

Artemisinin and Biomolecules: the Continuing Search for Mechanism of Action

Nkereuwem Edikpo¹ Samuel Ghasi² Adikwu Elias¹ and Nelson Oguanobi²

¹Department of Pharmacology, Faculty of Basic Medical Sciences, University of Port Harcourt, Port Harcourt, Choba, Rivers State, Nigeria and ²Department of Pharmacology and Therapeutics, Faculty of Medical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.

Abstract

This review was conceived with the aim of presenting a comprehensive account of the molecular interactions of artemisinin class of antimalarials and delineating it in the context of the different theoretical models of artemisinin mechanism of action. As the most rapidly acting schizontocide, artemisinin with its derivatives are in the forefront of the battle against malaria parasite. Forty one years after its extraction, the exact mechanism of action of artemisinin and its derivatives has not been unequivocally elucidated. Understanding its exact mode of action could help in new drug discovery that would circumvent the resistance mechanism of *P. falciparum*, present the additional and simpler tool of detecting and monitoring the spread of these resistant mutants by providing a surrogate marker of artemisinin resistance and, perhaps aid in the elimination of this menace. Artemisinin actions have been linked to the generation of radicals, which damage parasite vital biomolecules by alkylation, and oxidative stress in the microenvironment of its activation. Interference with the parasite heme-detoxification pathways, abolition of parasite mitochondrial inner membrane electropotential, inhibition of PfATP6 and apoptosis induction have all been documented as mediating artemisinin effects. At present, inhibition of PfATP6 as mediating artemisinin effects appears to attract the most attention. However, the picture is incomplete and sight should not be lost of equally strong and compelling facts implicating other

molecular targets and the biochemical basis of the well demonstrated phenomenon of quiescence of ring forms under artemisinin pressure.

Keywords: Artemisinin; Biomolecular Targets; PfATP6; Radicals; Hemozoin; Quiescence Mechanism

Introduction

Since the extraction of artemisinin in 1972 (1) and the subsequent availability of its semi-synthetic derivatives, artemisinin compounds have been at the forefront of the fight against malaria. In addition to its antimalarial effect, artemisinin exhibits several other biological effects but, the precise molecular target of artemisinin and its derivatives have not been unequivocally identified. As a result of this, more than 40 years after its extraction and identification, the search for the exact mode of action of artemisinin continues to attract huge volumes of research publication. This is not an isolated phenomenon as some therapeutic agents that came into existence through the traditional process of drug discovery and development (as opposed to rational drug design) had their mechanisms of action elucidated after decades of clinical usage. The quinoline antimalarial, chloroquine was synthesized in 1934 by Hans Andersang in Germany (2). It was more than half a century before its action was associated with the food vacuole of *plasmodium* in the early 1970s (3); Withering first described and standardized the use of foxglove leaves for the treatment of certain edematous patients in 1785 but, the molecular substrate of its action was not known until 1953 when inhibition of Na⁺/K⁺ ATPase was demonstrated as underlying its observed effects (4). The literature is replete with several diverse

Received 11/06/13; accepted 12/27/13

Correspondence: Dr. Nkereuwem Edikpo. Department of Pharmacology, Faculty of Basic Medical Sciences, University of Port Harcourt, Port Harcourt, Choba, Rivers State, Nigeria. Tel: +2348037896151 Email: uwemnkere@gmail.com

accounts of artemisinin molecular interactions. The aim of this review is therefore, to present a detailed account of the various theoretical models of artemisinin action from inception and, a summary of the most recent findings and current opinion on this important subject.

Much controversy shrouds the exact mechanism with which artemisinin compounds exert their diverse biological effects. Different models of artemisinin action have been published, each with experimental records as supporting evidence. Research findings on this issue are voluminous, diverse and often superficially contradictory, so much so that, tying the loose ends and producing a coherent and representative picture becomes at times, a daunting task. However, a systematic review of findings and propositions on this topic can conveniently be subsumed in two broad categories: Radical-dependent toxic and inhibitory effects of Artemisinin on plasmodial vital macromolecules and interference with hemoglobin degradation and heme detoxification pathways of *plasmodium*

Artemisinin and Free Radicals

When the molecular structure of artemisinin was deciphered in 1975 (1), it soon became apparent to the Scientific Community that an entirely new chemistry was being unleashed on the malaria parasite. Being structurally different from all previous antimalarials with its polycyclic nature and an embedded internal peroxide moiety, artemisinin assumed the status of a conundrum that persists to the present with regards to the molecular basis of its biological actions. Earlier works and experience with pre-existing peroxide compounds (5-9) may have provided the needed impetus, in a manner of speaking, to propel the radical-dependent theory of artemisinin action to prominence (10). The added ingredient of the identification of the 1,2,4-trioxane pharmacophore with its endoperoxide bridge as a sine qua non of antimalarial action (11, 12) may have further helped in directing research thinking and efforts along this line. In its basic form, this model of artemisinin action depicts the internal peroxide bridge of all clinically useful artemisinin compounds as a 'reactivity center' capable of reacting with any electron(s)-donating group to ultimately effect intramolecular electronic re-configuration. The net result is the decomposition of the peroxide bond and generation of oxygen- and carbon-centered radicals (activation), which kill the parasite via oxidation and

alkylation respectively, of parasite vital molecules. Krungkrai and Yuthavong (1987) (13) and Meshnick and colleagues (1989) (14); 1993 (15); 1994 (16) were some of the earliest groups of researchers to lay the groundwork for this proposition.

Activation of Artemisinin

Further refinements by subsequent researchers have added to the mechanistic frame of this theory. While most works emphasize the imperative of activating artemisinin for the generation of radical species, on which artemisinin actions vastly rest, controversy exists as to the precise nature and source of these 'activators.' Earlier groups working with peroxidic compounds (6, 7) amply demonstrated the ability of iron (Fe) to react with various peroxides and hydroperoxides to generate reactive oxygen species. While it has also been previously demonstrated that artemisinin reacts covalently with hemoglobin-bound iron (17-19), Creek et al (2009) (20) emphasized the improbability of this based on the demonstrated steric hindrance a bulky polycyclic artemisinin molecule encounters in approaching the heme pocket of hemoglobin. They argue that, probably such reaction may have occurred with formed heme following inadvertent hemoglobin disintegration during the experiment. Among the various pools of iron in the erythrocyte-hemoglobin, heme, hemozoin, cytochrome, etc., hemoglobin is the most abundant source of iron, and the redox status of embedded iron and the hemoglobin conformation fluctuate depending on the solvent, buffers and temperature used in *in vitro* studies (20). Zhang and Gerhard (2008) (21) demonstrated that, while artemisinin may react with these various pools of iron (free iron, heme, and deoxygenated and oxygenated hemoglobin, cytochrome), its reaction with heme-Fe²⁺ was the most efficient. Ferrous heme is probably the foremost activator of artemisinin (21-23) and the primary subcellular site of artemisinin activation is said to be the parasite food vacuole (24) where ferrous heme is in abundance as a result of hemoglobin uptake and degradation. However, it must be noted that artemisinin being an endoperoxide compound and having the general reactivity typical of its peroxidic class (10), the involvement of other metals or enzymes cannot be completely ruled out in its activation (25).

The activation of artemisinin is said to occur through reductive scission or open peroxide model (26). In reductive scission, Fe²⁺ interacts with

artemisinin donating electron that results in ring opening and formation of oxygen-centered radicals. These rearrange through primary and secondary carbon-centered intermediates to stable ring-contracted tetrahydrofuran acetate or 4-hydroxy deoxyartemisinin which have no antimalarial activities (10). The open peroxide model which appeals to Haynes et al (2007) (25) is akin to Fenton reaction involving Fe^{2+} and the internal endoperoxide moiety yielding secondary carbon-centered radicals and hydroxyl radicals without ring opening. These short-lived radicals are believed to damage parasite vital biomolecules leading to its death. The existential span of these radicals being in the milliseconds to nanoseconds range (27), their detection through routine method is not feasible. However, methods like cyclic voltametry and electron paramagnetic resonance spectroscopy after spin-trapping (with DMPO- 5,5-dimethyl-1-pyrroline-N-oxide) to provide for longer-lasting adducts have been deployed in the direct detection of these species (15). Quantitative ^{31}P NMR spin rapping techniques represents an improvement on their direct detection. Alternatively, EPR spectroscopy of spin-trapped inert, rearranged product of artemisinin activation (28), oxygen consumption and florescence technique (29), thiol reduction and measurement of oxidized glutathione (28) have all been used to demonstrate indirectly, the relevance of radical species in artemisinin biological actions. Further boost to this theory of artemisinin action lies in the fact that free radical scavengers abolish artemisinin activity (30), pro-oxidant agents (e.g., miconazole and doxorubicin) and anti-oxidants (e.g., catalase, dithiothreitol and alpha-tocopherol) enhance and diminish respectively, the antimalarial therapeutic efficacy of artemisinin (13). The dependence of artemisinin action on enhanced oxidative stress in the microdomains of its activation was further strengthened when Klonis et al (2011) (31) documented that hemoglobin uptake and digestion was necessary for its action. This dependence may explain the fact that blood stages of the parasite dependent on hemoglobin digestion (late ring forms and trophozoites) are more sensitive to artemisinin (32, 33) as opposed to early ring forms with no hemoglobin-digesting abilities. It is quiescence mechanism associated with these later forms that has been implicated in slow-clearing phenotypes in Thai-Cambodian border (34, 35).

