Chapter

Stable Artesunate Resistance in A Humanized Mouse Model of *Plasmodium falciparum*

Sheetal Saini, Rajinder Kumar and Rajeev K. Tyagi

Abstract

Plasmodium falciparum, the most devastating human malaria parasite, confers higher morbidity and mortality. Although efforts have been made to develop an effective malaria vaccine, stage- and species-specific short-lived immunity crippled these efforts. Hence, antimalarial drug treatment becomes a mainstay for the treatment of malaria infection in the wake of the unavailability of an effective vaccine. Further, there has been a wide array of antimalarial drugs effective against various developmental stages of *P. falciparum* due to their different structures, modes of action, and pharmacodynamics as well as pharmacokinetics. The development of resistance against almost all frontline drugs by *P. falciparum* indicates the need for combination therapy (artemisinin-based combination therapy; ACT) to treat patients with *P. falciparum*. A higher pool of parasitemia under discontinuous *in vivo* artemisinin drug pressure in a developed humanized mouse allows the selection of artesunate resistant (ART-R) *P. falciparum*. Intravenously administered artesunate, using either single flash doses or a 2-day regimen, to the *P. falciparum*-infected human blood chimeric NOD/SCID.IL-2R$\gamma^{-/-}$ immunocompromised (NSG) mice, with progressive dose increments upon parasite recovery, was the strategy deployed to select resistant parasites. Parasite susceptibility to artemisinins and other antimalarial compounds was characterized *in vitro* and *in vivo*. *P. falciparum* has shown to evolve extreme artemisinin resistance as well as co-resistance to antimalarial drugs. Overall, the present information shall be very useful in devising newer therapeutic strategies to treat human malaria infection.

Keywords: artemisinin, artesunate resistance, co-resistance, *Plasmodium falciparum*, humanized mouse model

1. Introduction

1.1 Malaria biology

Malaria is a leading parasitic disease caused by protozoans belonging to the genus *Plasmodium* (*P.*) when injected by the female mosquito (*Anopheles*) in humans during a blood meal. Out of 172 species of *Plasmodium* parasite, only five species are reportedly known to cause malaria infection: *Plasmodium falciparum, Plasmodium malariae, Plasmodium vivex, Plasmodium ovale*, and *Plasmodium knowlesi*. The parasite completes its sexual and asexual cycles in a very complicated manner [1]. *Plasmodium* gametocytes are taken up during the blood meal from the

human host and travel through salivary glands to the gut of *Anopheles* and continue the sexual multiplication. The haploid male and female gametes fertilize and give rise to zygote in the gut, which further grows and elongates into the ookinetes. Ookinetes following several replication processes become oocyst that transforms into sporozoites [2]. These sporozoites move from the gut to salivary glands and are released by the mosquitoes into the human bloodstream during a blood meal.

Once sporozoites are released in the bloodstream, the asexual development stage gets started [3]. Sporozoites traverse to their first incubation site, that is, hepatocytes (pre-erythrocytic stage), and later continues the asexual blood-stage infection inside red blood cells (RBCs). The asexual blood-stage infection results in clinical manifestations of the disease. Some sporozoites reach the liver within a few hours and penetrate the hepatocyte and undergo asexual replication known as exo-erythrocytic schizogony. Each sporozoite develops into a schizont containing 18–26 merozoites. The entire pre-erythrocytic phase lasts about 5–16 days depending upon the parasite species. RBCs are "center stage" and host the asexual development of *Plasmodium*. The merozoites (infective form) released from the liver recognize, attach, and enter the RBCs quickly. Merozoite passes through a developmental transformation beginning with a characteristic ring stage and leading to the formation of a multinucleated schizont within RBCs. Mature schizont ruptures to release the merozoites and continues the multiplication in RBCs. Each mature schizont contains around 18–26 merozoites, and these are released following the lysis of RBC to invade fresh RBCs. It is the lysis of RBCs that induces the bouts of fever and anemia in the infected individual, whereas, in *P. vivax* and *P. ovale* infection, a few merozoites remain in dormant stages for four months or year causing malaria relapse [4].

