Artemisinin-based combination with curcumin adds a new dimension to malaria therapy

Govindarajan Padmanaban*, V. Arun Nagaraj and Pundi N. Rangarajan

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

Malaria afflicts 300 million people worldwide, with over a million deaths every year. With no immediate prospect of a vaccine against the disease, drugs are the only choice to treat it. Unfortunately, the parasite has become resistant to most antimalarials, restricting the option to use artemisinins (ARTs) for effective cure. With the use of ARTs as the front-line antimalarials, reports are already available on the possible resistance development to these drugs as well. Therefore, it has become necessary to use ART-based combination therapies to delay emergence of resistance. It is also necessary to discover new pharmacophores to eventually replace ART. Studies in our laboratory have shown that curcumin not only synergizes with ART as an antimalarial to kill the parasite, but is also uniquely able to prime the immune system to protect against parasite recrudescence in the animal model. The results indicate a potential for the use of ART–curcumin combination against recrudescence/relapse in *falciparum* and *vivax* malaria. In addition, studies have also suggested the use of curcumin as an adjunct therapy against cerebral malaria. In this review we have attempted to highlight these aspects as well as the studies directed to discover new pharmacophores as potential replacements for ART.

Keywords: Artemisinin, combination therapy, curcumin, malaria.

CHLOROQUINE (CQ) is a wonder drug as an antimalarial in terms of its efficacy, minimal side effects and affordability. However, the development of widespread resistance to this molecule has been one of the main reasons for resurgence of *falciparum* malaria, especially in the African continent. Replacement drugs such as sulphadoxine-pyrimethamine (SP) could not sustain for long due to the development of rapid resistance in the parasite. Other drugs such as amodiaquine, mefloquine, atovaquone, etc. could not fit the bill due to a variety of reasons, including development of cross-resistance, side effects and cost considerations. Quinine, despite its toxicity and reported resistance development, continues to be a final option. In this gloomy picture with around 300 million malaria cases and a million deaths per year, the advent of the use of artemisinin (ART) derivatives has given hope of malaria containment. However, the requirement of a 7-day ART monotherapy has led to incomplete patient compliance. This together with the availability of spurious versions in the market has already raised concerns of possible resistance development to ART as well. This has led the World Health Organization (WHO) to insist on ART-based 3-day combination therapy to prolong the efficacy of this treatment and delay resistance development. Effective implementation of this strategy, together with measures to contain and protect against mosquito bites has already led to signs of a decreasing trend in malaria incidence in the African continent. However, malaria needs a constant vigil and there is a need to develop newer ART-based combinations and perhaps, an alternative to ART as well. Studies in our laboratory and elsewhere with animal models have indicated that a combination therapy with ART + curcumin can add a new dimension to malaria therapy in terms of its potential to prevent parasite recrudescence and relapse in *falciparum* and *vivax* malaria as well as to protect against cerebral malaria. In this review an attempt is made to provide an overview of the recent developments in this direction.

*For correspondence. (e-mail: geepee@biochem.iisc.ernet.in)

ARTs and their mechanism of action

ART, also known as qinghaosu, is extracted from the plant *Artemisia annua* or sweetworm wood, and Chinese scientists have made valuable contributions in the discovery and use of this molecule for the treatment of malaria[1]. Various derivatives of artemisinin prepared by Chinese and other scientists with improved pharmaceutical properties include artemether, arteether, dihydroartemisinin, artesunate, artemisone and artelinate (Figure 1). These ART derivatives are essentially sesquiterpene lactone endoperoxides and are potent antimalarials, killing CQ- and SP-resistant parasites. ARTs are particularly more active than any other antimalarial, reducing the number of parasites by approximately 10^4 per cycle[2].