Interaction of artemisinin compounds with parasite vital biomolecules has been postulated to underlie its biological actions. The reaction of activated

artemisinin with its plasmodial substrate is said to be covalent in nature (36-38). It is held that this interaction process either damages or inhibits parasite biomolecules vital to its survival. Several of these biomolecules have been isolated and characterized though, the functional significance of a few remain to be ascertained. First, artemisinin has also been documented as forming covalent adducts with isolated erythrocyte membrane (but not intact erythrocyte membrane) proteins- α - and β -spectrin, band 3, 4.1, 4.2 actins and, glyceraldehydes-3-phosphate dehydrogenase (39). Since artemisinin is known to reduce parasite-enhanced erythrocyte membrane fluidity (40) these covalent interactions with erythrocyte structural membrane proteins may, in addition to oxidative stress (41) underlie this observed effect of artemisinin.

Inhibition has also been reported with various plasmodial enzymes. Parasite hemoglobins (falcipain, plasmepsin, falcilysin) with which the parasite digests hemoglobin for its amino acid requirement have also been shown to be inhibited by artemisinin (42) even though the researchers' preoccupation was with the heme detoxification theory of artemisinin action. Histidine rich protein II (HRP II) and recently identified heme detoxification protein (HDP) have also been identified as subject of artemisinin inhibition (18, 42, 43). Heme, a primary and toxic product of hemoglobin degradation by malaria parasite has been demonstrated severally to form covalent adducts with artemisinin through which the heme detoxification into hemozoin is inhibited (20, 44). Hemozoin, the insoluble and relatively inert product of heme detoxification by malaria parasite is not spared the effect of artemisinin. While Hong and colleagues (1994) (37) reported just the covalent binding of dihydroartemisinin to hemozoin (*in vitro* and *ex vivo*) and their co-migration on thin layer chromatography, Pandey et al (1999) (42) noted that this interaction was accompanied by disintegration of hemozoin into monomeric ferric heme. A correlation was later established between heme-alkylating ability and antimalarial effect of endoperoxide antimalarials (23). This was strong for the Spiro- and Dispiro-1,2,4-trioxolane antimalarials but, not so strong for artemisinin, reflecting perhaps, multiple modes of action of the artemisinin class of antimalarials. Irrespective of the preceding authors' affiliations, their experimental findings do lend credence across the divide, in support of artemisinin interactional ability having applicability beyond the heme detoxification theory of artemisinin action. A

curious finding related to protein binding was the labeling of six parasite proteins by radio-labeled arteether, arteflene and dihydroartemisinin in a rather specific and covalent manner (45). SDS-PAGE electrophoresis demonstrated only 4 bands clearly (25 kDa, 50 kDa, 65 kDa, >200 kDa). The pharmacological relevance of this labeling experiment lies in the fact that the proteins were not the most abundant parasite proteins, clinically relevant drug concentrations were used and, expression of these proteins are not stage- or strain-specific. Moreover non-parasitized erythrocyte was not labeled and all three drugs exhibited the same binding qualities whereas, deoxyarteether (which contains no peroxide bridge) did not label parasite. The 25 kDa protein was later identified as *P. falciparum* translationally controlled tumor protein homologue (38) though its function remains undefined.

The SERCA Connection

Calcium homeostasis is an indispensable function in the plasmodial species. The concentrations of Ca^{2+} must be maintained within limits compatible with normal physiology. As extremes of this cation are incompatible with normal parasite physiology, the intraerythrocytic stages of malaria parasite have evolved means of maintaining intracellular concentrations of Ca^{2+} within acceptable limits which is said to be between 40nM and 100nM throughout the growth cycle (46). Two cytoplasmic store of Ca^{2+} have been identified- endoplasmic reticulum-like and acidocalcisome stores (47) with the former serving immediate needs and constantly fluctuating with external Ca^{2+} concentrations, while the later is the stable reservoir. While several Ca^{2+} transporters have been identified in *P. falciparum* (48), PfATP6, the *Plasmodium falciparum* SERCA (sarco-endoplasmic reticulum calcium ATPase) orthologue has gained pre-eminence because of the reviews it has received in connection with artemisinin molecular targets (4). SERCA, first identified in a mammalian skeletal muscle in 1961/62 was the first ATP-dependent Ca^{2+} transport system to be so characterized. It pumps 2Ca^{2+} from the cytosol into the endoplasmic reticulum (in response to raised cytosolic calcium concentrations) in exchange for $<4\text{H}^+$, leaving the inner space of the reticulum slightly alkaline (49). The *P. falciparum* SERCA orthologue (PfATP6) which shares 41%

identity and 65% similarity with its human counterpart is a 1228 amino acids protein (50). The essential structure of PfATP6 consists of 10 α -helical transmembrane spanning segment and three cytosolic domains- the effector (A), the phosphorylation (P) domains (both connected to the M domain) and the nucleotide-binding (N) domain which is connected to the P domain (49). The thapsigargin (and presumably, artemisinin) binding site is on the cytosolic side near the M3 end of a hydrophobic cavity formed by the M3, M5, and M7 transmembrane helices (51).

Although there are various Ca^{2+} -ATPases in *plasmodium*- PfATP1-3, 6, 7, and more recently, 4 (52) that are involved in Ca^{2+} homeostasis, PfATP6 is the only SERCA-type ATPase in *P. falciparum* (4). Previous works on malaria parasite by Marchesini et al (2000) (53) and on *T. gondii* by Moreno and Zhong (1996) (54) had demonstrated the overriding importance of SERCA in Apicomplexan calcium homeostasis. When Eckstein-Ludwig and colleagues (2003) (4) presented florescence imaging to demonstrate that artemisinin specifically binds to the topological domain of this calcium pump (endoplasmic reticulum-like organelle) at the thapsigargin binding site in *P. falciparum* and subsequently inhibiting it, the earlier documented importance of PfATP6 to plasmodial calcium homeostasis became reinforced. Using *Xenopus* oocytes as an expression system for PfATP6, the Eckstein-Ludwig team was able to demonstrate the inhibition of expressed PfATP6 (although at supratherapeutic concentration-1 μM), antagonism of artemisinin action by thapsigargin (a specific inhibitor of SERCA) and dependence of artemisinin action on activation by iron. Also demonstrated were a near-perfect correlation between pump inhibition and in vitro antimalarial actions of artemisinin compounds and, quite surprisingly, the lack of labeling of parasite food vacuole by artemisinin-BODIPY. The last point brings into question plasmodial food vacuole being the primary site of artemisinin action as held by Crespo et al (2008) (24). Though later groups (55, 56) failed to demonstrate sensitivity of PfATP6 to artemisinin, intense efforts both in terms of using PfATP6 as a molecular marker for artemisinin resistance and validation as authentic molecular target is ongoing. The correlation between certain PfATP6 polymorphisms and 'artemisinin resistance' is mixed, with some studies reporting association between certain PfATP6 phenotypes and artemisinin resistance (57, 58, 59, 60) while others could not demonstrate such

(61-64). These contradictory reports is hard to explain except in the highly polymorphic nature of this pump (64) some of which preceded the advent of 'artemisinin pressure' (65, 66) and the unresolved and contentious issue of what really constitutes 'artemisinin resistance' (67, 68). These apart, the question arises and still remains of how structurally diverse molecules like pyrimidinylpiperazine derivatives of artemisinin, some trioxolane derivatives and steroidal tetroxane mediate their antimalarial effect by binding into the tharpsigargin-binding cleft to inhibit parasite PfATP6 (25). A partially satisfying explanation comes from a molecular docking simulation of artemisinin (69) which suggests that activated artemisinin (more precisely, Fe^{2+} -artemisinin adduct) inhibits the Ca^{2+} pump through allosteric mechanisms.

