New Drug Discovery and Development in India to Counter Malaria

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4.1 Introduction

Malaria is a vector-borne disease caused by the *Plasmodium* spp. in humans and animals. This protozoan infection has immense social and economic impact in endemic regions across globe. An estimated 219 million cases of malaria were reported in 2017 with highest prevalence in Africa (92%), followed by South-East Asia (5%) and Eastern Mediterranean (2%) region. According to the World Health Organisation's (WHO) World Malaria Report 2018, 15 countries in sub-Saharan Africa and India contribute to 80% of the global malaria burden. India contributes 90% of total malaria cases in South-East Asia and ~50% of *P. vivax* cases worldwide. Despite rigorous efforts in malaria elimination programmes, there are intermittent barriers which affect the long-term implementation of intervention strategies. Further, the emerging cases of drug resistance against current frontline drugs have raised a health alarm underscoring the need for alternative antimalarial intervention strategies. Other impediments in malaria elimination programmes are socioeconomic disparity, suboptimal diagnosis and treatment which often lead to transplacental transmission of parasite in pregnant mothers, noncompliance to drug regimen

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which contributes to parasite recrudescence/relapse, and nonavailability of a potent vaccine against malaria. In the era of globalization, increased movement of people brings asymptomatic reservoir from endemic regions into non-endemic areas resulting in transmittance of resistance across geographical boundaries. Further, climate change has affected vector population size and density, vector survival, and parasite transmission rates.

For the treatment of malaria, WHO recommends Artemisinin combination therapy (ACT) regimens for 3-day treatment with following the drug combinations (1) artemether + lumefantrine; (2) artesunate + amodiaquine; (3) artesunate + mefloquine; (4) dihydroartemisin (DHA) + piperaquine (PPQ) (for children <25 kg weight) and artesunate + sulfadoxine-pyrimethamine. Delayed clearance upon treatment or "partial resistance" to Artemisinin has been detected in five countries of the Greater Mekong Subregion [Cambodia, Lao People's Democratic Republic (PDR), Myanmar, Thailand and Vietnam] (Imwong et al. 2017a, b; Ménard et al. 2018). Some areas of Greater Mekong Subregion have also reported resistance against ACT partner drugs, regardless of the presence of artemisinin partial resistance, thus leading to treatment failure. Delayed clearance time has been reported for dihydroartemisinin and piperaquine in Cambodia and South of Vietnam (Spring et al. 2015; Thanh et al. 2017); this is accompanied by significantly higher IC_{50}s of isolates with delayed clearance ($\geq$72 h) than those with normal clearance times for chloroquine, DHA, and PPQ (Thriemer et al. 2014). Resistance toward sulfadoxine-pyrimethamine has been reported in Republic of Congo (Alker et al. 2008), Sudan (A-Elbasit et al. 2006) and India (Kumar et al. 2015). There is also a geographical overlap of malaria co-infections with HIV and tuberculosis, with limited information available on how HIV or tuberculosis modifies therapeutic response to ACTs.

Even though India has made progress in controlling malaria, detection of malaria cases with co-infection of Dengue and Japanese Encephalitis Virus is of concern (Dev 2019; Sahu et al. 2016). Until 2016, the country remained under the category of malaria "controlling," not malaria "eliminating," nations (Newby et al. 2016). The National Strategic Plan for Malaria Elimination in India (2017–2022) has formulated effective disease management (diagnosis, preventive/curative interventions), surveillance (epidemiological, entomological) and awareness programmes to make India malaria-free by 2027. High-risk areas for malaria in India include Odisha, Chattisgarh, Jharkhand, Madhya Pradesh, Maharashtra, and north-eastern states (especially Assam, Tripura, Mizoram, and Meghalaya). Most cases of human malaria infections are reported for *P. falciparum* (prevalent in forest and peripheral areas) and *P. vivax* (prevalent in plains). The disease is transmitted by *Anopheles* spp. (*A. stephensi*, *A. culicifacies*, *A. fluviatilis,* etc.) which have heterogeneous distribution throughout the country. The various challenges for malaria elimination in India are: (a) shortage of skilled human resource to participate in and coordinate malaria elimination programmes, (b) limited access of health services at point-of-care (remote, conflict-affected or endemic areas), (c) ineffective vector control programmes and widespread resistance to insecticides, (d) movement of asymptomatic reservoir across states and international boundaries including from countries with reported artemisinin resistance, (e) lack of effective public–private partnership

for monitoring antimalarial drug quality, efficacy which are critical for sustenance of antimalarial initiatives.

The National Malaria Drug Policy (2013) of India recommends that *P. vivax* be treated with chloroquine for 3 days and primaquine for 14 days. However, primaquine is contraindicated in pregnant women, breastfeeding mothers, infants (<6 months age) and patients with G6PD deficiency. Uncomplicated *P. falciparum* cases are to be treated with ACT (artesunate for 3 days + sulphadoxine-pyrimethamine for 1 day). This is to be accompanied by single-dose primaquine preferably on day 2. Due to reports of resistance to sulphadoxine-pyrimethamine in North-Eastern states of India, artemether-lumefantrine is recommended in these regions (not recommended during the first trimester of pregnancy and for children weighing <5 kg). For severe/complicated malaria, parenteral treatment regimens with artesunate/artemether/arteether/quinine injections followed by area-specific oral ACT or quinine + doxycycline or clindamycin are recommended.

Globally, efforts are being made by government sponsored antiparasitic drug screening programmes in academic institutions including the "open source" drug discovery platforms. These are strengthened with support and coordination offered by the not-for-profit organization—Medicines for Malaria Venture, Geneva. The Wellcome Trust (UK), Consortium for Parasitic Drug Development (USA) and the Bill and Melinda Gates Foundation also fund initiatives toward development of safe and efficacious next-generation medicines against drug-resistant parasitic infections. Ongoing research efforts involve generation of new antimalarials with the following features: (1) rapid action, delayed development of resistance, (2) knowledge of mode of action, (3) oral delivery with preferably single-dose cure for improved compliance, (4) action on multiple parasite stages for transmission blocking, (5) action on the liver stage, especially hypnozoite in the case of *P. vivax*. Drug formulations for improved bioavailability and ease of use in comatose patients are also being developed.