Multiple mechanisms of action of ART have been proposed[3]. One line of thinking centres on ART interaction with heme-iron, generated in the food vacuole by hemoglobin digestion. The concepts range from scission of the endoperoxide bridge by reduced heme-iron[4], free-radical generation by alkylated heme adducts[5], alkylating and

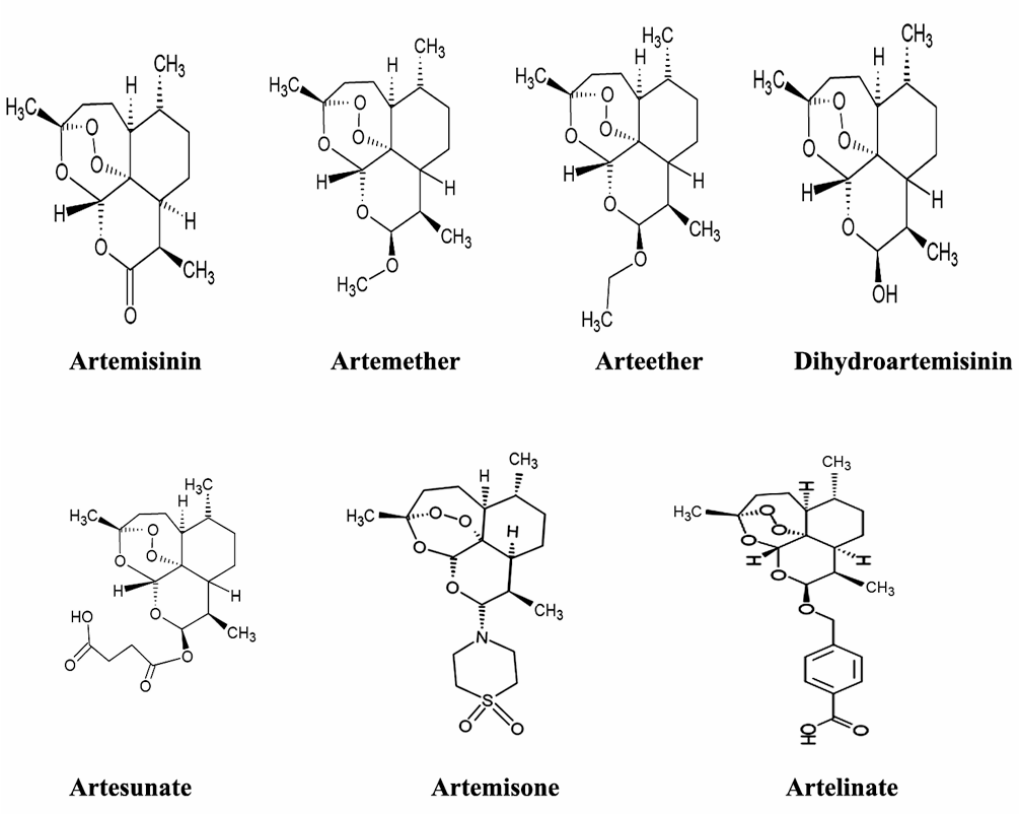

Figure 1. Structure of artemisinin and its derivatives.

oxidizing proteins, and interacting with iron–sulphur centres in enzymes and lipids[6,7]. These studies would suggest that ART can alkylate a variety of proteins[8]. However, there is also a view that ART has a specific target, namely PfATP6, a SERCA-type calcium-ATPase[9]. Single nucleotide polymorphisms (SNPs) in the gene associated with decreased susceptibility to ART have been reported from French Guiana and Senegal, suggesting causal association[10]. However, further analysis with more clinical samples has revealed a high degree of genetic diversity in this gene, making specific phenotype–genotype association rather tenuous[11,12].

There have also been other proposals to explain the mechanism of action of ART. Thus, it has been suggested that ART may be interfering with flavoenzyme function[13]. Yet another study has shown that the parasite mitochondrion is an important direct target, if not the sole one[14,15]. It has been suggested that ART and related endoperoxide-containing derivatives may interact with the parasite electron transport chain to generate local free radicals to inhibit mitochondrial function, leading to death of the parasite. Interestingly, while yeast and parasite mitochondria are susceptible to this action of ART, mammalian mitochondria are resistant. More evidence is becoming available to suggest the link between hemoglobin metabolism and ART action. It has been shown that endoperoxide antimalarials have an early effect on digestive vacuole morphology, without having any effect on that of the endoplasmic reticulum[16]. Recent studies have shown that ART inhibits hemoglobin uptake and this process coupled with hemoglobin hydrolysis is required for potent antimalarial activity[17]. It is likely that ART has more than one site of action at the molecular level.