The Mitochondrial Connection

The plasmodial mitochondrion, just as its apicoplast (70) evolved from an endosymbiotic event (71) and is structurally and functionally different from its mammalian counterpart. The *P. falciparum* mitochondrion varies in number and structure according to the developmental stage of the parasite, maturing from a single, small, round and double membrane-bound structure of the early ring forms to the branched and tubular-cristate form of the mature trophozoites with a low-density matrix (72). Though in higher eukaryotic organisms, the mitochondrion functions as the 'power house' of the cell, a hub of redox activities (73), a Ca^{2+} -store house (50) and a reservoir for biomolecular ingredients for programmed cell death (74), its function in the parasite is slightly different and has not been completely elucidated. Although the plasmodial mitochondrion possesses the cellular machinery for ATP synthesis via oxidative phosphorylation (75, 76) its contribution to the entire cellular ATP is considered insignificant as the parasite relies predominantly on anaerobic glycolysis (substrate level phosphorylation) for its energy needs (77). Also, an active and functional electron transport chain (a target of atovaquone) that maintains the electrochemical potential across the inner mitochondrial membrane ($\Delta\psi_m$) exists in the plasmodial mitochondrion. Unlike its mammalian counterpart (that functions predominantly in oxidative phosphorylation) it is believed to be an electron disposal system for dihydroorotate

dehydrogenase in pyrimidine biosynthesis. It also serves to generate the needed electrical potential across the inner mitochondrial membrane that is necessary for transport of metabolites across it (78).

As a result of the functional peculiarity of the parasite mitochondrion and perhaps, following on the heels of atovaquone's mitochondrial connection, researchers have long considered the function of this enigmatic organelle a possible target of rational drug designing (79, 80). Not surprising therefore, interference with mitochondrial function has been identified as one of the actions of artemisinin compounds. One of the first studies to identify the plasmodial mitochondrion as a target of artemisinin actions was by Li et al (2005) (81). The targeted deletion of NDE1 or NDI1 (genes encoding mitochondrial NADH dehydrogenases) in *Saccharomyces cerevisiae* conferred resistance to artemisinin inhibitory effect on this yeast while overexpression conferred enhanced sensitivity to artemisinin action. The expression of *P. falciparum* ortholog of these genes in the knock-out yeast is reported to have partially restored sensitivity to artemisinin effect. Mitochondrion was implicated because there was no inhibition by artemisinin in a medium with a fermentable source of carbon (anaerobic glycolysis which is entirely cytosolic) while a switch to a non-fermentable source of carbon (mitochondrial oxidation) revealed inhibition of yeast by artemisinin. Furthermore, components of the electron transport chain (Cu^{2+} , Fe-S, NADH dehydrogenase) were speculated to have been involved in artemisinin activation that resulted in the observed reactive oxygen species which ultimately led to the inner mitochondrial depolarization. This last action is believed to have abolished the electrochemical potential needed for pyrimidine biosynthesis, the absence of which resulted in parasite death. The essentials of this experiment was repeated 5 years later on *P. falciparum* (29) and confirmed the effect of artemisinin on parasite mitochondrial inner membrane potential via the locally generated reactive oxygen species, the specific and confined milieu in which artemisinin activation occurred according specificity on artemisinin effects. That the mitochondrion is not the sole artemisinin activation site with respect to its mitochondrial action is clear from a study that demonstrated the production of reactive oxygen species (although in reduced amount) in a cell line in which the electron transport chain was not functional (82).

Other Connections

The biological actions of artemisinin extend beyond its antimalarial effects. It has been documented by several researchers in various fields of studies as possessing antiangiogenic/antivasculogenetic, antierythropoietic, cytostatic and proapoptotic effects. These effects, in various combinations are said to underlie its neurotoxic, embryotoxic and its cytotoxic actions and, with few exceptions are said to be mediated by free radicals generated in the confined environment in which artemisinin activation occurs. This is discussed below.

Its effect on apoptosis has been well documented. Mercer and colleagues (2007) (83) reported the iron-dependent cytotoxic effect of dihydroartemisinin on certain leukemic cell lines. LC-MS analysis helped in detecting the stable rearrangement product of artemisinin activation which served as indirect evidence of free radical generation. Death of these cells was shown to be as a result of endoperoxide-derived radical activation of the mitochondria that ultimately resulted in caspase-dependent apoptosis. More recently, a similar result has been obtained on breast cancer cells (30) with the added fact that lysosomal generation of reactive species contributed to enhanced outer mitochondrial membrane permeabilization that triggered the observed apoptosis.

The role of endoperoxide-derived radicals in apoptosis induced by artemisinin compound was further highlighted by Handrick et al (2010) (84) who demonstrated that 'pretreatment with the lipophilic radical scavenger, vitamin E or the hydrophilic radical scavengers glutathione and N-acetylcysteine, reduced DHA-induced membrane oxidation and apoptosis, respectively.' Similar results have been documented by others (85, 86).

Selective toxicity of artemisinin compounds towards stem cells committed to erythroid lineage has been documented (87). Dihydroartemisinin selectively depleted CD34⁺ erythroid cells without affecting more mature erythroid cells, in a time- and dose-dependent manner. Subsequent experiment established the endoperoxide moiety as responsible for this effect while the attendant antiproliferative effect noticed on all stages of erythroid differentiation was determined by the nature of the substituent at C-10 (88). These effects were attributed to cell cycle arrest and apoptosis induction which have been confirmed as being dependent on artemisinin-induced oxidative stress (89).

Antivasculogenetic and antiangiogenic actions of

artemisinin compounds are well known (90). They are said to form the partial basis of their anti-tumor and embryotoxic effects. One of the primary observations giving rise to these has been the reduced expression and secretion of VEGF (vascular endothelial cell growth factor) and downregulation of its receptors (91). Similar effects have been observed with lymphangiogenesis (92) and are believed to be the result of endoperoxide-derived reactive oxygen species (90).

The strength of these experimental findings notwithstanding, the free radical theory of artemisinin action is not without some questioning observations; first, the proposition is incompatible with the unstable and short-lived radicals diffusing away from sites of activation to alkylate and damage parasite biomolecules. Secondly, the actions of free radicals are said to be indiscriminate (93) while artemisinin action is rather specific to the plasmodial target. A partially satisfactory response may reside in the fact that artemisinin is able to cross biological membranes and the compartment in which its activation occurs may afford a more restricted target of the reactive species generated. Furthermore, plasmodial anti-oxidant defense system is said to be relatively weak (94) compared to its more robust mammalian counterpart. Moreover, attention has been drawn to the following observations: certain amino derivatives of dihydroartemisinin react slowly with Fe²⁺ yet possess excellent antimalarial activity, certain trioxolane compounds produce abundant trapped C-centered radicals yet, possess poor antimalarial activities. Moreover, 10-deoxy-10-dihydroartemisinin, being an excellent antimalarial is completely decomposed in its reaction with Fe²⁺ without generating carbon-centered radicals. The relevance of carbon-centered radicals to the actions of artemisinin is at best tenuous (10, 25).

Artemisinin and Plasmodial Heme Detoxification

Heme Detoxification in Plasmodium

One of the earliest associations of artemisinin with the parasite's hemoglobin degradation pathway was made by Pandey et al (1999) (42) perhaps, drawing inspiration from the generally accepted mode of action of predecessor antimalarials, the quinolines-chloroquine, amodiaquine, desmethyl amodiaquine etc- which are reputed to exert their antimalarial effect by interfering with heme detoxification pathways in the intra-erythrocytic parasite (95). And spurred on by the previous documentation of heme-

alkylating ability of artemisinin (37), the desire to elucidate the mechanism of parasite death by this alkylation process inexorably led to discoveries that associated artemisinin with hemoglobin metabolism in the parasite (96).

Plasmodial species are hemoprotozoal parasites adopting a dual life-cycle that alternates between the invertebrate mosquito and a vertebrate host. During the later cycle, the intra-erythrocytic stages of the parasite literally 'swim in a sea of hemoglobin' on which it depend for part of its nutritional requirements. Genomic studies has revealed that, apart from enzymes for glycine-serine, cysteine-alanine, aspartate-17sparagines, proline-ornithine and glutamine-glutamate interconversions, evidence is lacking of biochemical machinery for the de novo biosynthesis of amino acids in *plasmodium* (97). Consequent upon this, it is generally accepted that, the intra-erythrocytic parasite is obligately dependent on host amino acids (derived from hemoglobin with or without supplementation from the serum) for protein biosynthetic functions (98) and probably for energy needs. To further underline the fundamental importance of host hemoglobin to the intra-erythrocytic parasite, Babbitt et al (2012) (99) reported that, in a culture of intra-erythrocytic *P. falciparum* depleted of isoleucine (the only amino acid essential to parasite that is not a component of hemoglobin) remained viable for 72 hours though metabolism, growth and development progressed at a hibernating rate. An earlier study with a similar focus by Liu et al (2006) (100) demonstrated the disastrous consequences on parasite survival by various hemoglobins gene deletion. The importance of hemoglobin to the intra-erythrocytic stages of the parasite can therefore, not be overemphasized.