4.2 Antimalarials: The India Story

The urgent need to develop indigenous new fast-acting schizonticides was recognized with the resurgence of malaria and rise in drug-resistant cases in the country in the 1970s. Control and management of complicated *P. falciparum* malaria cases was also a concern. Efforts were made to control malaria by employing vector control strategies as well as investing in new drug discovery efforts. Some significant developments (Fig. 4.1) are discussed here.

4.2.1 α/β Arteether: A Successful Intervention for Complicated Malaria

Focused effort toward new chemical entities (NCE) resulted in the development of α/β arteether, a semisynthetic derivative of artemisinin, as a joint programme of two

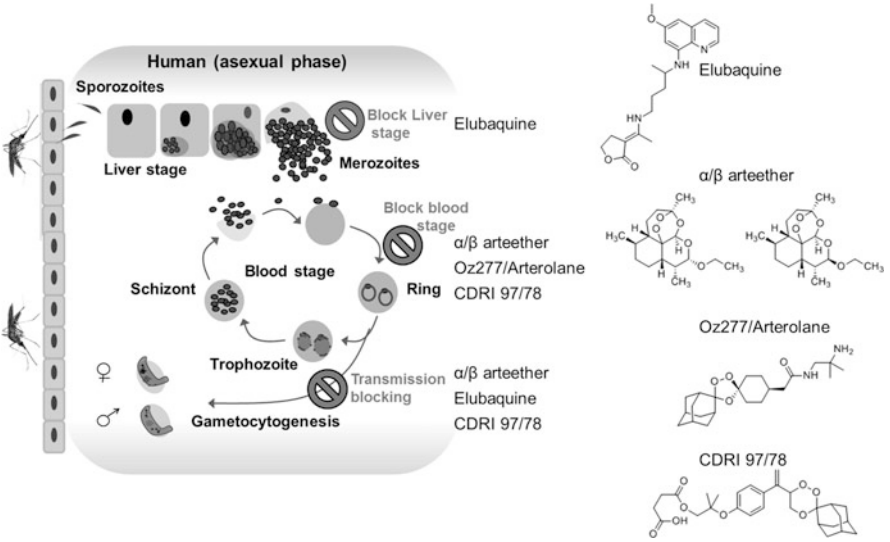

Fig. 4.1 Sites of antimalarial interventions of compounds discovered and developed in India. Out of these, α/β arteether and Oz277/arterolone are in market. Known sites of intervention in blood stage are; cytosol (antifolates: pyrimethamine, proguanil; tRNA synthetases: cladologs, eEF2: DDD107498), plasma membrane (ATP4 inhibitors: KAE609), mitochondrion (DHODH inhibitor: DSM265; Cytb inhibitor: atovaquone), apicoplast (translational inhibitors: doxycycline, clindamycin) and food vacuole (inhibitors for hemozoin formation: chloroquine, mefloquine; protease inhibitors: WEHI-842, MG132), vesicle trafficking (PI4K inhibitor: MMV390048, UCT943)

CSIR laboratories-CIMAP and CDRI (R.A. Vishwakarma et al. Indian Patent no. 173947). Arteether is a mixture of β and α anomers (70:30 ratio). It was first reported as a fast-acting schizonticide in the rodent parasite *P. yoelii nigeriensis* MDR screen, exhibiting curative efficacy at 5 mg/kg × 4 days, i.m. (Dutta et al. 1989a). Its curative efficacy was also established in primate malaria models: *P. knowlesi* (12 mg/kg × 5 days) (Bajpai et al. 1989), *P. cynomolgi* (5 mg/kg × 3 days) (Dutta et al. 1989b), *P. fragile*—a cerebral malaria model (5 mg/kg × 3 days) (Bajpai et al. 1989); α/β arteether is also gametocytocidal (Tripathi et al. 1996). Following successful preclinical safety evaluation of the injectable formulation and clinical safety in a Phase I study at CDRI, trials were conducted for uncomplicated and complicated *P. falciparum* malaria. Phase II clinical trial with 51 patients at the Ispat General Hospital, Rourkela established proof of concept of clinical antimalarial efficacy of α/β arteether at the dose of 150 mg once daily for 3 days (i.m.) that killed parasites in the blood between 1 and 3 days and cleared fever between 1 and 6 days with no side effects (Mishra et al. 1995). Phase III trials were carried out in more than 500 uncomplicated and complicated *P. falciparum* patients in seven centers across the country [Jawaharlal Nehru Hospital, Bhilai; Malaria Research Centre/Government Medical College, Jabalpur; Malaria Research Centre,

New Delhi; Lady Hardinge Medical College, New Delhi; Central Reserve Police Force Base Hospital, Guwahati; Regional Malaria Research Centre (ICMR), Dibrugarh, Assam; Tata Main Hospital, Jamshedpur; Ispat General Hospital, Rourkela] (Mohanty et al. 1997; Mukim et al. 2011). The cure rate was found to be 93.3% in uncomplicated cases. In the 211 complicated disease cases treated with α/β arteether, only 14 expired of which ten died within 2 days before completing the 3-day arteether therapy (Asthana et al. 2001).

α/β arteether was approved for marketing by the Government of India, licensed to Themis Chemicals, and marketed as E-Mal in 1997. Its safety and 100% efficacy was confirmed in post-marketing surveillance in 2003. E-Mal was included in the National Drug Policy on Malaria, Ministry of Health and Family Welfare, Govt. of India in 2010 and continues as preferred treatment for complicated/cerebral malaria cases in India. The drug is also being exported to seven nations in Africa. From the single brand E-Mal in 1997, α/β arteether grew to 175 products in June 2016. This expansion resulted in a desirable fall in its market price (from ~Rs. 400 in 1997 to ~Rs. 80 in 2016), making α/β arteether an affordable antimalarial for public health.