1.2 Malaria vaccine

Globally, malaria is one of the leading causes of mortality (0.3–2.3%), and Africa and Asia are highly endemic regions among the other continents [5]. According to World Health Organization, approximately 299 million cases of malaria and 400,000 deaths have been reported worldwide in 2019 [6]. A variety of antimalarial drugs are available but the emergence of resistance has been a major setback in containing malaria infection. Currently, Mosquirix (RTS,S/AS01) developed by GlaxoSmithKline is the only vaccine available in the market against malaria. RTS,S/AS01 is an engineered vaccine comprising of the genes from the outer protein of *P. falciparum* circumsporozoites and a portion of a hepatitis B surface antigen with chemical adjuvant to boost the immune responses. Mosquirix reduced malaria cases in children to nearly half [7] and has had 40% efficacy in children receiving four vaccine doses [8]. However, the efficacy dropped to 26% in children receiving only three vaccine doses and 33% efficacy was observed during the first year in infants (up to 3 months old). Further, the effectiveness of Mosquirix is only for one year and failed to provide long-term protection but could be combined with chemotherapy to prevent the transmission of malaria in low-endemic regions [9].

A variety of vaccines focusing on irradiated sporozoites is under trial, which may give exposure to the array of antigens and help induce protective immunity against malaria [1].

1.3 Drug-based therapy

Antimalarial drugs are the most commonly used treatment option for malaria in tropical regions. Three types of antimalarial drugs are currently available *viz.* aryl amino alcohol compounds (quinine, lumefantrine, chloroquine, amodiaquine, mefloquine, etc.), antifolate compounds (pyrimethamine and sulfadoxine), and artemisinin

and derivatives [10]. *Plasmodium* parasites have a complex life cycle that involves a mammalian and an invertebrate host. All the symptoms are caused by the repeated rupture and penetration of erythrocytes by the asexual blood-stage parasites (merozoites). Hence, most of the antimalarial drugs target the asexual erythrocytic stage of the parasite. As per WHO's Model List of Essential Medicines (MLEM), currently, there are 14 curative drugs for the treatment of malaria (treatment postinfection) and 6 prophylactic medicines (treatment before infection), either single or in combination [11]. Curative drugs for *P. falciparum* are mostly artemisinin-based combinations with artemisinin derivative (short half-life) in combination with partner drug(s) with a different mechanism of action (longer half-life). Out of 14 curative drugs, chloroquine is used for *P. vivax*, primaquine is used for *P. vivax* and *P. ovale* both, and the rest 12 are used for treating *P. falciparum* malaria.

1.3.1 Quinine

First isolated from the bark of the cinchona tree in 1820, and it remained one of the most effective malaria treatment options till the early 2000s [12]. Even today, quinine is obtained entirely from its natural source due to its difficult synthesis of the active molecule. Resistance for quinine was first reported in the 1980s, and since 2006, not being used as a frontline antimalarial drug [12]. However, the drug is still present on the WHO's list of essential medicines and is used wherever artemisinins are not available [11]. Quinine has blood schizonticidal and gametocytocidal activity against *P. vivax* and *P. malariae*. Quinine also inhibits heme polymerase activity (required to convert toxic heme into nontoxic hemazoin) and, hence, leads to the accumulation of heme (cytotoxic substrate) in parasites.

1.3.2 Chloroquine

It was used to treat all forms of malaria infections with fewer side effects in the 1940s [13]. Blood stage of *P. vivax*, *P. ovale,* and *P. malariae*, sensitive strains of *P. falciparum*, and gametocytes of *P. vivax* are sensitive to chloroquine. It is highly effective in controlling acute malaria infection as compared to quinine. Moreover, it has been efficient and safer to use to treat sensitive cases. The first resistance case was reported in the 1950s and by the time, many malaria parasites have developed resistance against chloroquine. As per MLEM, chloroquine is used as a curative and prophylactic drug against *P. vivax* in the regions where resistance is not known to evolve (Central American regions) [11].