It is estimated that the parasite ingests a whopping 75% (101) and degrades 25% (45) of host cell hemoglobin. Plasmodial species have evolved to possess excellent, although redundant biochemical pathways for hemoglobin degradation. These consist of various proteases belonging to the aspartic [Plasmeprin I, II, IV and histo-aspartic protease (HAP)], cysteine (Falcipain 1, 2 and 3) and metalloprotease (Falcilysin) families of proteases (100) which degrade hemoglobin into its component parts- globin and heme- in the parasite's food vacuole. Many of these have been characterized (102, 103, 104, 105) and exhibit differences in their maximal rate of hemoglobin degradation depending on the prevailing pH and compartmentalization. It is generally assumed that, short peptides resulting

from the cooperative actions of these hemoglobins are exported to the cytosol where various neutral aminopeptidases act to generate individual amino acids (101). Level of expression of the hemoglobins is highest at the trophozoite stage, followed by the schizonts and then, the ring stage (106).

Unfortunately for the parasite, as catabolism of hemoglobin proceeds, free, soluble heme accumulates in the parasite food vacuole as a toxic by-product. Several papers have reported the inherent toxicity of free heme both, to the parasite (107) and to host components (108). Free heme has been shown to be toxic to host proteins (109) and DNA (110) causing strand scission in the later case. Specifically, heme has been shown to interact in an inhibitory manner with parasite glyceraldehydes-3-phosphate dehydrogenase (PfGAPDH), glutathione reductase (PfGR), thioredoxin reductase (PfTR) and glutaredoxin among others (111). These are enzymes of anti-oxidant defenses for the parasite. Heme toxicity has been attributed to its bioreactivity and pro-oxidant characteristics mediated via generation of toxic free radicals in the Fenton and Haber-Weiss reactions (112). The mammalian system averts this pro-oxidant cascade predominantly through the heme oxygenase system (113) and various anti-oxidant defense systems. *P. falciparum* modulates the ferric heme-induced oxidative stress predominantly through the agency of PfGAPDH, PfGR and glutaredoxin. It has been suggested that the undue sensitivity of PfGAPDH to heme-induced oxidative stress is a cunning adaptation to divert metabolic flux through the pentose monophosphate shunt in order to generate more reducing equivalents in the face of accumulating ferric heme (111).

Though heme detoxification through the heme oxygenase system had been considered in the past to be totally nonexistent in malaria parasite (114, 115), a more recent publication (116) has reported the identification of a nuclear gene in *P. falciparum* that codes for an apicoplast-targeted heme oxygenase (PfHO). The recombinant version of HO is said to catabolize heme into bilirubin IX α . Sartorello et al (2010) (117) demonstrated this and the hitherto unknown heme-scavenging property of *P. falciparum* (maximally exhibited at the trophozoite stage) into the food vacuole for polymerization. However, others (113, 118) have argued that since PfHO lacks the histidine-binding residue of higher eukaryotic HO, it may subserve other functions different from heme detoxification. This view is predicated on the later

group's inability to demonstrate any difference in the canonical product of HO (biliverdin/or bilirubin) between infected and uninfected erythrocytes. Also, they could not detect endogenous biliverdin in *P. falciparum* using a fluorescent-labeled biliverdin-binding protein while detecting exogenous biliverdin. These two preceding points and the fact that that expressed recombinant PfHO exhibited low heme-binding activity and lacked heme-degrading ability bring into contention the primary function of PfHO. Whether a functional heme oxygenase system exists in the malaria parasite is, at most debatable at present. It must however be noted that, the malaria parasite not being extinct is an undeniable evidence of having evolved a survival strategy that circumvents the toxic effect of accumulated by-product of its nutritional chemistry. The most studied of these has been the conversion of heme into hemozoin (or malaria pigment)- a dark brown, irregular, inert and insoluble crystalline structure found in the food vacuole of malaria parasite. In fact, on the basis of finding an identical pigment in mosquito, Ronald Ross was led into making the astounding discovery that mosquito transmits malaria (119). Aside of biomineralization of heme into haemozoin which detoxifies ~95% of heme in the parasite (120) and is considered the default and dominant pathway of heme detoxification in *plasmodium*, peroxidative (121) and glutathione-mediated (122) degradation of heme have been found to be operative in the parasite. Ultra-structural studies in the early 1990s (123) helped decipher its crystalline nature. Haemozoin consists of dimers of ferriprotoporphyrin IX with the ferric (Fe^{3+}) iron of one molecule forming a coordinate bond with the propionate carboxyl group of a second molecule and each of this basic unit being held together through hydrogen bond in an elongating stack (101). Different pathways of hemozoin formation have been documented. These include spontaneous biomineralization (124), histidine rich protein (HRP)-mediated, neutral lipid-mediated haemozoin formation. More recently, a heme detoxification protein (HDP) has been reported that coexists in the food vacuole of *P. falciparum* with various hemoglobins in a ~200kDa complex (44) and is reputed to be a thousand times more efficient than HRP II and lipids in heme polymerase activities (113). HDP had previously been described as being secreted out of the parasite before being endocytosed into the parasite's food vacuole (115)-an odd and energy-sapping process that may suggest additional function(s) of this protein.

The foregoing account of plasmodial amino acid sourcing and utilization, heme generation and detoxification activities characterizes the parasite as having evolved vastly redundant biochemical pathways that seek to frustrate any perturbation (from within it or from the host) of its established, cozy intra-erythrocytic habitat.

Artemisinin Intrusion

Artemisinin and its derivatives have been apportioned multiple points of interaction within the parasite's hemoglobin utilization pathways. The corresponding multiplicity of artemisinin interaction to match the redundancy in parasite's hemoglobin utilization and heme detoxification pathways, might underline artemisinin parasitocidal potency vis-à-vis the more restricted interaction of the quinoline antimalarials with these pathways. The principal action of artemisinin is said to be the inhibition of heme polymerization (42). It is this action, adherents argue, that leaves accumulated heme with all its bioreactivity and oxidative machinery assaulting the parasite into a well deserved premature demise. The major targets of artemisinin action in this regard has been the inhibition of heme detoxification protein (HDP) and histidine rich protein II (HRP II), and the degradation of intravacuolar hemozoin into monomeric ferric heme. The inhibitory action on hemozoin formation extends to spontaneous polymerization and neutral lipid-effected polymerization. Pandey et al (1999) (42) were among the first group to demonstrate the inhibitory effect of artemisinin on heme polymerization. In an *ex vivo* experiment utilizing *P. yoelii* lysate (plasmodial lipids) and HRP II, they were able to demonstrate the inhibition of heme polymerization by artemisinin. They also documented the breakdown of already formed hemozoin by artemisinin (in a concentration-dependent manner) and inhibition of hemoglobin catabolism into heme and globin. The relevance of immediately preceding finding may lie with plasmodial starvation rather than with heme toxicity. Prior to this, Hong et al (1994) (37) had demonstrated the covalent interaction of artemisinin with heme in malaria hemozoin both *in vitro* and *in situ* and, more recently Robert et al (2013) (125) have demonstrated heme-alkylating ability of artemisinin and its correlation with *P. yoelii* nigeriensis strains sensitivity.

The mechanism of inhibition of HRP II was further explained by Kannan and colleagues (2005) (18). In their model of interaction, they posit that, the

earlier proposed primary alkylation of protein-free heme by artemisinin to form 'haemart' which in turn was responsible for interaction with, and subsequent inhibition of HRP II-dependent polymerization (96) was slower at physiological conditions than direct interaction of artemisinin with globin-bound heme. This interaction, they claim, yields 'haemartoglobin' with subsequent conversion to haemart which inhibitory action prevents heme polymerization. It must be noted that, this interaction of artemisinin primarily with hemoglobin is hard to reconcile with the reported stability (non-reactivity) of artemisinin derivatives and 1,2,4-trioxolanes with oxyhemoglobin reported by Creek et al (2009) (20). The controversy notwithstanding, the baseline fact remains that, artemisinin-heme adducts are believed by adherents of this theory of artemisinin mechanism of action to inhibit HRP II.

Artemisinin inhibitory action on heme polymerization also extends to HDP, a more recently characterized and more efficient heme-polymerizing protein than HRP II (44). HDP was demonstrated to exist with hemoglobins in a ~200kDa complex in the food vacuole of intra-erythrocytic parasite. Again, artemisinin was shown to inhibit the heme polymerase and hemoglobin-degrading activity of this complex. Prior to the discovery that HDP exists in a complex with hemoglobins, Jani et al (2008) (115) had also demonstrated the inhibitory effect of artemisinin on this protein.