4.2.2 Primaquine Derivative Elubaquine/Aablaquine for *P. vivax*

Primaquine (PQ), an 8-aminoquinoline that was first synthesized in the USA in 1946 remained the only transmission blocking antimalarial till the recent launch of Tafenoquine (Lacerda et al. 2019). Although PQ is effective against all exoerythrocytic forms of the parasite, its low efficacy against endo-erythrocytic parasites necessitates co-administration with a blood schizonticide in a 14-day treatment for anti-relapse therapy and radical cure of *P. vivax* (or *P. ovale*)-infected patients (Vale et al. 2009). However, PQ is contraindicated in infants, pregnant women and causes hemolytic anemia in individuals with G6PD deficiency. The latter is a special problem in India as populations residing in malaria-endemic regions of eastern India also have a higher incidence of G6PD deficiency. Another adverse effect of PQ is methemoglobinemia, a pathological condition arising from abnormal accumulation of methemoglobin, the product of auto-oxidation of the hemoglobin iron core.

A primaquine derivative, elubaquine/bulaquine/aablaquine (CDRI 80/53) was developed at CDRI, Lucknow. The drug had proven gametocytocidal efficacy in monkeys infected with *P. cynomolgi*; a single administration of elubaquine at 1.25 mg/kg blocked parasite oocyst development after 24 h and at 3.75 mg/kg within 5 h (Puri and Dutta 2005), thus preventing transmission through infected *Anopheles stephensi* mosquitoes. Gametocytocidal activity of elubaquine is more potent and faster than PQ. The improved safety profile of the drug over PQ is indicated by low methemoglobin accumulation in human volunteers (CDRI document on 80/53 1997). After 7 days of administration, elubaquine-induced methemoglobin levels ranged between 2.29% and 3.02%, whereas identically administered PQ increased methemoglobin from 3.97% to 16.32%.

Phase II clinical trials with elubaquine, carried out on 697 patients infected with *P. vivax*, showed that the drug given orally for 5 days at 25 mg/kg had a similar

pattern of relapse as PQ at 15 mg/kg (Valecha et al. 2001). A safety and tolerability comparison of PQ and elubaquine conducted on 141 *P. vivax*-infected patients in Thailand (Krudsood et al. 2006) reported that four G6PD-deficient patients treated with PQ experienced a significant fall in hematocrit beyond the 7 day treatment period, whereas the three elubaquine-treated G6PD deficient patients did not exhibit a significant change in hematocrit leading to the conclusion that elubaquine does not cause clinically significant hemolysis. Pharmacokinetic analysis of elubaquine in different animal species has suggested species-specific differences (Mehrotra et al. 2007). The improved G6PD-related safety profile of elubaquine needs to be validated in a larger patient pool together with generation of its complete in vivo pharmacokinetic and metabolite profile for understanding the observed differences with PQ. Elubaquine was licensed to Piramal Enterprises Ltd. in 1999. It is not being marketed at present.

The most recent addition to the drug arsenal against *P. vivax* relapse is tafenoquine, an 8-aminoquinoline marketed by GlaxoSmithKline. Although its long half-life (2–3 weeks) allows a single oral dose to clear hypnozoites, it still has the problem of causing hemolysis in G6PD deficient patients. The United States Food and Drug Administration (FDA) approved single-dose tafenoquine for radical cure (prevention of relapse) of *P. vivax* malaria in 2018. Initial development of tafenoquine (WR 238605) was done by the Walter Reed Army Research Institute, Washington DC during which time collaborative primate malaria studies were conducted at CDRI, Lucknow and showed that tafenoquine was more effective at ED_{50} level compared to PQ (Puri and Dutta 2003).

Exploration of quinolines for new antimalarials continues. A series that acts on both chloroquine-sensitive and -resistant parasites was identified (Indian patent 2291/DEL/2013) and a molecule from the series with demonstrated efficacy in the *P. yoelii* N67-mouse model and *P. cynomolgi*-Rhesus macaque model is currently under preclinical safety evaluation at CDRI (unpublished data). Several 4-aminoquinolines with in vitro activity against chloroquine-resistant *P. falciparum* and curative efficacy in *P. berghei*-infected BALB/c mice have also been recently reported (Singh et al. 2016).

4.2.3 Synthetic Endoperoxides

As ACTs started gaining ground as first-line treatment for malaria in many parts of the world, malaria chemotherapy moved to counter the vagaries of *Artemisia annua* production, supply chain and extraction costs. Progress was made using the endoperoxide sesquiterpene artemisinin and its derivatives (artemether, arteether, artesunate etc.). Chemists at CDRI synthesized substituted 1,2,4-trioxanes that were extensively screened against MDR *P. yoelii nigeriensis* rodent parasite followed by efficacy evaluation in *P. cynomolgi* and *P. knowlesi* in rhesus monkey model (Singh et al. 2007a, b) (US Patent, 6316493). The gametocytocidal action of two molecules in this series was also confirmed. CDRI 97/78 and CDRI 99/411 were identified as potent oral antimalarials, found safe in safety pharmacology and

toxicology studies and subsequently licensed to IPCA Pharmaceuticals for clinical development. CDRI 97/78 is an equipotent hemisuccinate derivative of CDRI 97/63 and is rapidly metabolized to the latter. The compound was approved for Phase I clinical trials in India; Phase Ia (single ascending dose safety and PK) conducted in PGIMER, Chandigarh found the compound safe in human volunteers (Shafiq et al. 2014).