1.3.3 Amodiaquine

It was first synthesized in 1948 [14] and used in combination with artesunate for treating uncomplicated *P. falciparum* malaria (Camoquine® or Coarsucam™) [15]. The mechanism of action of amodiaquine is similar to that of chloroquine involving inhibition of hemozoin formation [16].

1.3.4 Pyrimethamine and sulfadoxine

In the early 1950s, Elion G and Hitchings G developed pyrimethamine [17] and Elion, Hitchings, and Black won the joint Nobel Prize in Physiology or Medicine (1988) for "their discoveries of important principles for drug treatment." Sulfadoxine was developed in the early 1960s [18] and in 1981, the pyrimethamine and sulfadoxine combination was approved for the treatment of malaria. The emergence of high levels of resistance against the combination of pyrimethamine

and sulfadoxine led to its discontinuation as a prophylactic drug since both drugs inhibit the parasite's folate biosynthesis pathway (dihydrofolate reductase, DHFR and dihydropteroate synthetase, respectively) [19].

1.3.5 Primaquine

Primaquine, an 8-aminoquinoline, was first used in early 1950s. 8-aminoquinolines eliminates mature *P. falciparum* gametocytes, exo-erythrocytic (hepatic) stage of all *Plasmodium* species, and prevents the relapse cases of *P. vivax* and *P. ovale* showing suboptimal blood-stage activity. Despite its remarkable antimalarial properties, primaquine is reportedly known to confer severe side effects [20].

1.3.6 Piperaquine

It was developed as a part of the Chinese National Malaria Elimination Programme in the 1960s [21]. Although China used this drug as a replacement for chloroquine, the emergence of resistance against piperaquine prohibited its use as monotherapy. Currently, this is used with a partner drug with DHA (Eurartesim®) as a combination therapy [22]. It binds to heme-containing species by blocking heme detoxification and acts through getting accumulated in the digestive vacuole [14].

1.3.7 Doxycycline

In the early 1960s, Pfizer Inc. (USA) invented doxycyclin, a synthetically derived broad-spectrum bacteriostatic agent from *Streptomyces* sp. Doxycyclin is an efficacious prophylactic drug and, in combination with a partner drug, is highly effective as a curative drug for *Plasmodium* infection. Doxycycline is particularly used as a preventive drug in the regions with chloroquine and multidrug-resistant (MDR) *P. falciparum* malaria. It is not recommended for pregnant women and children below 8 years of age, but adverse effects were scarcely reported [23].

1.3.8 Mefloquine

American Army developed mefloquine in the 1970s [24] and is still part of the MLEM. Initially introduced for the treatment of chloroquine-resistant malaria, mefloquine has been used as a curative (in combination with artesunate) and prophylactic drug. In the late 1980s, resistance cases were reported for mefloquine [25]. Further, antimalarial action is mediated by disrupting the hemoglobin digestion in the asexual erythrocytic stage of the parasite [14]. The rendered adverse effects on the central nervous system prohibited its use as an antimalarial drug [26].

1.3.9 Artemisinin and its derivatives

Tu Youyou first isolated artemisinin in 1971, from a traditional Chinese medicine plant *Artemisia annua* [27], and was conferred Nobel Prize (2015) in Physiology or Medicine for "her discoveries concerning a novel therapy against malaria." Artemisinin and its derivatives (artemether, artesunate, and arteether) are metabolized to its active compound DHA and are effective against all MDR forms of *P. falciparum*. Artemisinins acts in multiple ways including the generation of free radicals after being activated by heme, which, in turn, destroys proteins essentially required for the parasite growth and development [28]. Additionally, its action is associated with upregulation of unfolded protein response (UPR)

pathways [29] and downregulation of *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K) [30] and Ca2+ transporter (PfATP6) [31]. Artemisinins are crucial to fight the battle against malaria with artemisinin combination therapy (ACT) accounting for the majority of current antimalarial treatments [22], and in the late 2000s, emerging artemisinin resistance was noticed in Southeast (SE) Asia [5, 32].