ART-based combination therapies

Despite the potent antimalarial action of ART, it suffers from poor pharmacokinetic properties and short half-lives. This has necessitated the use of ART monotherapy for seven days, resulting in poor compliance. This together with the availability of sub-standard drugs in the market has necessitated the development of ART-based combination therapies (ACTs) to be used in a 3-day therapy[18], comprising ART together with a slowly eliminated companion blood-stage schizonticide. This treatment reduces the parasite burden by 10^8, leaving only a small fraction of the parasite burden to be abolished by the second drug. The ARTs also exert activity against gametocytes, reducing the probability of transmission. A meta-analysis of individual patient's data from 16 randomized trials consisting of 5948 patients that studied the effects of the addition of artesunate to the standard treatment regimen of *Plasmodium falciparum* malaria

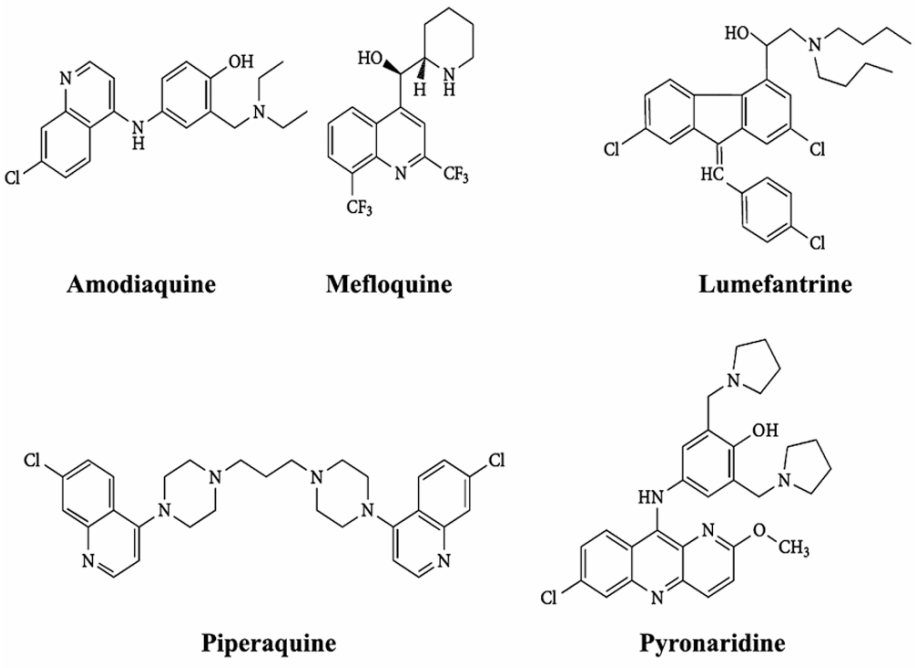

Figure 2. Structure of partner antimalarials.

indicated substantially reduced treatment failure, recrudescence and gametocyte carriage[19].