Another fully-synthetic peroxide, OZ277 (RBx11160, Arterolane, of the Dispiro 1,2,4-Trioxolane series) was synthesized by Vennerstrom and coworkers (Vennerstrom et al. 2004) (U.S. Patent number 7371778) as a fast-acting blood schizonticide. Arterolane exhibited single-dose curative efficacy against *P. berghei* ANKA infection in mice at 30 mg/kg dose. OZ277 is also effective against artemisinin-resistant *P. falciparum* Cam3.I^{R539T} parasites (isolate from Cambodia with K13-propeller mutation R539T) at pharmacologically relevant concentrations (Baumgartner et al. 2017). A multicenter, randomized Phase II trial of Arterolane with 230 patients from four centers in Thailand, India, and Tanzania (mainland and Zanzibar) who received 50, 100, or 200 mg of arterolane (once daily for 7 days) showed that it was rapidly acting, effective, and safe (Valecha et al. 2010). A subsequent Phase III trial (Toure et al. 2016) with fixed-dose combination of arterolane maleate with piperaquine phosphate showed that the combination had comparable efficacy with the artemether-lumefantrine combination for treatment of uncomplicated *P. falciparum* malaria. Arterolane-piperaquine (Synriam) was launched by Ranbaxy in 2012 and is currently marketed by SunPharma. Artefenomel (OZ439), a novel trioxolane, was designed to bypass the initial low exposure liability related with OZ277 (Charman et al. 2011). OZ439 is currently a front-runner candidate in combination with ferroquine to allow for once-daily dosing in fewer doses than the current 3-day ACT regimen (Sanofi and MMV partnership).

4.2.4 Exploration of Leads from Traditional Knowledge

The fact that two major antimalarial compounds—quinine and artemisinin were isolated from plants as a result of scientific exploration of traditional knowledge has provided impetus to further reconnaissance of plant-derived molecules with clues from Ayurveda/Unani systems and folk medicine. Although several reports using plant extracts for assaying antimalarial activity have been published (Bagavan et al. 2011; Kantamreddi et al. 2009; Panda and Luyten 2018; Shankar et al. 2012; Simonsen et al. 2001), there has been little progress in identifying bioactive molecules.

The Central Council of Ayurveda and Siddha patented Ayush-64, a combination of four plants namely *Alstonia scholaris* (aqueous extract of bark), *Picrorhiza kurroa* Royle (aqueous extract of rhizome), *Swertia chirata* (aqueous extract of whole plant), and *Caesalpinia crista* Linn (powder of seed pulp) for its antimalarial activity. However, when the combination was tested in *P. vivax* patients in a non-crossover, randomized clinical trial at the Malaria Research Centre in collaboration with National Anti-Malaria Programme (Valecha et al. 2000), the results

showed a much lower cure rate with Ayush-64 (1 g oral dose, three times a day for 5–7 days) as compared to chloroquine (1500 mg oral, over 3 days). Leaf paste of *Nyctanthes arbor-tristis* Linn ("Parijat" or "Harsingar") was tested in 120 *P. vivax* and *P. falciparum* patients at the MA Podar Hospital, Mumbai. The paste given orally three times a day for 7–10 days was reported to cure 92 (76.7%) patients within 7 days, another 20 patients by 10 days, and the remaining had to be cured by standard antimalarial therapy (Karnik et al. 2008). Activity-guided RPHPLC fractionation of the ethanol extract of *Nyctanthes* leaves suggested that more than one active compound defines potency and that iridoid glycosides are the most probable phytoconstituents responsible for anti-plasmodial activity (Kumari et al. 2012).

The dried rind of *Punica granatum*, promoted as OMARIA, is being used in Odisha for prophylaxis and cure of malaria (Lekana-Douki et al. 2012). In a study conducted in Italy, the preparation was subjected to activity-guided fractionation of the methanolic extract of fruit rind and in vitro activity was found to be associated with fractions enriched in tannins-punicalagins, ellagic acid and its glycoside. However, both the methanolic extract and the fraction did not show in vivo efficacy in the murine malaria model (Dell'Agli et al. 2009). This was attributed to lower bioavailability and possible conversion of ellagitannins into inactive metabolites in mice. The lack of efficacy in the mouse malaria model makes it difficult to take forward a preclinical analysis of OMARIA, although it continues to be used to treat patients in Odisha.

Curcumin was reported as an antimalarial by Reddy et al. (2005) as it inhibited chloroquine-resistant *P. falciparum* growth in culture (IC_{50} of 5 μM) and reduced parasitemia by 80–90% when administered orally (100 mg/kg for 5 days) to *P. berghei*-infected mice. Subsequently, a combination of α,β-arteether (E-Mal) and curcumin was shown to result in better survival rates of *P. berghei*-infected mice, and a 3-day oral regimen of curcumin with a single injection of α,β-arteether at 750 μg or 1.5 mg per mouse led to 100% survival and complete protection of animals against recrudescence (Nandakumar et al. 2006), possibly through enhancement of TLR-2 mediated innate immune response (Vathsala et al. 2012). Further, a PLGA-based nanoformulation of curcumin was developed to improve therapeutic index (Dende et al. 2017); PLGA-curcumin improved bioavailability of curcumin and had comparable efficacy with native curcumin at a 15-fold lower concentration; PLGA-curcumin prevented the breakdown of the blood–brain barrier and inhibited the sequestration of parasitized-RBCs and $CD8^+$ T cells in the brain. It also inhibited mRNAs for inflammatory cytokines and chemokine receptor CXCR3 and activated the anti-inflammatory cytokine IL-10 and was proposed for use as an adjunct in antimalarial therapy.

4.2.5 Formulations

Novel drug delivery approaches have been attempted for increasing the bioavailability of drugs, reducing dose or providing an alternative route of administration. A polymeric lyotropic liquid crystalline formulation of arteether-lumefantrine gave a

prolonged release of both drugs and conferred complete protection with no mortality at 1/40th of the therapeutic dose in *P. berghei* ANKA infected mice, suggesting the possibility of single-shot therapy (Dawre et al. 2018). Arteether nanoemulsions and self-nanoemulsifying drug delivery systems have also been tested (Dwivedi et al. 2015, 2014). Atovaquone that targets parasite mitochondrial cytochrome B is marketed in combination with proguanil as Malarone (GSK). Atovaquone nanoparticles formulation given with proguanil showed approximately two-fold improved bioavailability in rats compared to Malarone (R) with significant dose reduction in Peter's 4-day suppressive tests in mice (Darade et al. 2018). The bioavailability of lumefantrine in a solid dispersion formulation is enhanced many folds when assessed in a randomized, open-label study in healthy volunteers (Jain et al. 2017). Nanostructured lipid carriers (NLC) for artemether-lumefantrine oral therapy with significant reduction in dose have been formulated (Prabhu et al. 2016b) and an NLC formulation of the drug combination has also been made for intravenous therapy in cerebral malaria patients (Prabhu et al. 2016a). A solid self-microemulsifying drug delivery system (SMEDDS) formulation of artemether–lumefantrine capable of maintaining plasma concentration of lumefantrine above the minimum effective concentration for approximately 4 days has been reported (Bhandari et al. 2017). A long circulatory PEGylated liposomal formulation of the gametocytocidal ionophore maduramicin has shown enhanced antiparasitic activity compared to the free drug (Raza et al. 2018).

