. falciparum*, the *in vitro*-produced gametocytes and test compound are subcultured in fresh medium at 37 °C for 24 h and fed to mosquitoes. Correspondingly, blood taken from *P. berghei*-infected mice is heparinized, added to the test compound and fed to mosquitoes. The starved mosquitoes are fed the gametocytes for 30 min at 37 °C. Engorged mosquitoes are maintained at 28 °C, 60–80 % relative humidity for 8–12 days. The mosquitoes are dissected and their midgut is stained with 0.2 % mercurochrome and the numbers of oocysts

formed are counted. The *in vivo* sporogony assay uses *P. berghei* gametocytes formed in mice. Using the whole blood from mouse showing 10 % *P. berghei* parasitemia, mice are infected. After the mice have produced gametocytes, they are treated with drugs under testing. Such mice are anaesthetized and starved. *A. stephensi* mosquitoes are allowed to feed on them for 30 min. The engorged mosquitoes are maintained and examined for oocyst formation as in FMA (Blagborough et al. 2013; Delves et al. 2012a, b).

Information on activities of many putative next-generation antimalarials listed in Tables 3 and 4 with regard to malaria transmission is now known, since the above assays became available. The molecules of Tables 3 and 4 that have been found to possess significant antitransmission activity fall into three groups: (a) The following 16 compounds possess both gametocytocidal (blockading of mature gametocytes) in human blood stage and sporogonocidal (blockading of sporogony in mosquito) activities: albitiazolium, C10-trioxane, DSM265, ELQ300, 4CF3 phenyl, fosfidomycin, GNF-156, ketotifen, methylene blue, NITD-609, NMT inhibitor, NPC-1161B, OZ439, sulphide-3-artesanilide, torin-2 and usnic acid. In this group, several molecules such as ELQ300, methylene blue and usnic acid possess multiple antitransmission activities: gametocytocidal, anti-exflagellation, anti-fertilization, anti-ookinete formation and anti-oocyst development. (b) A total of six compounds possess gametocytocidal property: ACT-21365, cladosporin, decoquinatate, NSC-1158011, tafenoquine and quinine dimer. (c) Pyrimethamine and mefloquine comprise the sporogonocidal group. Besides, several compounds of known biological activities have been reported to be sporogonocidal (Fig. 10). This group includes bumped kinase inhibitor (BKI)-1. This orally bioavailable compound selectively inhibits the parasite's calcium-dependent protein kinase 4 and thereby microgametocyte exflagellation as well as oocyst formation get inhibited (Ojo et al. 2012). A number of HSP90 inhibitors (Fig. 10), mostly anticancer agents, have been found to possess sporogonocidal activity: alvespimycin (analogue of benzoquinone antibiotic geldanamycin), maduramycin (monoglycoside polymer of *Actinomadura rubra* fungus), MMV019406, MMV666125, narasin (derivative of salinomycin from *Streptomyces aureofaciens*), NSC-174938 and NVP-AYU922 (isoxazole amide) (Sun et al. 2014). *Plasmodium* specifies about 5,500 genes. Molecules that can target any of the essential *Plasmodium* protein/RNA selectively are expected to possess gametocytocidal and/or sporogonocidal property.

Molecules active against asexual liver and blood stages, gametocytogenesis and sporogony

Now that assays are available for testing activities against all the four important stages of *Plasmodium* life cycle in human and mosquito hosts, it has been possible to identify molecules that

are prophylactic against malaria as well as cure malaria. Such molecules of great interest included in Tables 1, 3 and 4 are as follows: albitiazolium, C10-trioxane, decoquinate, ELQ300, 4CF3 phenyl, GNF156, ketotifen, NITD609, NMT inhibitor, NPC-1161B, primaquine, sulphide-3-artesanilide, tafenoquine, torin-2, WEHI-916 and usnic acid. The following 11, of these 16 molecules, have been found to be active also against hypnozoites: albitiazolium, decoquinate, ELQ300, GNF156, NITD609, NMT inhibitor, NPC-1161B, primaquine tafenoquine, torin-2 and usnic acid. Among these, only primaquine and tafenoquine demonstrate G6PD liability, and others have distinct targets and represent novel chemical scaffolds. Thus, it appears that there is a good scope for developing new combination of drugs that will cure and eliminate malaria pleiotropically.