Several combinations of ART derivatives with a second drug such as chloroquine, amodiaquine, mefloquine, lumefantrine, SP, piperaquine, dapsone–proguanil, atovaquone–proguanil, etc. have been tried. However, WHO currently recommends the following four ART-containing combinations: (1) artesunate + amodiaquine, (2) artesunate + SP, (3) artesunate + mefloquine, and (4) artemether + lumefantrine. In addition, evidence is available to add another combination to the list, namely dihydroartemisinin + piperaquine[20] (Figure 2). Combination of artesunate + amodiaquine demonstrates a better overall efficacy than amodiaquine alone[21]. Amodiaquine is closely related to CQ and perhaps, acts through a similar mechanism of inhibiting the heme-detoxication process. However, the combination shows a high degree of pharmacokinetic mismatch leading to prolonged exposure of parasites to low doses of amodiaquine and its active metabolites, which may facilitate development of resistant parasites. Moreover, amodiaquine is reported to induce neutropenia. Amodiaquine is not currently recommended in India as an antimalarial because of its toxicity. Artesunate + SP combination produced promising results in the Gambia[22], though in subsequent WHO-led trials in African children, the combination showed disappointing results[19]. Artesunate + mefloquine combination has been used in Thailand for almost a decade with excellent results[23,24]. The drug has also been tried in South America with success[25]. Despite development of resistance to mefloquine monotherapy, mediated by *Pfmdr1* gene amplification, the combination has remained effective. The disadvantages of this combination are its cost, adverse drug reaction and pharmacokinetic mismatch of each drug. Artemether + lumefantrine (Coartem) is the only fixed-dose ART-containing formulation registered after internationally recognized guidelines and prioritized by WHO for therapy against CQ- and SP-resistant parasites. The combination appears to be safe and well tolerated[26], though one study showed irreversible hearing impairment[27]. It also suffers from the same disadvantage of pharmacokinetic mismatch and a complex 3-day regimen consisting of six doses at 0, 8, 24, 36, 48 and 60 h that may be difficult to comply. It is also considered inferior to the artesunate + mefloquine combination[28,29]. There is also considerable debate on the possibility of resistance development to lumefantrine in Africa. Yet another combination that holds promise is dihydroartemisinin + piperaquine (Artekin)[30,31]. Piperaquine is a bisquinoline, structurally related to CQ. Pyronaridine, an acridine-type Mannich base, has been extensively studied in China and has been shown to have striking activity against *P. falciparum*. Artesunate–pyronaridine combination has shown excellent results in Phase III clinical trial[32]. An interesting feature of the partner drugs is that they all seem to act primarily through interaction with heme/hemozoin in the food vacuole, generated from hemoglobin digestion. Resistance is known to most of the combining partners, although ACT is functional and effective. WHO has provided guidelines for the treatment of malaria, indicating the dose regimen and evaluation of the efficacy of each combination under a given set of conditions[20].

ART + curcumin combination for malaria therapy

As already indicated, a general principle in ACT is to have a partner drug with a longer half-life than ART, so that the residual parasites not cleared by ART are eliminated by the partner drug. However, resistance of the parasite to the partner drug and concentrations of the partner drug below threshhold levels are issues to be dealt with in the long-term use of these ACTs. In this context, studies in our laboratory have shown that a new combination therapy with ART + curcumin is unique, with potential advantages over the known ACTs. Both the drugs have short half-lives and no resistance is known to curcumin, a dietary component from turmeric, a widely used Indian spice. Interestingly, curcumin, in addition to having a direct killing effect as an antimalarial, is also able to prime the immune system against *Plasmodium berghei* in a murine model, rendering the combination to act like a therapeutic vaccine. Thus, this combination has unique potential to prevent parasite recrudescence and relapse. It is well known that immunity in *falciparum* malaria is short-lived and *vivax* malaria poses problems of relapse even after several months. Studies in the murine model have indicated a potential for the use of ART + curcumin combination to prevent recrudescence and relapse, in addition to the known efficacy of ACTs in parasite clearance. These results are described in detail elsewhere[33] and a brief summary is provided here.

Studies in our laboratory have established the antimalarial activity of curcumin. Our studies had earlier shown that curcumin has modest antimalarial activity[34]. However, recent studies have claimed that oral delivery of curcumin bound to chitosan nanoparticles is able to completely cure *Plasmodium yoelli*-infected mice[35]. Interestingly, we found that curcumin in combination with α,β-arteether (AC treatment) was effective in preventing recrudescence, seen with ART monotherapy[36]. This study was carried out in mice infected with *P. berghei* (NK65, non-cerebral strain) for 24 h before drug treatment. The mechanism of action of AC treatment is of interest, for which studies were carried out over the last 5 years in mice infected with *P. berghei* (ANKA, cerebral strain) for 72 h before drug treatment. Thus, mice were infected for 72 h with *P. berghei* and then received a single injection of arteether alone (AE treatment, 750 μg), or along with three oral doses (5 mg/dose) of curcumin (AC). Cumulative data with around 400 animals are available. Infected, untreated animals died between 5 and 6 days. In AE treatment, recrudescence started around 20 days and the animals died around 23/24 days. The mortality was around 95%. In AC treatment, over 95% of the animals survived. Estimation of curcumin content in blood and tissues indicated a short half-life. Therefore, ART + curcumin represents a combination of two drugs with half-lives of a few hours. Thus it was of interest to unravel the short-term and long-term effects of curcumin in this regard. Real-time PCR analysis for parasite 18SrRNA was carried out to get an accurate measure of parasitemia using a correlation between C_t values from real-time PCR and parasitemia as assessed using the Giemsa stain. The studies indicate that the AC treatment is able to clear the parasite in the blood faster than the AE treatment.