Specific targeting to infected RBCs has been attempted by using chitosan particles derivatized with dehydroascorbic acid (DHA) (Shafi et al. 2017). DHA competes with glucose for binding to the mammalian glucose transporter GLUT-1 which is over-expressed in *Plasmodium*-infected human RBCs. The chitosan-DHA particles were superior in terms of uptake and extent of preferential targeting to infected RBCs in vitro. Specific targeting to infected RBCs is not usually required as antimalarial drugs in use reach plasma concentrations high enough to sustain therapeutic threshold concentrations in erythrocytes; however, targeting to infected cells can result in dose reduction and aid in overcoming efflux-mediated drug resistance.

4.3 Exploring New Biological Targets for Drug Discovery and Design

Parasite resistance to antimalarial drugs is generated by accumulation of mutations which result in (1) reduced uptake or increased efflux of drug, (2) altered protein–drug interaction, (3) metabolic bypass of targeted pathway, and (4) upregulation of stress–response pathways. Concerted efforts are required to develop a comprehensive understanding (biochemical, structural, and molecular) of critical pathways to evaluate druggability of validated biological targets and design specific inhibitors. In the subsequent sections, we discuss some of the potential drug targets/pathways that have been investigated by researchers in India.

4.3.1 Fatty Acid Synthesis (FAS-II Pathway)

The mammalian host has type I fatty acid synthases (FAS-I) which are multifunctional proteins whose distinct domains catalyze different steps involved in fatty acid synthesis. In contrast, the malaria parasite possesses type II fatty acid synthesis system (FAS-II) whereby individual reactions are catalyzed by separate FAS enzymes. The FAS-II pathway involves preparation, initiation, and elongation phases for fatty acid synthesis with the parasite relict plastid, the apicoplast, being the site of FAS-II mediated synthesis. Surolia and coworker (Surolia and Surolia 2001) reported the antimalarial activity of Triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol]. Triclosan is an antimicrobial biocide that is known to inhibit fatty acid synthesis in bacteria (McMurry et al. 1998). Triclosan exhibited IC_{50} value of 0.7 μM in in vitro *P. falciparum* cultures and curative dose of 38 mg/kg in the *P. berghei*-mouse model. The authors also reported that Triclosan specifically inhibits enoyl-ACP reductase which is required for fatty acid synthesis in apicoplast (Surolia and Surolia 2001). Mutational analysis of enoyl-ACP revealed differences in the binding of Tricolsan to parasite and bacterial enzyme (Kapoor et al. 2004). Through structure-based approach, 2′-subsituted analogs of Triclosan were designed and tested for their blood stage antimalarial activities; some of the scaffolds showed nanomolar activities in enzyme based inhibition of *Pf*ENR and were similar to Triclosan-mediated enzyme inhibition (Kapoor et al. 2009). Another class of enoyl-ACP reductase inhibitors, bromo-benzothiophene carboxamides were designed, but their antimalarial activities were not better than Triclosan (Banerjee et al. 2011). Gene knock-out studies have subsequently revealed that FASII is dispensable in blood stages; however, it is critical for liver stages and sporozoite development in the mosquito midgut. This suggested that the observed blood stage antimalarial activity of FAS-II inhibitors was likely to be due to alternative biological targets (Shears et al. 2015), although inhibition of growth of late liver stage parasites by Triclosan was reported (Singh et al. 2009). Nevertheless, a comprehensive SAR for FAS-II inhibitors might aid in identification of inhibitors of parasite multiplication in the liver and survival of hypnozites in *P. vivax* (Schrader et al. 2013).

4.3.2 Aminoacyl tRNA Synthetases

Plasmodium has active protein translation in the cytoplasm and also in its organelles—the mitochondrion and apicoplast. A critical component of the translation machinery is aminoacyl tRNA synthetases (aaRS) that function to charge tRNA with the cognate amino acid for its incorporation in the growing polypeptide chain. All parasite aaRS are nuclear-encoded and localize to the parasite cytoplasm (16 aaRS), apicoplast (15 aaRS), mitochondrion (1aaRS); four aaRS exhibit dual localization in the cytoplasm and apicoplast (Habib et al. 2016). Structure elucidation of aaRS led to structure-based design and evaluation of specific inhibitors (Khan et al. 2011, 2013a, b). Initial screening of small-molecule library led to identification

of hit molecules with promising methionyl-tRNA synthetase inhibition and antimalarial activities. This included REP3123 and REP8839 which are known to inhibit bacterial methionyl-tRNA synthetases and also exhibit antimalarial activity (IC_{50} values ~150 nM) (Hussain et al. 2015). Over the years, in silico and structural biology based approaches have helped to map the druggable site for aaRS (Manickam et al. 2018). For instance, ATP binding site inhibitors (cladosporin), molecules binding to 3′-end of tRNA pocket (halofuginone and borrelidin) and molecules binding to tRNA editing site (benzoxaborole) have been investigated (Goodman et al. 2016; Jain et al. 2015; Khan et al. 2014). Amongst these, cladosporin (an antifungal metabolite) has shown potent antimalarial activity against blood and liver stages of the malaria parasite ($IC_{50} < 100$ nM). The scaffold includes 6,8-dihydroxylisocoumarin ring joined to tetrahydropyran group with a methyl moiety and is known to specifically target lysyl-tRNA synthetase (KRS) over the mammalian homolog (Khan et al. 2014). Recently, a library of stereoisomers of cladosporin (cladologs) were synthesized and assessed for their antimalarial activity (Das et al. 2018). With systematic changes at chiral centers, the authors could map the critical attributes which increased the antimalarial potency of cladologs with specificity over human KRS. In a recent study, another cladosporin-based scaffold showed potent in vivo efficacy with ED_{90} of 1.5 mg/kg (oral administration) in rodent malaria model along with selective inhibition of *Pf*KRS over human KRS (Baragana et al. 2019). Cytosolic and organellar tRNA synthetases, thus offer an exciting opportunity for identification of novel antimalarials.