1.3.10 Lumefantrine

Chinese antimalarial research effort "Project 523" that led to the discovery of artemisinin also synthesized lumefantrine in 1976 [33]. Currently, lumefantrine is used in combination with artemether. Lumefantrine is known to inhibit the transcriptional and translational pathway of malaria parasites [16].

2. Artemisinin resistance

One of the greatest challenges in achieving malaria control is antimalarial drug resistance (**Figure 1**). It has been associated with the malaria dissemination to new areas and resurgence in areas where the disease had been eliminated from. The situation is worsened by the incomplete treatments as it puts massive drug selection pressure on *P. falciparum* parasites, and hence, it helps evolve resistance against all frontline drugs. Chloroquine resistance leads to the resurgence and spread of malaria for decades in most countries in the 1960s [34]. Pyrimethamine, amodiaquine (chloroquine analog), arylaminoalcohols mefloquine, and halofantrine too suffered reduced efficacy in the 1980s, spreading resistant parasites. WHO recommended the combined use of two or more compounds with different modes of action to provide necessary cure rates and delay the development and spread of resistance. With low mixed-strain transmission rates, Southeast (SE) Asia was historically the first region to show resistance to frontline drugs [32]. Resistance to chloroquine, mefloquine, and sulfadoxine-pyrimethamine was initially seen in SE Asia [35, 36]. Artemisinin-based combination therapies (ACTs) have been seen as effective in controlling malaria. ACTs were a first-line treatment option for malaria since the early 2000s and were quickly adopted worldwide [34].

The mechanism of action of most antimalarials depends on targeting a single pathway/molecule, for example, DHFR-mediated folate synthesis (sulfadoxine-pyrimethamine), cytochrome bc1 (atovaquone), and heme detoxification (chloroquine). The artemisinin(s) binds to an array of parasite proteins and influences multiple cellular processes including glycolysis, translational pathway, and cell cycle regulation [37–40]. Some studies also suggest that artemisinin may target and depolarize the mitochondrial membrane potential [41, 42]. Due to these functional

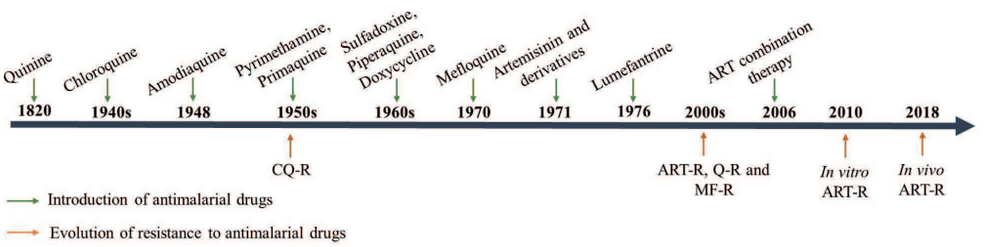

Figure 1.
Timeline for the introduction of antimalarial drugs and emergence of resistance. Image illustrates the approximate timeline for the discovery of antimalarial drugs and evolution of resistance. In vitro and in vivo (huRBCs-reconstituted human blood chimeric/humanized mice) induction of artsunare resistance in laboratory strains of P. falciparum *to confirm the emergence of resistance. CQ-R, chloroquine resistance; ART-R, artemisinin resistance; Q-R, quinine resistance; MF-R, mefloquine resistance.*

attributes, artemisinin eliminates the asexual blood stage and early sexual gametocyte forms of *P. falciparum* parasites at low concentrations (nM) regardless of its short half-life (<1 h). To overcome the half-life issue, artemisinin and its derivatives (artemether, artesunate, and dihydroartemisinin; DHA) are used in combination with a drug of longer half-life. In Southeast Asia (SE), primary artemisinin derivative combinations used are artesunate with mefloquine and DHA with piperaquine, whereas in Africa artemether with lumefantrine and artesunate and amodiaquine. In the late 2000s, emerging artemisinin resistance was noticed in SE Asia [5, 32] and the most problematic situation currently is the rapid increase in failure rates of DHA used in combination with piperaquine. Later have been the first-line treatment and the preferred ACT in most of the SE Asia [43, 44]. Artemisinin resistance enchants potential pressure on partner drugs to work quickly and effectively in case of efficacy failure of artemisinin. *P. falciparum* Kelch 13 (Pf-Kelch13) (located on chromosome 13) is shown to be primarily responsible for artemisinin resistance. Kelch13 protein is involved in intraerythrocytic parasite development and many other cellular processes including hemoglobin endocytosis responsible for parasite growth and antimalarial activity of artemisinin. K13 mutations in parasites mediate artemisinin resistance through the reduced killing potential of artemisinin drugs. Moreover, mutations in K13 drive the enhanced removal of damaged proteins by the parasite [45].