Cui *et al.*[37] have shown that curcumin enhances reactive oxygen species (ROS) generation in *P. falciparum* cultures in the concentration range 20–100 μM, and there is inhibition of histone acetylation. We have found that AC treatment is more potent than curcumin or AE in inhibiting hemozoin formation and enhancing ROS generation in *P. berghei*-infected mice[33]. Thus, it appears that the short-term effect of AC is mediated through ROS generation in the presence of heme iron.

However, parasites were not cleared from the liver or spleen in AE or AC treatments as assessed using RT–PCR for parasite 18SrRNA. Thus, these exoerythrocytic parasites could be the reservoirs for subsequent recrudescence in blood. Interestingly, in the AE treatment recrudescence levels reached around 60% parasitemia and the animals died around 23/24 days after infection. In the AC treatment there was a transient increase in parasitemia to 2–3%. These parasites disappeared in about 24 h, and the animals were healthy and survived for a life time. Blood from recrudescing animals after AE treatment could reinfect naïve animals and kill them in 5/6 days. Blood from recrudescing AC-treated animals did not infect naïve animals.

Thus, curcumin has a long-term priming effect on the immune system under these conditions. The striking increase in spleen mass during recrudescence in AE treatment was completely prevented in AC treatment. The striking increase in spleen weight in AE treatment after an initial decrease started around 14 days. Microbial products, including those from the parasite are known to activate the innate immune pathway, leading to INFγ-mediated protection in self-clearing parasite model or a surge of INFγ, leading to death of mice in the *P. berghei* (ANKA) model causing cerebral malaria. In the latter context, IL-10 becomes the anti-inflammatory cytokine offering protection. In view of the report that TLR2 agonists induce IL-10 in antigen-presenting cells (APCs) and IL-10 is involved in antibody generation, efforts were made to quantify these parameters in the spleen and serum. On the 14th day, among the parameters tested, there was a striking increase in mRNA levels for TLR 2, IL-10 and IgG-subclass in spleen from AC-treated animals compared to AE-treated animals. AC treatment resulted in a decrease in INFγ and IL-12 mRNA levels. This picture was reflected in serum levels of cytokines and IgG-subclass antibody levels. Soon after infection there was an increase in INFγ as well as IL-10 levels. But, AC treatment resulted in a striking increase in IL-10

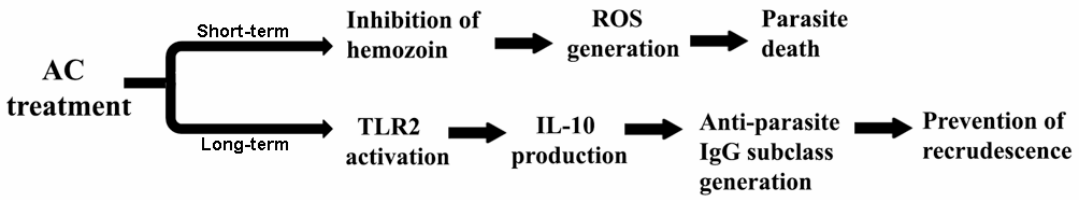

Figure 3. Model to explain the mechanism of action of arteether–curcumin (AC) combination therapy in *Plasmodium berghei*-infected mice.

levels and a decrease in INFγ levels. Interestingly, there was an increase in almost all anti-parasite IgG-subclass antibody levels in the serum. Serum from recrudescing AC-treated animals reacted with several parasite proteins in the Western blot analysis, when compared to the preparation from AE-treated animals. All these changes correlated with the protection of animals during the recrudescence phase. In order to establish the role of TLR2 and IL-10 in the protection offered in the AC treatment, experiments were carried out with knock-out animals. It was found that the AC-treatment was not able to offer protection during the recrudescence phase in TLR2$^{-/-}$ and IL-10$^{-/-}$ animals. IL-10 injection to AE-treated or AC-treated TLR2$^{-/-}$ animals, was able to prolong survival for a few days[33].