A recent finding further underlining the importance of heme detoxification pathways to the action(s) of artemisinin has been the upregulation of alternative pathway of heme detoxification (glutathione-dependent heme detoxification) in artemisinin-resistant *P. yoelii* (126). The suggestion here is that in artemisinin-sensitive plasmodial species, heme-detoxification through the hemozoin-formation pathway is of some relevance to artemisinin antimalarial actions. This finding notwithstanding, there are strong objections to the heme-detoxification theory of artemisinin action. It has been held as evidence against the hemozoin theory of artemisinin action that, early ring forms of the parasite and non heme-generating parasites like toxoplasmal and trypanosomal species which are incapable of heme-polymerizing abilities are killed by the artemisinins. These are strong arguments! However, it must be reflected that, in the Greater Mekong region where resistance to the artemisinin was noticed, ring forms constituted one of the least sensitive (to the artemisinins) stages of the parasite (34) and that, increasing sensitivity was noticed from

mid ring to late ring stages just as hemoglobin digestion was just beginning to manifest (33). It has also been noted (127) that in the aqueous environment that characterizes the *in vitro* experiments that seem to support this theory, both artemisinin and dihydroartemisinin are unstable, giving rise to ring-opened products which are capable of reacting with heme molecules axially to inhibit hemozoin formation. Using the HPIA (heme polymerization inhibitory activity) and BHIA (β -hematin inhibitory activity) assays they were able to demonstrate that, 10-deoxodihydroartemisinin, an excellent antimalarial could not inhibit hemozoin formation in both assay. This is because it lacks oxygen at C-10: possessing lesser tendency towards ring-opening because of this, it could not interact with heme via π - π bonding or even axially as is the case of artemisinin and dihydroartemisinin. They postulated that the dose-dependent inhibition in the HPIA assay (and not BHIA assay) by artemisinin and dihydroartemisinin may reflect axial interaction with heme by their ring-open product- an action totally unrelated to antimalarial activity. They also pointed out that, the heme prosthetic group of phase-1 metabolizing enzymes only hydroxylates the periphery of artemisinin leaving the endoperoxide intact- additional point subjugating heme-artemisinin interaction into pharmacodynamic irrelevance. A review by Krishna et al (2006) (11) on this all-important topic while questioning the sensitivity of some of the methods employed by some of the proponents of this theory noted that, heme-artemisinin interaction occurring *in vitro* 'does not mean that it actually occurs in that crucially important biological context where drug action takes place.' To this group of researchers, while it is not doubtful that artemisinin group of antimalarials interact with heme and hemozoin, the interaction is seen as part of the artemisinin-activation scenario and not the molecular target that underlie the biological effects of artemisinin (128). In addition, non-heme Fe^{2+} artemisinin appears to be more favored in artemisinin activation by contemporary researchers (128).

REFLECTION

Since the demonstration by Eckstein-Ludwig et al (2003) (4) that artemisinin specifically inhibits *Plasmodium falciparum* SERCA orthologue (PfATP6) expressed in *Xenopus laevis* oocytes, interest in this calcium pump as a molecular basis of artemisinin action has been on the ascendancy as reflected in extensive literature it has attracted (25,

57, 58, 59, 60, 69, 129). Attempts at specifying and validating PfATP6 as the molecular target of artemisinin diverse biological actions and therefore, the basis of 'artemisinin resistance' have been inconclusive. While abundant evidence has been marshaled to implicate PfATP6 in artemisinin actions at least, at the basic science level, epidemiological and genomic studies have not consistently linked PfATP6 haplotypes with artemisinin biological effects. Krishna et al (2010) (129) have provided elaborate review to reconcile these inconsistencies to a reasonable extent. First, attention has been drawn to the S769N mutation in PfATP6 linked to increase in IC₅₀ of artemether in French Guiana over a three-year period. This association could not be repeated with dihydroartemisinin in some African patients (130) and with artemether, artesunate and dihydroartemisinin in a line of transgenic *P. falciparum* harboring this mutation (63). Krishna et al (2010) (129) believe that, this particular mutation and indeed, others might serve to modulate sensitivity to the artemisinin (instead of directly interfering as is the case with PfCRT) with the genetic background of the parasite playing important role to sensitivity. This scenario is akin to the role played by Pfmdr1 in chloroquine resistance. The Authors also pointed to the role of artemisinin in 'triggering calcium-dependent secretion of cellular invasion proteins and altered intracellular calcium oscillations' (131). These, they posit clearly implicate the artemisinin in plasmodial Ca²⁺ homeostasis probably involving other mechanisms that may not be reflected in the amino acid sequence of its target, PfATP6.

But, reports implicating other molecular targets in artemisinin biological actions are written in prints too bold to be discountenanced: the enhanced oxidative stress generated by artemisinin (28), the enhancement of heme toxicity by artemisinin (132), the 'elevation of the antioxidant defense network, and increased expression of many chaperones in dihydroartemisinin-resistant parasites (133) are worthy of note. The upregulation of alternative pathway of heme detoxification (glutathione-dependent heme detoxification) in artemisinin-resistant *P. yoelii* (126) and the possible involvement of the quiescence mechanism in artemisinin recrudescence and resistance (32, 33, 34) are too significant to be confined to the dustbin of history. The last point (quiescence mechanism) is particularly intriguing. The difficulty in reconciling the delayed clearance phenotypes earlier noticed in some spots in the Greater Mekong subregion with

the lack of a corresponding increase in IC₅₀ of artemisinins (134) was perplexing and contributed to the debate of what really constitutes artemisinin resistance (67, 68). Klonis et al (2013) (33) partially resolved this contradiction by demonstrating a vastly increased IC₅₀ of artemisinin and dihydroartemisinin on young ring forms (6-20 hours post-invasion) of resistant *P. falciparum* isolates in a tightly synchronized *in vitro* system that delivered artemisinin and dihydroartemisinin at clinically relevant concentrations in 4 hourly pulses. This entanglement of resistant phenotypes of *P. falciparum* with events traceable to the ring stages, the observation that several metabolic activities is reduced in early ring stages but reversed at the schizonts stage (35), the inchoate sensitivity of late ring forms just as hemoglobin digestion is just taking root have prompted the consideration that perhaps, the artemisinin class of antimalarials may have different mechanisms of action or activation, each specific to every stage of its intraerythrocytic cycle (135).

CONCLUSION

This review concludes on the note that, though Ca²⁺ homeostasis and PfATP6 appear to be the most 'beautiful bride' among research Scientists (and justifiably so) in their quest for the molecular target of artemisinin actions, sight should not be lost of the other 'brides' for these are yet, early days for artemisinin. With very many confounding facts that call for further consideration and research, it is imprudent to totally disregard any experimental lead. For all we know, its peroxidic reactivity casting a web of *in vitro* interaction across a wide spectrum of plasmodial biomolecules, the artemisinin class of antimalarials may have more than one molecular target at clinically relevant concentrations.

Acknowledgements

We acknowledge the fatherly role of Professor Paul O. Okonkwo formerly of Department of Pharmacology and Therapeutics, University of Nigeria Nsukka, Nigeria. He has been instrumental in the overall process that led to the production of this manuscript. We commend the patience and encouragement of our Wives and Children during the long hours we spent at the table researching this article. We also acknowledge herein, the authors whose works we quoted. We commend them all to God's blessings.

Conflict of interests

The authors declare no conflicts of interest.