4.3.3 DNA Gyrases

DNA gyrases (type II Topoisomerases) play a critical role in maintenance of DNA topology during bi-directional *ori* replication of the circular apicoplast genome in the parasite (Nagano et al. 2014; Weissig et al. 1997). The *P. falciparum* nuclear genome encodes GyrA and GyrB proteins that harbor an N-terminus signal and transit sequence for apicoplast targeting. GyrA has DNA cleavage and DNA wrapping domains, while GyrB has an N-terminus ATPase domain with DNA binding domain and GyrA-interacting domain at the C-terminus. Bacterial DNA gyrase inhibitors (ciprofloxacin, novobiocin) also inhibit parasite growth. Novobiocin was shown to inhibit ATPase activity of GyrB, albeit with binding affinity different from bacterial gyrase probably due to the presence of unstructured domains in *P. falciparum* GyrB. Novobiocin treatment specifically altered apicoplast DNA integrity in comparison to nuclear and mitochondrial DNA (Raghu Ram et al. 2007). Similarly, coumermycin was shown to inhibit the ATPase activity of *P. falciparum* GyrB in a dose-dependent manner (Dar et al. 2007). This confirmed that apicoplast DNA replication can be a potential site of intervention. Recently, acriflavine with known antibacterial and anticancer activity has been shown to have anti-plasmodial activity in both in vitro *P. falciparum* culture and the *P. berghei*-mouse model. Acriflavine impairs DNA replication by specifically inhibiting apicoplast GyrB

(Dana et al. 2014). Other DNA gyrase inhibitors include coumarin-triazole based compounds (Yadav et al. 2018); however, their comprehensive SAR and evaluation of in vivo efficacy is required.

4.3.4 Noncanonical Structures at Telomeric Ends

Besides protein targets, noncanonical nucleic acid secondary structures especially at telomeric ends can be sites for intervention. Telomeric and sub-telomeric regions have high enrichment of G-rich sequences which have the propensity to form G-quadruplex structures in *Plasmodium* (Bhartiya et al. 2016). Telomere architecture and dynamics of the parasite and host have remarkable differences in telomere length (*Plasmodium* 1–2 kb, human 10–15 kb), telomerase size (*P. falciparum* telomerase is 2.5 times larger than the human homolog), and diversity of proteins binding to telomeric and sub-telomeric regions (Figueiredo and Scherf 2005; Figueiredo et al. 2005). Therefore, disturbing telomere structure and function through G-quadruplex interacting ligands can be explored as an alternative strategy for antimalarial intervention. The life of drugs that target critical parasite biosynthetic pathways is limited by the progressive accumulation of mutations in protein targets leading to drug resistance. Hence, a probable advantage of pharmacological targeting of noncanonical structures in telomeric ends can be a delay in emergence of resistance, as acquiring mutations that disturb secondary structures in telomeres is likely to have a dramatic fitness cost. G-quadruplex interacting ligands like bisquinolinium derivatives of 1,8-naphthyridine and pyridine, bisbenzimidazole carboxamide derivatives, benzothiazole hydrazones of furylbenzamides show in vitro antimalarial activity with reasonable selectivity index (Anas et al. 2017). These ligands cause shortening of parasite telomeres, alter the transcriptional dynamics of sub-telomeric genes and induce DNA damage. However, a comprehensive SAR is essential to identify "best in class" so that they can be taken forward for evaluation of in vivo efficacy.

4.3.5 ATP Transporters

Amongst various transporters, parasite plasma membrane localized *Pf*ATP4 has emerged as a promising drug target (Dennis et al. 2018). This is a P-type ATPase that converts energy from ATP hydrolysis into cation transport (sodium export is coupled with proton import). Multiple chemotypes like KAE609 (spiroindolone), MMV007275 and MMV0011567 (carboxamides from MMV malaria box), NF-Pf4492 (aminopyrazole), 21A092 (pyrazoleamide), and (+)-SJ733 (dihydroisoquinolone) identified by international groups showed promising inhibition of *Pf*ATP4 activity and parasite growth. Of these, KAE609 (also known as cipargamin) kills *P. falciparum* and *P. vivax* parasites in vitro at EC_{50} values $<$10 nM (Rottmann et al. 2010; White et al. 2014). KAE609 also inhibits gametocyte and oocyst development in mosquitoes. KAE609 has rapid parasite clearing

ability and good pharmacokinetic–pharmacodynamic properties and has progressed through Phase I and IIa clinical trials (Leong et al. 2014). In India, molecular docking studies revealed that 1,3-benzoxazine derivatives of phytophenol eugenol and isoeugenol bind to *Pf*ATP4. These compounds also showed promising antimalarial activity due to disruption of sodium homeostasis (Sharma et al. 2018). A recent report has indicated that disturbing AMP homeostasis in *Plasmodium* by overexpression of adenosine 5′ monophosphate deaminase (AMPD) is inimical to parasite survival suggesting that allosteric activators of *Pf*AMPD could be designed and evaluated as antiparasitic agents (Nagappa et al. 2019).