Triple artemisinin-based combination therapies (TACTs) and mass drug administration (MDA) are proposed to combat artemisinin resistance [46, 47]. Combining more than two drugs with different modes of action prevents the chances of multidrug resistance and its spread. MDA targets the asymptomatic malaria receptacles that may serve as hotbeds for the transmission and perseverance of resistant parasites. TACT efficacy is currently in the second phase; with underway Tracking Resistance to Artemisinin Collaboration II (TRAC II) multiple-site study. DHA, piperaquine and mefloquine, and artemether and lumefantrine along with amodiaquine combinations have shown promising results and might help to defer the evolution of artemisinin resistance. Moreover, TACTs might reinstate the artemisinin sensitivity in the areas prevalent for artemisinin resistance [48].

2.1 Experimental induction of artesunate resistance *in vitro* and *in vivo* (human RBCs-reconstituted NSG mice [humanized mice])

Various studies have been performed to understand artemisinin (ACT) resistance being evolved in laboratory strains of *P. falciparum in vitro* and in human blood chimeric mice. Human studies suggest single-dose artemisinin-induced dormant parasites in *P. falciparum* 3D7 or K13-infected strains of *P. falciparum*-infected patients. These parasites are most likely a reservoir for recrudescent infection following artemisinin mono- and combination therapy (ACT). Artemisinin-resistant *P. falciparum* may be experimentally selected following different regimens *in vitro*. The selected resistant parasites could employ different mechanism(s) of action to escape drug pressure for extended survival. Witkowski et al. [49] submitted laboratory *P. falciparum* (F32-Tanzania) to artemisinin pressure for over 3 years/100 cycles to select artemisinin-resistant parasites. These artemisinin-resistant parasite could survive up to 7000× of the initial IC50 of artemisinin (~10 nM) with unaltered chemosensitivity. Further, under high artemisinin pressure, parasites were arrested at the ring stage and re-gained their sensitive phenotype when drug pressure was removed. This unstable resistance phenotype questions the experimental generation of resistant phenotype [49]. Chavchich et al. [50] findings directly associated the development of resistance against artemisinin

derivatives in *P. falciparum* strains (W2 and TM91C235) with the pfmdr1 gene and protein expression. However, there were no changes seen in these markers when D6 parasites were submitted to a similar drug pressure [50]. These findings were attested by Tucker et al. [51]. After continuous exposure of DHA for 1–2 months (320 nM maximum), Cui et al. were able to generate DHA-resistant *P. falciparum* strains 7G8, Dd2, HB3, and D10 but 3D7. DHA resistance was not seen limited to the ring stage but also occurred in trophozoites and schizonts like artemisinin [52]. Rocamora et al. [53] generated artemisinin-resistant *P. falciparum* parasite lines from 6A and 11C clones of the 3D7 strain of *P. falciparum*. Resistant clones displayed a significant decrease in artemisinin sensitivity within 1.5 months of selection and showed the enhanced cellular response against oxidative stress (antioxidant defense) and protein damage (unfolded protein response; UPR) [53]. Major pathways associated with UPR against artemisinin resistance are reported to be *Plasmodium* reactive oxidative stress complex (PROSC) and TCP-1 ring complex (TRiC) [29].