REFERENCES

1. Tu Y. The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine. *Nat Med* 2011;17:19-22.
2. Srikanth BA, Ali NMA, Babu SC. Chloroquine Resistant Malaria. *J Adv Sci Res* 2012;3:11-14.
3. Schlesinger PH, Krogstad DJ, Herwaldt BL. Antimalarial Agents: Mechanisms of Action. *Antimicrob Agents Chemother* 1988;32:793-98.
4. Eckstein-Ludwig U, Webb RJ, van Goethem IDA et al. Artemisinins target the SERCA of *Plasmodium falciparum*. *Nature* 2003;424:957-61.
5. Dockrell HM, Playfair JHL. Killing of Blood-Stage Murine Malaria Parasites by Hydrogen Peroxide. *Infect Immun* 1983;39:456-59.
6. Clark IA, Hunt NH, Cowden WB, Maxwell LE, Mackie E J. Radical-mediated damage to parasites and erythrocytes in *Plasmodium vinckei* infected mice after injection of t-butyl hydroperoxide. *Clin Exp Immunol* 1984;56:524-30.
7. Kepler JA, Philip A, Lee YW, Musallam HA, Carroll FI. Endoperoxides as potential antimalarial agents. *J Med Chem* 1987; 30:1505-09.
8. Chamulitrat W, Takahashi N, Mason RP. Peroxyl, Alkoxy, and Carbon-centered Radical Formation from Organic Hydroperoxides by Chloroperoxidase. *J Biol Chem* 1989;264:7889-99.
9. Vennerstrom JL, Acton N, Lin AJ, Klayman DL. Peroxides as oxidant antimalarials. *Drug Des Deliv* 1989;4:45-54.
10. Krishna S, Uhlemann A, Haynes RK. Artemisinins: mechanisms of action and potential for resistance. *Drug Resist Updat* 2004;7:233-44.
11. Krishna S, Woodrow CJ, Staines HM, Haynes RK, Mercereau-Puijalon O. Re-evaluation of how artemisinins work in light of merging evidence of in vitro resistance. *Trends Mol Med* 2006;120:200-05.
12. Meshnick SR, Taylor TE, Kamchonwongpaisan S. Artemisinin and the Antimalarial Endoperoxides: from Herbal Remedy to Targeted Chemotherapy. *Microbiol Rev* 1996;60:301-15.
13. Krungkrai SR, Yuthavong Y. The antimalarial action on *Plasmodium falciparum* of qinghaosu and artesunate in combination with agents which modulate oxidant stress. *Trans R Soc Trop Med Hyg* 1987;81:710-14.
14. Meshnick SR, Tsang TW, Lin FB et al. Activated oxygen mediates the antimalarial activity of qinghaosu. *Prog Clin Biol Res* 1989;313:95-104.
15. Meshnick SR, Yang Y, Lima V, Kuypers F, Kamchonwongpaisan S, Yuthavong Y. Iron-dependent Free Radical Generation from the Antimalarial Agent Artemisinin (Qinghaosu). *Antimicrob Agents Chemother* 1993;37:1108-1114.
16. Meshnick SR. The mode of action of antimalarial endoperoxides. *Trans R Soc Trop Med Hyg* 1994;88:31-32.
17. Selmezi K, Robert A, Claparols C, Meunier B. Alkylation of human hemoglobin Ao by the antimalarial artemisinin. *FEBS Lett* 2004;556:245-48.
18. Kannan R, Kumar K, Sahal D, Kukreti S, Chauhan VS. Reaction of artemisinin with haemoglobin: implications for antimalarial activity. *Biochem. J* 2005;385:409-418.
19. Messori L, Gabbiani C, Casini A, Siragusa M, Vincieri FF, Bilia AR. The reaction of artemisinins with hemoglobin: a unified picture. *Bioorg Med Chem* 2006;14:2972-7.
20. Creek DJ, Ryan E, Chairman WN. et al. Stability of Peroxide Antimalarials in the Presence of Human Hemoglobin. *Antimicrob Agents Chemother* 2009;53:3496-3500.
21. Zhang S, Gerhard GS. Heme activates artemisinin more efficiently than hemin, inorganic iron, or hemoglobin. *Bioorg Med Chem*. 2008;16:7853-61.
22. Sibmooh N, Udomsangpetch R, Kijjoa A, Chantharakasri U, Mankhetkorn S. Redox Reaction of Artemisinin with Ferrous and Ferric Ions in Aqueous Buffer. *Chem Pharm Bull* 2001;49:1541—6.
23. Creek DJ, Charman WN, Chiu FCK et al. Relationship between Antimalarial Activity and Heme Alkylation for Spiro- and Dispiro-1,2,4-Trioxolane Antimalarials. *Antimicrob Agents Chemother* 2008;52:1291-96.
24. Crespo MDP, Avery TD, Hanssen E et al. Artemisinin and a Series of Novel Endoperoxide Antimalarials Exert Early Effects on Digestive Vacuole Morphology. *Antimicrob Agents Chemother* 2008;52:98-109.
25. Haynes RK., Chan WC, Lung C, et al., The Fe²⁺-Mediated Decomposition, PfATP6 Binding, and Antimalarial Activities of Artemisone and Other Artemisinins: The Unlikelihood of C-Centered Radicals as Bioactive Intermediates. *ChemMedChem* 2007;2:1480-97.
26. O'Neill PM, Barton VE, Ward SA. The Molecular Mechanism of Action of Artemisinin—The Debate Continues. *Molecules* 2010;15:1705-21.
27. Kobayashi A, Watanabe H, Ozawa K, Hayashi H, Yamazaki N. Oxygen-derived free radicals related injury in the heart during ischemia and reperfusion. *Jpn Circ J* 1989;53:1122-31.
28. Wu Y, Chen H, Jiang K et al. Interaction of biomolecules with qinghaosu (artemisinin) and its derivatives in the presence of ferrous iron- an exploration of antimalarial mechanism. *Pure Appl Chem* 1999;71:1139-1142.
29. Wang J, Huang L, Li J et al. Artemisinin Directly Targets Malarial Mitochondria through Its Specific Mitochondrial Activation. *PLoS ONE* 2010; 5:e9582.
30. Hamacher-Brady A, Stein HA, Turschner S et al., Artesunate Activates Mitochondrial Apoptosis in Breast Cancer Cells via Iron-catalyzed Lysosomal Reactive Oxygen Species Production. *J Biol Chem* 2010;286:6587-6601.
31. Klonis N, Crespo-Ortiz MP, Bottova I et al. Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and digestion. *Proc Natl Acad Sci U S A* 2011;108:11405-10.
32. Witkowski B, Lelievre J, Barragan MJL et al.

- Increased Tolerance to Artemisinin in *Plasmodium falciparum* Is Mediated by a Quiescence Mechanism. *Antimicrob Agents Chemother* 2010;54:1872-77.
33. Klonis N, Xie SC, McCaw J et al. Altered temporal response of malaria parasites determines differential sensitivity to artemisinin. *Proc Natl Acad Sci USA* 2013;110:5157-62.
 34. Saralamba S, Pan-Ngum W, Maude RJ et al. Intrahost modeling of artemisinin resistance in *Plasmodium falciparum*. *Proc Natl Acad Sci USA* 2011; 108: 397-402
 35. Mok S, Imwong M, Mackinnon MJ et al. Artemisinin resistance in *P. falciparum* is associated with altered temporal pattern of transcription. *BMC Genomics* 12:391 doi: 10.1186/1471-2164-12-391.
 36. Asawamahesakda W, Benakis A, Meshnick SR. The interaction of artemisinin with red cell membranes. *J Lab Clin Med* 1994a;123:757-62.
 37. Hong Y, Yang Y, Meshnick SR. The interaction of artemisinin with malarial hemozoin. *Mol Biochem Parasitol* 1994;63:121-28
 38. Bhisutthibhan J, Pan X, Hossler PA et al. The *Plasmodium falciparum* Translationally Controlled Tumor Protein Homolog and Its Reaction with the Antimalarial Drug Artemisinin. *J Biol Chem* 1998;273:16192-8.
 39. Asawamahesakda W, Ittarat I, Chang C, McElroy P, Meshnick SR., Effects of antimalarials and protease inhibitors on plasmodial hemozoin production. *Mol Biochem Parasitol* 1994b;67:183-191.
 40. Sherman IW, Greenan JR. Altered red cell membrane fluidity during schizogonic development of malarial parasites (*Plasmodium falciparum* and *P. lophurae*). *Trans R Soc Trop Med Hyg.* 1984;78:641-44.
 41. Sibmooh N, Pipitaporn B, Wilairatana P et al. Effect of artemisinin on lipid peroxidation and fluidity of the erythrocyte membrane in malaria. *Biol Pharm Bull* 2000;23:1275-80.
 42. Pandey AV, Tekwani BL, Singh RL, Chauhan VS. Artemisinin, an Endoperoxide Antimalarial, Disrupts the hemoglobin Catabolism and Heme Detoxification Systems in Malaria Parasite. *J Biol Chem* 1999;274:19383-8.
 43. Kannan R, Sahal D, Chauhan VS. Haem-artemisinin adducts are crucial Mediators of the ability of artemisinin to inhibit haem polymerization. *Chem Biol* 2002;9:321-32.
 44. Chugh M, Sundararamana V, Kumar S et al. 2013, Protein complex directs hemoglobin-to-hemozoin formation in *Plasmodium falciparum*. *Proc Natl Acad Sci USA* 2013;110:5392-7.
 45. Asawamahesakda W, Ittarat I, Pu Y, Ziffer H, Meshnick SR. Reaction of Antimalarial Endoperoxides with Specific Parasite Proteins. *Antimicrob Agents Chemother* 1994c;38:1854-58.
 46. Garcia CR, Dluzewski AR, Catalani LH, Burtin R, Hoyland J, Mason WT. Calcium homeostasis in intraerythrocytic malaria parasites. *Eur J Cell Biol* 1996;71: 409-13.
 47. Alleva LM, Kirk K. Calcium regulation in the intraerythrocytic malaria parasite *Plasmodium falciparum*. *Mol Biochem Parasitol* 2001;117:121-128.
 48. Moreno SNJ, Docampo R. Calcium regulation in protozoan parasites. *Curr Opin Microbiol* 2003;6:359-64.
 49. Brini M, Carafoli E. Calcium Pumps in Health and Disease. *Physiol Rev* 2009;89:1341-78.
 50. Nagamune K, Moreno SN, Chini EN, Sibley LD. Calcium Regulation and Signaling in Apicomplexan Parasites. *Subcell Biochem* 2008;47:70-80.
 51. Xu C, Ma H, Inesi G, Al-Shawi M, Toyoshima C. Specific Structural Requirements for the Inhibitory Effect of Thapsigargin on the Ca^{2+} ATPase SERCA. *J Biol Chem* 2004;279:17973-9.
 52. Krishna S, Woodrow C, Webb R. et al. Expression and Functional Characterization of a *Plasmodium falciparum* Ca^{2+} -ATPase (PfATP4) Belonging to a Subclass Unique to Apicomplexan Organisms. *J Biol Chem* 2001;276:10782-7.
 53. Marchesini N, Luo S, Rodrigues CO, Moreno SN, Docampo R., Acidocalcisomes and a vacuolar H^{+} -pyrophosphatase in malaria parasites, *Biochem J* 2000;347: 243-53.
 54. Moreno SN, Zhong L. Acidocalcisomes in *Toxoplasma gondii* tachyzoites. *Biochem J* 1996;313:655-59.
 55. Card D, Pozza A, Arnou B et al. Purified E255L Mutant SERCA1a and Purified PfATP6 Are Sensitive to SERCA-type Inhibitors but Insensitive to Artemisinins. *J Biol Chem* 2010;285:26406-16.
 56. Arnou B, Montigny C, Morth JP et al. The *Plasmodium falciparum* Ca^{2+} -ATPase (PfATP6): insensitive to artemisinin, but a potential drug target. *Biochem Soc Trans.* 2011;39:823-31.
 57. Uhlemann AC, Cameron A, Eckstein-Ludwig U et al. A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. *Nat Struct Mol Biol* 2005;12:628-9.
 58. Bertaux L, Quang LH, Sinou V, Thanh NX, Parzy D. New PfATP6 Mutations Found in *Plasmodium falciparum* Isolates from Vietnam. *Antimicrob Agents Chemother* 2009;53:4570-1.
 59. Pillai D, Lau R, Khaimar K et al. Artemether resistance in vitro is linked to mutation in PfATP6 that also interact with mutations in PfMDR1 in travelers returning with *Plasmodium falciparum* infections. *Malar J* 2012;11:131.
 60. Pulcini S, Staines HM, Pittman JK et al. Expression in Yeast Links Field Polymorphisms in PfATP6 to in Vitro Artemisinin Resistance and Identifies New Inhibitor Classes. *J Infect Dis* 2013;208:468-78.
 61. Imwong M, Dondorp AM, Nosten F et al. Exploring the Contribution of Candidate Genes to Artemisinin Resistance in *Plasmodium falciparum*. *Antimicrob Agents Chemother* 2010;54:2886-92.
 62. Adhin MR, Labadie-Bracho M, Vreden SG. Status of potential PfATP6 molecular markers for artemisinin resistance in Suriname. *Malar J* 2012;11:322.
 63. Cui L, Wang Z, Jiang H et al. Lack of association of the S769N mutation in *Plasmodium falciparum* SERCA (PfATP6) with resistance to artemisinins. *Antimicrob Agents Chemother* 2012b;56:2546-52.
 64. Miao M, Wang Z, Yang Z et al. Genetic Diversity and Lack of Artemisinin Selection Signature on the *Plasmodium falciparum* ATP6 in the Greater Mekong