Thus, AC-treatment is able to offer protection through priming the immune system. This is done through the activation of TLR2, leading to a surge in IL-10 and anti-parasite antibody production. Our model for the short-term and long-term effects of AC treatment is depicted in Figure 3. These studies have significant implication for preventing recrudescence and relapse in *falciparum* and *vivax* malaria. As an immunomodulator, curcumin in combination therapy, perhaps, has the potential to be tested in the case of other infections and as an adjuvant as well.

Curcumin as an adjunct therapy against cerebral malaria

It has been reported that 7% of the *falciparum* malaria cases progress to cerebral malaria (CM)[38]. CM is characterized by neurological features, and the underlying pathogenesis is due to sequestration and adherence of parasitized erythrocytes to the brain microvasculature that is accompanied by over production of type-I pro-inflammatory cytokines followed by upregulation of endothelial adhesion molecules[39]. Curcumin has profound anti-inflammatory activity and might exert its therapeutic effects in the case of CM by inhibiting NF-κB activation, followed by downregulation of proinflammatory cytokine production and expression of cytoadhesion molecules on endothelial cells[40]. With the use of the CM model in mice, it has been shown that curcumin is able to prevent CM and delay death of animals by about 10 days[41]. It has been suggested that administration of an immunomodulator such as curcumin along with an anti-plasmodial drug would be effective in severe malaria. This is precisely the strategy used in our study to prevent paraite recrudescence and relapse, and the underlying mechanism of anti-inflammatory cytokine (IL-10) activation discussed earlier[33] is likely to play an important role in combating CM as well.

Future challenges

An important issue with curcumin is its poor bioavailability and rapid metabolism, although its efficacy against a variety of diseases ranging from cancer, diabetes, arthritis, cardiovascular and neurological diseases has been documented[42]. The various curcumin metabolites are given in Figure 4. Several attempts are underway to improve bioavailability through the use of preparations such as liposomes, phospholipid complexes, nanoparticles and with piperine adjuvant to inhibit curcumin metabolism[43]. However, the performance of all these curcumin preparations are yet to be assessed in humans, and most of the data available in the literature are with rodents.

More than 100 countries have moved into using ACTs, and this provides a challenge to produce adequate amounts of good quality ART in different parts of the globe. The present formulations available are heavily subsidized and it would be a challenge to sustain availability at affordable costs. Heavy demand would spur inferior preparations hitting the market[44]. Indications for resistance development to ACTs in the field are already available[45,46]. It also appears that in addition to the development of resistance to ART, inefficacy of the partner would also contribute to treatment failure. ARTs do have neurotoxicity and manifestation of the side effects would depend on the route of administration, lipid solubility and other factors. Efforts are also underway to prepare derivatives to reduce lipid solubility, enhance efficacy and decrease neurotoxicity (Table 1)[44]. To this should be added the effort to produce artemisinic acid through synthetic biology approach involving microbial production. This was achieved in the laboratory of Keasling, where it was shown that an artemisinic acid-producing strain of *Saccharomyces cerevisiae* can be constructed by engineering the endogenous farnesyl phosphate pathway and expressing the *Artemisia annua* genes,