Rodrigues et al. [54] generated artesunate- and mefloquine-resistant *P. chabaudi* parasite in Balb/c mice and confirmed that resistance could be generated against combination drugs even when both drugs are administered simultaneously [54]. Maslachah et al. used *P. berghei* ANKA-infected Swiss mice and observed repeated passages of artemisinin-treated parasites in mice increased the effective dose of artemisinin from 50% to 90% with the reduced parasite clearance time and recrudescence time. Additionally, repeatedly artemisinin exposed parasites showed dormancy and vacuole formation [55]. Humans share >85% similarity with murines, but for the complexity of the cellular system and specificity of the human immune system, *in vivo* mouse models are far close to an ideal model for studying human malaria parasites. Rodent parasites are used as surrogates for human parasites, but the genetic differences between rodent and human parasites make it difficult to correlate with human studies. Human pathogens, which do not infect other animal species, require an animal model that could reconstitute or replicate the human immune system [56]. Fortunately, the mouse-human chimeric animals present a viable preclinical *in vivo* model to study the interaction of the human immune system with infectious agents. Immunodeficient mice engrafted with human RBCs (humanized mice) support the development of asexual blood-stage infection of *P. falciparum* [57, 58]. Artesunate-resistant (ART-R) *P. falciparum* (Uganada, Palo Alto Marburg) was experimentally selected by submitting discontinuous artesunate pressure on *P. falciparum*-infected humanized NOD/SCID IL-2R$\gamma^{-/-}$ immunocompromised mice reconstituted with human erythrocytes (huRBC). This humanized animal model supported the incremental increase in artesunate dosage to select 100 times ART-R parasites (240 mg/kg) to that of clinical dose (2.4 mg/kg). ART-R phenotype exhibited two patterns of IC50 in the selected parasites *in vivo*. Further, the first-stage phenotype showed substantial resistance to artemisinin *in vivo* (400×) without a shift seen in IC50, and the second-stage resistance phenotype showed an absence of response to a very high artemisinin dose (3200×) with a shift in IC50 and co-resistance to quinine, halofantrine, and amodiaquine. This phenotype was further demonstrated as having high-grade and stable artemisinin resistance phenotype. This is the first report of its kind [59], wherein a humanized mouse model was developed and a stable ART-R phenotype was achieved. Moreover, mimicking the clinical double doe regime (for consecutive two days and 24 h apart), 41% survival was observed with the highest dose of artemisinin (80 mg/kg) in contrast to 80% survival in the single-dose protocol (240 mg/kg) (**Figure 2**) [59]. There have been humanized mouse models to study asexual blood and liver stage infection of *P. falciparum* [57, 58, 60–62] and *P. vivax* [63, 64].