Subregion. PLoS ONE 2013;8: e59192

65. Tanabe K, Zakeri S, Palacpac NMQ et al. Spontaneous Mutations in the Plasmodium falciparum Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} -ATPase (PfATP6) Gene among Geographically Widespread Parasite Populations Unexposed to Artemisinin-Based Combination Therapies. Antimicrob Agents Chemother 2011;55:94-100.
66. Fernandez-Martinez A, Mula P, Cravo P et al. Characterization of the Plasmodium falciparum Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} -ATPase Gene in Samples from Equatorial Guinea before Implementation of Artemisinin-Based Combination Therapy. Am J Trop Med Hyg 2013;88:43-7.
67. Fairhurst RM, Nayyar GML, Breman JG et al. Artemisinin-Resistant Malaria: Research Challenges, Opportunities, and Public Health Implications. Am J Trop Med Hyg 2012;87:231-41.
68. Krishna S, Kremsner PG, Antidogmatic approaches to artemisinin resistance: reappraisal as treatment failure with artemisinin combination therapy. Trends Parasitol 2013;29:313-17.
69. Shandilya A, Chacko S, Jayaram B, Ghosh I. A plausible mechanism for the antimalarial activity of artemisinin: A computational approach. Sci Rep 2013;3:2513.
70. McFadden GI. The apicoplast. Protoplasma 2011;248: 641-50.
71. Lang BF, Gray MW, Burger G. Mitochondrial genome evolution and the origin of eukaryotes. Annu Rev Genet 1999;33:351-97.
72. Torrentino-Madamet M, Desplans J, Travaillé C, Jammes Y, Parzy D. Microaerophilic Respiratory Metabolism of Plasmodium falciparum Mitochondrion as a Drug Target. Curr Mol Med 2010;10:29-46.
73. Kakkar P, Singh BK. Mitochondria: a hub of redox activities and cellular distress control. Mol Cell Biochem 2007;305:235-53.
74. Jayapal V. Fundamentals of Medical Immunology. 5th ed. New Delhi: Medical Publishers; 2007.
75. Uyemura SA, Luo S, Moreno SNJ, Docampo R, Oxidative Phosphorylation, Ca^{2+} Transport, and Fatty Acid-induced Uncoupling in Malaria Parasites Mitochondria. J Biol Chem 2000;275:9709-15.
76. Nina PB, Morrissey JM, Ganesan SM et al. ATP Synthase Complex of Plasmodium falciparum: Dimeric Assembly in mitochondrial membranes and resistance to genetic disruption. J Biol Chem 2011;286:41312-22.
77. Mather MW, Vaidya AB. Mitochondria in malaria and related parasites: ancient, diverse and streamlined. J Bioenerg Biomembr 2008;40:425-33.
78. Painter HJ, Morrissey JM, Vaidya AB. Mitochondrial Electron Transport Inhibition and Viability of Intraerythrocytic Plasmodium falciparum. Antimicrob Agents Chemother 2010;54:5281-7.
79. Fidock DA, Rosenthal PJ, Croft SL, Brun R, Nwaka S. Antimalarial Drug Discovery: Efficacy Models for Compound Screening. Nature 2004;3:509-20.
80. Grellier P, Depoix D, Schrével J, Florent I. Discovery of New Targets for Antimalarial Chemotherapy. Parasite 2008;15:219-25.
81. Li W, Mo W, Shen D et al. Yeast model uncovers dual roles of mitochondria in the action of artemisinin. PLoS Genet 2005;1:329-34.
82. Mercer AE, Copple IM, Maggs JL, O'Neill PM, Park BK. The Role of Heme and the Mitochondrion in the Chemical and Molecular Mechanisms of Mammalian Cell Death Induced by the Artemisinin Antimalarials. J Biol Chem 2010;286:987-96.
83. Mercer AE, Maggs JL, Sun X et al. Evidence for the involvement of Carbon-centered Radicals in the Induction of Apoptotic Cell Death by Artemisinin Compounds. J Biol Chem 2007;282:9372-82.
84. Handrick R, Ontikatz T, Bauer K et al. Dihydroartemisinin Induces Apoptosis by a Bak-dependent Intrinsic Pathway. Mol Cancer Ther 2010;9:2497-2510.
85. Lu YY, Chen TS, Wang XP, Li L. Single-cell analysis of dihydroartemisinin-induced apoptosis through reactive oxygen species-mediated caspase-8 activation and mitochondrial pathway in ASTC-a-1 cells using fluorescence imaging techniques, J Biomed Opt 2010;15:e046028.
86. Xiao F, Gao W, Wang X, Chen T. Amplification activation loop between caspase-8 and -9 dominates artemisinin-induced apoptosis of ASTC-a-1 cells. Apoptosis 2012;17:600-11.
87. Finaurini S, Ronzoni L, Colancecco A et al. Selective toxicity of dihydroartemisinin on human CD34+ erythroid cell differentiation. Toxicology 2010;276:128-34.
88. Finaurini S, Basilico N, Corbetta Y et al. Dihydroartemisinin inhibits the human erythroid cell differentiation by altering the cell cycle. Toxicology 2012;300:57-66.
89. Ward S. Final Report-ARTEMIP (The safety pharmacology of Artemisinins when used to reverse pathophysiology of malaria in pregnancy). Liverpool: School of Tropical Medicine; 2013 Jan. Project No.: 200805. Available from http://cordis.europa.eu/search/index.cfm?fuseaction=result.document&RS_LANG=EN&RS_RCN=14053832&q=
90. Li Q, Weina P, Hickman M, editors. 2013 The Use of Artemisinin Compounds as Angiogenesis Inhibitors to Treat Cancer (Internet). Silver Spring, USA: INTECH; 2013 (cited 2013 Sep 15). Available at <http://dx.doi.org/10.5772/54109>.
91. Chen HH, Zhou HJ, Wu GD, Lou XE. Inhibitory Effects of Artesunate on Angiogenesis and on Expressions of Vascular Endothelial Growth Factor and VEGF Receptor KDR/flk-1. Pharmacology 2004;71:1-9.
92. Wang J, Zhang B, Guo Y et al. Artemisinin inhibits tumor lymphangiogenesis by suppression of vascular endothelial growth factor C. Pharmacology 2008;82:148-55.
93. Riley PA. Free radicals in biology: oxidative stress and the effects of ionizing radiation. Int J Radiat Biol 1994;65:27-33.
94. Bozdech Z, Ginsburg H. Antioxidant defense in Plasmodium falciparum - data mining of the transcriptome. Mol Cell Pharmacol 2013;5(2):75-89.