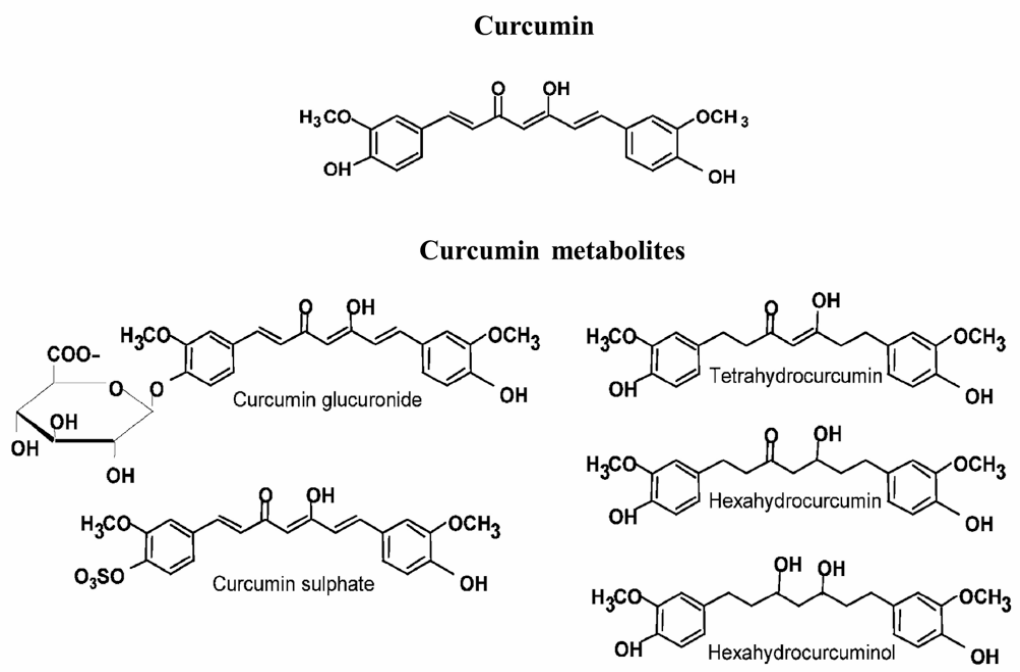

Figure 4. Structure of curcumin and its metabolites.

Table 1. Derivatives of artemisinin

Known artemisinin derivatives	Semi-synthetic artemisinin derivatives
Artemisinin	11-Azaartemisinins
Dihydroartemisinin	Fluorinated artemisinins
Artesunate	Carbon C10 functionalized derivatives
Artemether	Carbon C15 functionalized artemisinins (including artemisitene)
Arteether	Dihydroartemisinin esters, carbonates and ethers
Artemisinic acid from the plant	Carbon C10 alkyl and aryl derivatives (including artelinate)
	Carbon C10 amino derivatives

amorphadiene synthase, CYP71AV1 and its redox partner. By engineering the bacterial, yeast and plant genes, copious amounts of the intermediate amorphadiene have been produced in the microbial system for eventual conversion to artemisinic acid that is finally converted to ART chemically [47].

What are the options when full-fledged resistance develops to endoperoxide compounds? At the moment the situation is bleak with no alternative in the basket that can replace ARTs. However, active research is being pursued to identify newer targets and molecules, as reviewed by Wells *et al.*[48]. One approach continues to be to improve upon established antimalarials such as dihydrofolate reductase inhibitors or inhibitors of the electron transport chain such as atovaquone or 4-aminoquinolines or aminoalcohols. An example of such an effort is the design of a dual function acridone, which combines the heme-targeting character of acridones and a chemosensitizing component that counteracts resistance to quinoline antimalarial drugs[49].

Another approach is to embark on new targets based on knowledge of genome sequence and differential gene expression in different parasite stages of development. This may lead to molecules acting on known targets in the human for which a counterpart may exist in the parasite. Structurally related compounds can be tested against the parasite target. Such rational design approach has led to molecules inhibiting enzymes of nucleoside biosynthesis such as DHOD[50,51] transition states in adenosine deaminase[52] and purine nucleoside phosphorylase[53,54], or potential pathways in the apicoplast[55]. Another approach is to use successful targets in other therapeutic areas and then design molecules that can show specificity to the parasite. Examples of such targets are kinases, histone deacetylases and proteases. Another development has been high-content screening against paraite viability in 384 and 1536 well formats with large natural product and synthetic compound libraries. A success story in this approach is the identification of spiroazepineindole class of compounds as antimalarials with a favourable pharmacologic

profile from a starting library of around 12,000 compounds. Lead optimization studies have led to spiroindolone NITD609, showing pharmacokinetic properties compatible with once-daily oral dosing with single-dose efficacy in the rodent parasite[56]. The compound acts through a distinctly different mechanism of rapidly inhibiting parasite protein synthesis.

Most of the efforts are towards targeting the erythrocytic stage, and more strategies are needed to target the liver stage, gametocytes and the hypnozoite stage in *P. vivax*. One would like to hope that curcumin as an immunomodulator would prove to be an ideal combining partner with different classes of antimalarials.

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ACKNOWLEDGEMENTS. Studies in the authors' laboratory were supported by the Department of Biotechnology, New Delhi. G.P. was supported by the NASI-Chair. V.A.N. is a DST Ramanujan Fellow.