4.3.6 Heme Biosynthesis and Detoxification Pathways

Plasmodium possesses a heme biosynthesis pathway despite having access to host-derived heme (Nagaraj and Padmanaban 2017; Sato et al. 2004; Surolia and Padmanaban 1992). Parasite-synthesized heme serves as a cofactor for mitochondrial cytochromes that support electron transport. The heme biosynthesis pathway is not essential in blood stages, but is critical for liver and mosquito stages of the parasite life cycle (Goldberg and Sigala 2017; Ke et al. 2014; Nagaraj et al. 2013). Heme biosynthesis inhibitors will be ineffective in blood stages but might serve as transmission blockers and/or prophylactic agents. Researchers have argued that heme biosynthesis may not be a good drug target (Koreny et al. 2013). In fact, proteins involved in heme detoxification are good drug targets to explore. Two major antimalarials chloroquine and artemisinin act on heme polymerization and chloroquine also inhibits hemoglobin degradation (Chugh et al. 2013). *Pf*HDP (heme detoxification protein), a major protein involved in hemozoin formation (nontoxic crystalline form of heme) has been suggested as a critical drug target. Through screening of Maybridge library, the identified hit ML-2 (1-(3,4-dihydronaphthalen-2-yl)-4-[3-(trifluoromethyl)phenyl]piperazine) showed dose-dependent parasite inhibition (Gupta et al. 2017). Quinolines and peroxide-based compounds have been shown to inhibit hemozoin formation (Fong and Wright 2013; Pandey et al. 1999; Verma et al. 2016). Some synthetic peroxides that show promising antimalarial activity against chloroquine-sensitive and resistant strains include 1,2-dioxane; 1,2,4-trioxanes; 1,2,4-trioxalanes and their hybrid molecules like trioxaquines (Chauhan et al. 2010; Yadav et al. 2014).

4.3.7 Proteases/Disaggregases

Plasmodium spp. have evolved efficient protein degradation machinery which helps in maintenance of protein homeostasis through efficient removal of misfolded or aggregated species. For instance, Clp proteases (ATP-dependent disaggregase machinery) are localized in different parasite organelles (mitochondria and apicoplast) (El Bakkouri et al. 2010; Jain et al. 2013). In silico screening of inhibitors of Clp proteases and comprehensive SAR will help in identification of potential hits

(Mundra et al. 2017). Other proteases include subtilisin-like protease, cysteine protease (falcipain), aspartyl protease (plasmepsin). These proteases are involved in hemoglobin digestion, protein trafficking, parasite invasion of RBC and egress from infected RBCs and hepatocytes (Moura et al. 2009; Prasad et al. 2012). In light of the existing knowledge about their enzymatic reactions and 3D-structure, these proteases are being explored as drug targets (Deu 2017; Mishra et al. 2019; Nasamu et al. 2017; Pino et al. 2017). Collaborations between ICGEB-New Delhi and researchers in Canada and Italy have identified initial hits targeting falcipain-2 and apicoplast ClpP protease (Chakka et al. 2015; Mundra et al. 2017; Rizzi et al. 2011). Isoquinolines, hydroxyethylamines-based active pharmacophores have been designed against plasmodial proteases (Batra et al. 2003; Gupta et al. 2017; Singh et al. 2019). Molecular dynamics based approach is also employed for design of PEXEL-based mimetics against parasite plasmepsin (Bedi et al. 2016). Through structure-guided drug discovery, KNI compounds initially discovered as inhibitors of HIV protease were modified to inhibit vacuolar plasmepsins (Mishra et al. 2018). KNI scaffold consists of allophenylnorstatine [[(2S,3S)-3-amino-2-hydroxy-4-phenylbutyricacid] (Apns)]. Alkylamino analog and phenylacetyl tripeptides exhibited promising antimalarial activity with minimal toxicity in human cells (Mishra et al. 2018). Apart from proteases, the ubiquitin-proteasome machinery of the parasite is being explored as a target for antimalarial intervention. A peptidyl inhibitor MG132 with a P2 leucine inhibited both cysteine protease and ubiquitin-proteasome system activities in parasite extracts and also strongly inhibited recombinant falcipains (Prasad et al. 2013). MG132 was highly selective for inhibition of *P. falciparum* indicating the possibility of generating dual-target inhibitors of malaria parasites.

4.3.8 Other Unique Pathways/Targets

Additional biological pathways explored for future antimalarial intervention include proteins involved in genome maintenance. For instance, *Pf*Alba (Acetylation lowers binding affinity) superfamily proteins whose acetylation state affects their DNA binding ability and consequently affects transcription have been studied. *Pf*Alba3 interacts with the epigenetic modifier Sir2a and occupies telomeric/sub-telomeric regions including the *var* gene promoters for transcriptional regulation of antigenic variation/virulence genes (Goyal et al. 2012, 2016). Similarly, another DNA binding protein, Origin Recognition Complex (ORC) binds to the sub-telomeric regions and regulates the expression of antigenic variation genes (Deshmukh et al. 2012; Gupta et al. 2008). Telomere/sub-telomere structure and function is also influenced by the epigenetic machinery (histone acetylases, deacetylases, methylases, and demethylases). Phylogenetic analyses of *Plasmodium* histone acetylases and deacetylases suggests that many of them are close to prokaryotic acetylases and deacetylases (Kanyal et al. 2018), and thus may be further evaluated as targets.

Research groups are also investigating protein folding, assembly and processing machineries. This includes the diverged protein folding machinery which has

acquired potential noncanonical roles to provide survival advantage to parasite (Bhartiya et al. 2015). Amongst the diverse class of chaperones or heat shock proteins (HSPs), HSP40 family has structurally diverse domains, exhibit differential organellar localization (few even exported into RBC), and execute noncanonical functions (Behl et al. 2019; Kulzer et al. 2012; Kumar et al. 2010). Establishing assays for their biochemical (protein folding and oligomer remodeling) activities will help to validate compounds which specifically inhibit parasite chaperones.