Single-dose safe drug candidates active against drug-resistant malaria to provide protection against reinfection

One of the major obstacles in malaria control programmes has been non-compliance of prescribed schedule of drug intake by patients. Underdosing has been associated with the development of resistance against the first-generation antimalarial drugs. To eradicate malaria, there is a need for drugs that should possess the following features: curing of malaria in a single dose by having high activity against liver and blood asexual stages and gametocytogenesis in humans and/or sporogony in mosquitoes; long in vivo half life; rapid onset of action against plasmodia sensitive or resistant against first-generation antimalarials; resistance to resistance development; and selectivity against parasite targets thus providing safety, especially for children and pregnant women, and least liability. The candidate molecules possessing at least some of such properties are C10-thioacetal, decoquinate, ELQ-300, GNF156, sulphide-3-artisanilide and torin-2. Molecules like decoquinate, ELQ300, GNF156 and torin-2 combined with each other and with suitable quick acting drugs should in the future provide cure as well as long-term prophylaxis against malaria and open possibilities of eradicating malaria.

Finding of targets of known antimalarials and for innovative new antimalarials

Flannery et al. (2013a, b) have reviewed the genetic methods that can be used for defining the targets of known antimalarials. The molecules listed in Tables 3 and 4 are only a small fraction (<0.15 %) of the total already shown to be active against asexual blood stage of malaria parasites. Many of the molecules remaining to be characterized for their biological

properties are expected to be active against multiple stages of human and mosquito parts of life cycles of different malaria-causing species of *Plasmodium*. If the targets of such molecules, among the products of ~5,500 genes that are reported to be present in genomes of *Plasmodium* species, become known, then it may be possible to derive from them altered forms of molecules that are relatively more effective and safer antimalarials. A list of important targets already identified for which novel antimalarial chemical scaffolds are desired is given in Table 5, and the methodology of discovering targets of active compounds is outlined below. It is already exemplified in the section in the discovery of mutations in K13 propeller domain of the PF3D7_134700 gene associated with the development of resistance against artemisinin. The procedure involves in vitro evolution of parasite lines resistant to an antimalarial. Replicated cultures containing 2×10^9 cells are exposed stepwise to increasing levels of an antimalarial starting from IC_{50} of the parent line and reaching to $2-5 \times IC_{50}$ or in single step at $2-10 \times IC_{50}$ concentration. The compound-added cultures are grown for 12 weeks. The presence of live and growing parasites in them is indicative of evolvment of resistance in the culture. Independent isolates from replicated cultures exposed to low and high concentrations of the antimalarial are purified and rechecked for the degree of resistance. The parental and resistant lines are whole genome sequenced to identify changes in the genome of resistant lines. Common mutations in sequences of resistant lines recovered from replicates, identified by whole genome resequencing, are held responsible for resistance development (Langmead et al. 2009; Li et al. 2008, 2009; Samarakoon et al. 2011; Bopp et al. 2013). Usually, resistance is found associated with non-synonymous base substitutions or small deletions in the promoter or structural parts of specific gene(s) or changes in a number of active copies of specific gene(s). Mutation in the promoter of a gene may be the cause of under- or over-expression and that in the structural parts may cause abolition or change in the activity of the gene product. The presence of gene in the decreased or increased number of copies results in under- or over-production of the gene product. Characterization of the life cycle of a mutant in model system(s) identifies the phenotype of the mutation. Reverse genetic analysis also contributes to phenotype description (Nkrumah et al. 2006; Yu et al. 2008; Straimer et al. 2012). Differential stage-specific transcriptomic and proteomic analyses on separated parasites of in vivo/in vitro-grown mutants and bioinformatics too help in the revelation of the phenotypic properties of mutants. In vitro interaction studies between target macromolecules of the parasite and drug molecule will allow derivation/designing of new molecules. This area of research is in its infancy, and emphasis on it is bound to make antimalarial molecules effective in the control/elimination/eradication of malaria.

Table 5 Some molecular targets identified for innovative antimalarial drug development