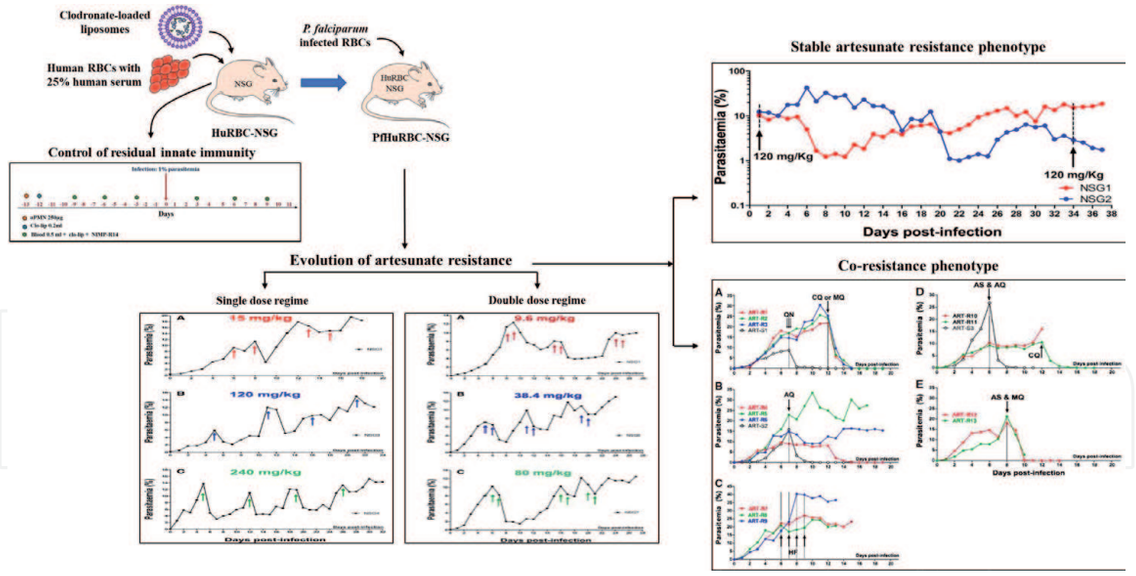

Figure 2.
Experimental selection of artesunate-resistant P. falciparum in a huRBCs-reconstituted NSG mice (PfhuRBC-NSG). NOD.Prkdcscid Il2rg$^{-/-}$ (NSG) mice non-adaptively controlled residual immune responses and reconstituted with human RBCs (HuRBCs) were used to study of asexual blood-stage infection of P. falciparum. *The artesunate resistance (ART-R) was developed by the stepwise selection in experimental humanized mice using single/flash dose (the highest dose: 240 mg/kg) and double-dose (the highest dose: 80 mg/kg) regimens. The stability of the resistance (ART-R) phenotype was attested in vivo by removing ART pressure for one month and then submitting the parasite to ART pressure. ART-R parasites exhibited co-resistance to quinine (QN), amodiaquine (AQ), and halofantrine (HF), and a combination of artesunate (AS) and AQ. These ART-R parasites showing cross-resistance were susceptible to chloroquine (CQ) and mefloquine (MQ).*

3. Conclusion and future perspectives

Human malaria infection remains a biggest challenge to humanity and extensive research is *en route* to find newer therapeutic options to treat this systemic infectious disease, *P. falciparum*. Basic research has been focused to decipher the mechanisms of infection of *Plasmodium* species and biochemical, genomics, proteomics, and metabolomics pathways. This information is used to advance the applied research wherein incessant efforts are being made to design vaccines and drugs against this menace. An effective vaccine development against *P. falciparum* has been a cumbersome process as it takes longer durations than drug therapies. A variety of drugs are developed acting against all developmental stages of different species. The development of drug resistance is affected by various genetic factors (mutations in genes involved in drug transport and metabolism) as well as environmental factors (drug pressure, the geographical distribution of the parasite, etc.). Currently, artemisinin and its derivatives are the cornerstones of effective malaria therapy regimens. These drugs are used in "artemisinin-based combination therapy" for treating malarial infections where artemisinin or its derivatives are given in combination with another unrelated partner drug. Despite ACTs, a parasite is gaining resistance against almost all drugs at a frightening pace suggesting an urgent need to devise novel antimalarial drug(s). Advanced techniques in genomics and proteomics help in developing novel drugs and drug targets. Since single-drug therapy may lead to problems such as ineffective parasite clearance and development of resistance, newer drug combinations are also being developed to clear parasites at even lower doses.

In the end, we developed humanized mice (huRBCs-reconstituted NSG mice) and selected stable ART-resistant *P. falciparum* that showed co-resistance to amodiaquine, quinine, and halofantrine. If resistance to artesunate and artemisinins

evolves at such a speed along with co-resistance to quinine and other antimalarials, we would be left with no satisfactory option for treating severe malaria and a compromised choice of treatments for uncomplicated malaria. Indeed, the current dependence on ARTs for both uncomplicated and severe malaria, together with a lack of viable therapeutic alternatives, is a compromising situation. We believe this may have dire consequences and would cripple efforts to achieve malaria control globally. Therefore, novel approaches are needed to devise newer drug and their targets to address this drug resistance issue.

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Author's contribution

RKT, SS, and RK contributed to conceptualization and writing; SS, RK, and RKT contributed to writing—review and editing. All authors have read and agreed to the final version of the manuscript.

Conflict of interests

Authors declare no conflict of interests exists. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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