The biogenesis of [Fe-S] clusters by the SUF pathway has also been proposed as a unique target, as this pathway is absent in humans but is conserved in bacteria, protozoa, and plants. In the malaria parasite, components of the SUF and ISC systems are largely encoded by the nucleus and localize to the apicoplast and mitochondrion, respectively (Kumar et al. 2011). The SUF pathway is essential for parasite survival in the blood and the mosquito stages (Charan et al. 2017; Gisselberg et al. 2013). No inhibitors of the SUF pathway are currently known but the possibility of chemically inhibiting the first enzyme (cysteine desulfurase SufS) of the pathway has been suggested (Charan et al. 2014). Besides biochemical characterization, genetic tools for generation of knock-out (KO) parasite strains are being employed to assess the essentiality and phenotypes of KO parasite. These attempts give insights into biological roles of uncharacterized *Plasmodium* proteins which may help identify new targets for future drug discovery/design (Al-Nihmi et al. 2017; Jaijyan et al. 2016; Mastan et al. 2017).

4.4 Repositioning of Molecules for Antimalarial Activity

The possibility of repurposing FDA-approved drugs for other uses such as antimalarials has been addressed by several groups. Screening of drug libraries in blood stage *P. falciparum* culture and liver stage in *P. berghei* model identified potential drug candidates for malaria (Chong et al. 2006; Derbyshire et al. 2012). These potential candidates belonged to diverse categories such as proton pump inhibitor, immunosuppressant, antihypertensive, ovulatory stimulant, antimicrobial, bone resorption inhibitor with varied IC_{50} values ranging from nanomolar (cyclosporine: 17 nM; telmisartan: 25 nM), submicromolar (clomiphene citrate: 0.219 μM; raloxifene hydrochloride and pentamidine isethionate: ~0.5 μM) and micromolar (lopinavir, rotanavir, azithromycin:~2.0 μM) (Pazhayam et al. 2019). Though the primary drug targets of these candidates are known in humans, in some cases their corresponding orthologs are absent in the malaria parasite. This suggests novel mechanisms of drug action in parasites. These drug candidates can be further evaluated for multistage activity (liver stage and gametocytocidal activity). Ivermectin, currently used against lymphatic filariasis, inhibits *Plasmodium* nuclear import/export (Panchal et al. 2014) and kills the parasite in the mosquito and human liver stages (Mendes et al. 2017). Drug repurposing efforts can help fast-track the optimized leads into next-generation antimalarials. Apart from this, open source drug discovery efforts supported by MMV and GSK have provided access to chemically diverse libraries (Malaria Box and Pathogen Box) to determine mode

of action and generate SAR around specific hit molecules. A recent study has used the Malaria Box compounds and identified novel modes of action such as defects in apicoplast segregation, inhibition of host cell egress/invasion with future work to be focused on target identification (Subramanian et al. 2018).

4.5 Gaps and Future Measures

Drug discovery for infectious diseases such as malaria requires an intense and sustained effort for generating novel active chemical scaffolds as leads for new drugs to counter emerging drug resistance. It is therefore imperative that a consolidated effort involving medicinal chemists with computational and parasite biologists is made to explore new validated protein targets for designing molecules and generating compound libraries around specific active sites or protein-protein interfaces. Screening models for evaluation in murine malaria exist in several laboratories in the country and the primate malaria model (*P. cynomolgi*—a model for *P. vivax* like relapse malaria, and *P. knowlesi*) is maintained at CDRI, Lucknow and used to evaluate new leads. The absence of a continuous in vitro *P. vivax* culture system presents constraints; also, the diversity of *P. vivax* strains in schizont/hypnozoite ratios and relapse times presents a challenge to standardization. Newer models such as humanized-(athymic) nude mouse that support the replication of asexual blood stages of *P. falciparum* in human erythrocytes would aid in evaluation of efficacy against falciparum malaria and pharmacokinetics/pharmacodynamics of leads (McCarthy et al. 2016a). It will also be of use in setting up a model for assaying drug-induced hemolysis in G6PD-deficient human blood which is a major issue in the widespread use of 8-aminoquinolines such as primaquine and tafenoquine.

In recent years, evaluation of clinical efficacy of drug candidates after completion of preclinical studies has been conducted in malaria-naïve volunteers infected with blood stage parasites or sporozoites. This combined Phase I/early Phase II study helps to rapidly obtain human pharmacokinetic and pharmacodynamics data and also identifies the correct dosing regimen for causal prophylaxis and/or cure (McCarthy et al. 2016b, 2011; Nyunt et al. 2009; Sulyok et al. 2017). Another advantage of the controlled human malaria infection (CHMI) approach, with infections initiated by mosquito bite or blood stage parasites, is that if volunteers are screened for antimalarial immunity in advance, higher parasite clearance rates due to preexisting immunity will not lead to an overestimation of drug efficacy (Stanisic et al. 2018). CHMI induced by *P. falciparum* blood stages for assessment of parasite clearance by schizonticidal drugs also allows precise quantitation of the number of parasites initiating infection in each study volunteer. However, adopting this clinical trial mode in resource-poor nations poses ethical challenges. A study on volunteers in a controlled infection trial in Kenya (Njue et al. 2018) revealed that financial compensation was among the strongest motivations for participation in the trial raising the possibility of exploitation of communities. If such CHMI trials are to happen in India, new ethical guidelines defining the threshold of risks must combine with a strong rationale for conducting the trial.

Since interest in the search for new antimalarials, or for that matter drugs for most infectious diseases including tuberculosis, is primarily restricted to public-funded institutions in India, government-aided discovery and development efforts must be strengthened. Existing pre-clinical regulatory test facilities need upgradation and should be made more accessible so that costs of developing anti-infectives are minimized. Identifying an industry partner for taking a "drug product" through clinical trials remains challenging. Since the fruits of this enterprise are not necessarily high on profit, privately owned pharmaceutical industry keeps away. Serious consideration must also be given to public sector manufacturing for drugs for infectious diseases. There are lessons to be learnt from the remarkable success of E-Mal for which scientists took only process patents for isolation and synthesis. Initially licensed to one company, it is now produced by more than 180 companies. The accompanying dramatic fall in prices of E-Mal (Misra 2017) ensures that patients who need it are also able to afford it. It is for this reason that antimalarials and drugs for other infectious diseases such as tuberculosis and leishmaniasis have to be considered "public goods" and not "commodities," and renewed effort must be made to bring together research groups with diverse expertise to work toward identification and development of new drugs and formulations.

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