S. no.	Target	Affected life cycle stage in		Reference(s)
		Human host	Mosquito host	
1	Actin-myosin motor system	Liver stage		Derbyshire et al. (2012a, b)
2	AP2-G protein	Gametocytogenesis		Kafsack et al. (2014), Sinha et al. (2014)
3	ATPase 4	All stages, including hypnozoites	Sporogony	Spillman et al. (2013), Smith et al. (2014)
4	ATP binding cassette (ABC) transporter	Blood and liver stages	Sporogony	Eastman et al. (2013)
5	Calcium-dependent protein kinase 4		Gamete exflagellation	Ojo et al. (2012)
6	Calpain	Blood stage		Choi et al. (2013)
7	CAX transporter		Sporogony	Guttery et al. (2013), Straimer et al. (2012)
8	CRT digestive vacuole transmembrane protein transporter			Papakrivos et al. (2012)
9	DXR (1-deoxy-D-xylulose-5-phosphate reductoisomerase)	Blood stage		Goble et al. (2013)
10	Export protein 1 and upregulated sporozoite protein 4 of parasitophorous vacuole membrane	All stages	All stages	Sun et al. (2014), Hanson et al. (2013)
11	Falcipain-2	Blood stage		Rizzi et al. (2011)
12	FAS-II	All stages	All stages	Lauinger et al. (2013)
13	GAP-50		Gametogenesis	Simon et al. (2013)
14	Heme biosynthesis	Liver stage	All stages	Nagraj et al. (2013)
15	Histone deacetylase	Blood stage		Mukherjee et al. (2008)
16	HSP90		Sporogony	Sun et al. (2014)
17	Hypoxanthine-xanthine-guanine phosphoribosyl transferase	All stages	All stages	Gardiner et al. (2009)
18	Kelch-like protein phosphate		Sporogony	Guttery et al. (2012a, b)
19	Lysyl tRNA synthetase	Liver and blood stages		Hoepfner et al. (2012)
20	MDM2	Liver and blood stages including hypnozoites		Kaushansky et al. (2013)
21	N-myristoyltransferase	All stages	All stages	Goncalves et al. (2012), Wright et al. (2014)
22	PF3D7_134700	Blood stage	Exflagellation	Ariey et al. (2014)
23	Phosphoethylamine methyl-transferase	Gametocytogenesis		Bobenchick et al. (2013)
24	Phosphatidyl inositol-4-kinase	Gametocytogenesis	All stages	McNamara et al. (2013)
25	Plasmepsin aspartate proteinase(s)	Blood stage		McKay et al. (2011)
26	Pyrimidine biosynthesis in mitochondria	All stages	All stages	Nilsen et al. (2013), Duffy and Avery (2013a, b)
27	PTP2	Gametocytogenesis		Regev-Rudzki et al. (2013)
28	R10-2 kinase	Blood stage		Parveen et al. (2013)
29	Reticulocyte-binding protein homologue-1	Blood stage		Gao et al. (2013)
30	ROM1 protein	Liver and blood stages		Vera et al. (2011)
31	Shewanella-like protein phosphatase		Sporogony	Patzewitz et al. (2013)

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Concluding remarks

The significant reduction in malaria cases from 515 million in 2002 to 219 million in 2013 is thought to be fragile in

view of the emergence and spread of resistance against artemisinin, the principal drug of ACT that was combating malaria, in Southeast Asia. The migration of ART-resistant parasites to other malaria endemic countries requires

immediate countermeasures. The perception of possible retreat in malaria control programme has reinvigorated research interest in multiple fronts of human-parasite-mosquito interactions. In the above discussion, some of the important recent progress and avenues of new research have been delineated. Virtues and deficiencies of the putative antimalarial vaccines RTS,S and AMA1 make their synergetic use in the future possible. The new malaria diagnostic technique REEAD is both time and cost effective for usage in field conditions. The DNA marker identified in ART-resistant parasites will complement REEAD in tracking of artemisinin-resistant malaria patient for their treatment to block further spread of such malaria. Progress towards blocking of malaria transmission includes discovery of mosquito repellents that are manifold more effective than DEET, construction of bacteria-infected mosquitoes in which the parasite is killed, suggested use of ivermectin molecules in ACTs to cure malaria as well as kill mosquitoes that may bite treated patients and discovery of antimalarial molecules of long half life for use as prophylactics. Development of imaginative high-throughput screens have provided a vast library of antimalarial molecules as well as means to discover drugs that singly or in combination are able to cure simple as well as relapsing malaria by activity against liver and blood stages of parasite in human and annulling sporozoite production in mosquitoes, thus blocking transmission of malaria between humans and also serving as prophylactic against reinfections in the immediate future. Genome sequencing and annotation and proteomic, biochemical and biophysical and chemical technologies now offer means for discovering antimalarials against specific parasite gene products, allowing the synthesis of innovative molecules that will be safe, cure malaria, stop parasite transmission and thus help in the achievement of the projected goal of eradicating malaria by 2035.

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Conflict of interest This is to certify that there is no clash of interest involved in the publication of this manuscript entitled “New insight-guided approaches to detect, cure, prevent and eliminate malaria” authored by Sushil Kumar, Renu Kumari and Richa Pandey and this has the consent of all concerned.

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