- Malar J 2004;3:23 doi: 10.1186/1475-2875-3-23.
95. Sullivan DJ, Matile H, Ridley RG, Goldberg DE. A Common Mechanism for Blockade of Heme Polymerization by Antimalarial Quinolines. *J Biol Chem* 1998;273:31103-7.
 96. Kannan R, Sahal D, Chauhan VS. Haem-artemisinin adducts are crucial Mediators of the ability of artemisinin to inhibit haem polymerization. *Chem Biol* 2002;9:321-32.
 97. Gardner MJ, Hall N, Fung E et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 2002;419:498-511.
 98. Saiki E, Nagao K, Aonuma H et al. Multivariable analysis of host amino acids in plasma and liver during infection of malaria parasite *Plasmodium yoelii*. *Malar J* 2013;12:19 doi 10.1186/1475-2875-12-19.
 99. Babbitt SE, Altenhofen L, Cobbold SA et al. *Plasmodium falciparum* responds to amino acid starvation by entering into a hibernatory state. *Proc Natl Acad Sci USA* 2012;109:e3278-87.
 100. Liu J, Istvan ES, Gluzman IY, Gross J, Goldberg DE. *Plasmodium falciparum* ensure its amino acid supply with multiple acquisition pathways and redundant proteolytic enzyme systems. *Proc Natl Acad Sci USA* 2006;103:8840-5.
 101. Tekwani BL, Walker LA. Targeting the Hemozoin Synthesis Pathway for New Antimalarial Drug Discovery: Technologies for In Vitro β -Hematin Formation Assay. *Comb Chem High Throughput Screen*. 2005;8:63-79.
 102. Salas F, Fichmann J, Lee GK, Scott MD, Rosenthal PJ. Functional expression of falcipain, a *Plasmodium falciparum* cysteine proteinase, supports its role as a malarial hemoglobinses. *Infect Immun* 1995;63:2120-5.
 103. Luker KE, Francis SE, Gluzman IY, Goldberg DE. Kinetic analysis of plasmepsins I and II aspartic proteases of the *Plasmodium falciparum* digestive vacuole. *Mol Biochem Parasitol* 1996;79:71-8.
 104. Eggleston KK, Duffin KL, Goldberg DE. Identification and characterization of falcilysin, a metallopeptidase involved in hemoglobin catabolism within the malaria parasite *Plasmodium falciparum*. *J Biol Chem* 1999;274:32411-47.
 105. Wyatt DM, Berry C. Activity and inhibition of plasmepsin IV, a new aspartic proteinase from the malaria parasite. *Plasmodium falciparum*, *FEBS Lett*. 2002;513:159-62.
 106. Pattanaik P, Jain B, Ravindra G et al. Stage-specific profiling of *Plasmodium falciparum* proteases using an internally quenched multispecificity protease substrate. *Biochem Biophys Res Commun* 2003;309:974-9.
 107. Orjih AU, Banyal HS, Chevli R, Fitch CD. Hemin Lyses Malaria Parasite. *Science* 1981;214:667-9.
 108. Tracz MJ, Alam J, Nath KA. Physiology and Pathophysiology of Heme: Implications for Kidney Disease. *J Am Soc Nephrol* 2007;18: 414-20.
 109. Aft RL, Mueller GC. Hemin-mediated DNA strand scission. *J Biol Chem* 1983;258:12069-12072.
 110. Aft RL, Mueller GC. Hemin-mediated oxidative degradation of proteins. *J Biol Chem* 1984; 259: 301-305.
 111. Campanale N, Nickol C, Daubenberger CA et al. Identification and Characterization of Heme-interacting Proteins in the Malaria Parasite, *Plasmodium falciparum*. *J Biol Chem* 2003;278:27354-61.
 112. Waller KL, Lee S, Fidock DA. 2004 Molecular and Cellular Biology of Chloroquine Resistance *Plasmodium falciparum*, in Waters A. P., Janse C. J. (ed), *Malaria Parasites: Genomes and Molecular Biology*, Horizon Scientific Press, P. 505
 113. Van Dooren GG, Kennedy AT, McFadden GI. The use and abuse of heme in apicomplexan parasites. *Antioxid Redox Signal* 2012;17:634-56.
 114. Francis SE, Sullivan DJ, Goldberg DE. Hemoglobin Metabolism in the Malaria Parasite *Plasmodium falciparum*. *Annu Rev Microbiol* 1997;51: 97-123.
 115. Jani D, Nagarkatti R, Beatty W et al., HDP—A Novel Heme Detoxification Protein from the Malaria Parasite, *PLoS Pathog* 2008; 4(4): e1000053.
 116. Okada k. The novel heme oxygenase-like protein from *Plasmodium falciparum* converts heme to bilirubin IXalpha in the apicoplast. *FEBS Lett* 2009; 583:313-19.
 117. Sartorello R, Budu A, Bagnaresi P et al. In vivo uptake of a haem analogue Zn protoporphyrin IX by the human malaria parasite *P. falciparum*-infected red blood cells. *Cell Biol Int* 2010;34:859-65.
 118. Sigala PA, Crowley JR., Hsieh S, Henderson JP, Goldgerg DE. Direct Tests of Enzymatic Heme Degradation by the Malaria Parasite *Plasmodium falciparum*. *J Biol Chem* 2012;287:37793-807.
 119. Ross R. On some Peculiar Pigmented Cells Found in Two Mosquitos Fed on Malarial Blood. *Br Med J*. 1897;2:1786-8.
 120. Egan TJ, Combrinck JM, Egan J et al. Fate of haem iron in the malaria parasite *Plasmodium falciparum*. *Biochem J* 2002;365:343-7.
 121. Loria P, Miller S, Foley M, Tilley L. Inhibition of the peroxidative degradation of haem as the basis of action of chloroquine and other quinoline antimalarials. *Biochem J* 1999;339:363-70.
 122. Ginsburg H, Famin O, Zhang J, Krugliak M. Inhibition of glutathione-dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action. *Biochem Pharmacol* 1998; 56(10): 1305-1313.
 123. Slater AFG, Swiggard WJ, Orton BR et al. An iron-carboxylate bond links the heme units of malaria pigment. *Proc Natl Acad Sci USA* 1990;88:325-9.
 124. Stiebler R, Hoang AN, Egan TJ, Wright DW, Oliveira MF. 2010 Increase on the Initial Soluble Heme Levels in Acidic Conditions Is an Important Mechanism for Spontaneous Heme Crystallization In Vitro. *PLoS ONE* 5:e12694.
 125. Robert A, Claparols C, Witkowski B, Benoit-Vical F. Correlation between *Plasmodium yoelii nigeriensis* susceptibility to artemisinin and alkylation of heme by the drug. *Antimicrob Agents Chemother* 2013;57:3998-4000.
 126. Witkowski B, Lelievre J, Nicolau-Travers M et al. Evidence for the Contribution of the Hemozoin Synthesis Pathway of the Murine *Plasmodium yoelii* to the Resistance to Artemisinin-Related Drugs. *PLoS ONE* 2012;7:e32620.
 127. Haynes R, Monti D, Taramelli D, Olhario P. Artemisinin Antimalarials Do Not Inhibit Hemozoin

- Formation. Antimicrob Agents Chemother 2003;47:117.
128. Meshnick SR. Artemisinin and Heme. Antimicrob Agents Chemother 2003;47:2712-3.
129. Krishna S, Pulcini S, Fatih F, Staines H. Artemisinins and the biological basis for the PfATP6/SERCA hypothesis. Trends Parasitol 2010;26:517-23.
130. Cojean S, Hubert V, Le Bras J, Durand R. Resistance to dihydroartemisinin. Emerg Infect Dis 2006;12:1798-9.
131. Nagamune K, Beatty WL, Sibley LD. Artemisinin induces calcium-dependent protein secretion in the protozoan parasite *Toxoplasma gondii*. Eukaryot Cell 2007;6:2147-56.
132. Wei N, Sadrzadeh SM. Enhancement of hemin-induced membrane damage by artemisinin. Biochem Pharmacol 1994;48:737-41.
133. Cui L, Wang Z, Miao J et al. Mechanisms of in vitro resistance to dihydroartemisinin in *Plasmodium falciparum*. Mol Microbiol. 2012a;86:111-28.
134. Dondorp AM., Nosten F, Yi P et al. Artemisinin resistance in *plasmodium falciparum* malaria. N Engl J Med 2009;361:455-67.
135. Sullivan DJ. Timing is everything for artemisinin action. Proc Natl Acad Sci USA 2013;110:4866-7.