Published in final edited form as:
Curr Opin Infect Dis. 2011 December ; 24(6): 570–577. doi:10.1097/QCO.0b013e32834cd3ed.

Recent clinical and molecular insights into emerging artemisinin resistance in *Plasmodium falciparum*

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Abstract

Purpose of review—Artemisinin-based combination therapies (ACTs) have been deployed globally with remarkable success for more than 10 years without having lost their malaria treatment efficacy. However, recent reports from the Thai–Cambodian border reveal evidence of emerging resistance to artemisinins. The latest published clinical and molecular findings are summarized herein.

Recent findings—Clinical studies have identified delayed parasite clearance time as the most robust marker of artemisinin resistance. Resistance has only been documented from Southeast Asia and has been observed in isolates that show no significant decrease in drug susceptibility *in vitro*. Genetic investigations have yet to uncover robust molecular markers. In-vitro studies have identified parasite quiescence or dormancy mechanisms that protect early 'ring-stage' intra-erythrocytic parasites against short-term artemisinin exposure. This might be achieved by reducing the rate of hemoglobin degradation, important for artemisinin bioactivation.

Summary—Should ACTs fail, no suitable alternatives exist as first-line treatments of *P. falciparum* malaria. Intensified efforts are essential to monitor the spread of resistance, define therapeutic and operational strategies to counter its impact, and understand its molecular basis. Success in these areas is critical to ensuring that recent gains in reducing the burden of malaria are not lost.

Keywords

artemisinin-based combination therapy; drug resistance; malaria; molecular markers; parasite clearance times; *Plasmodium falciparum*; recrudescence; treatment failure

Introduction

In 2006, the WHO officially recommended that artemisinin (ART)-based combination therapies (ACTs) be adopted as first-line treatment of uncomplicated malaria caused by *Plasmodium falciparum* [1]. This recommendation came in response to the global spread of resistance to the former first-line antimalarials chloroquine and sulfadoxine–pyrimethamine

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Conflicts of interest
There are no conflicts of interest.

[2,3•]. The global implementation of ACTs (Fig. 1) [1], along with vector control measures including insecticide-treated bednets and indoor residual spraying, has helped to considerably reduce the disease burden in many endemic countries. In the past decade, over 40 countries have reported a greater than two-fold reduction in malaria cases (http://www.who.int/malaria/world_malaria_report_2010/en/index.html). Despite these achievements, malaria continues to have a devastating impact, with an estimated 780 000 deaths and over 225 million cases in 2009. The greatest burden falls on young children residing in sub-Saharan Africa [4••].

ART, long used in Chinese traditional medicine, was found in the early 1970s to have potent antimalarial activity [5,6]. This was soon followed by its chemical identification as a sesquiterpene peroxide and the development of methods to produce the derivatives artesunate, artemether (ATM), and the active metabolite dihydroartemisinin (DHA). Defining properties of these ART derivatives include their ability to very rapidly decrease numbers of asexual blood stage parasites, their activity against the broadest range of intra-erythrocytic developmental stages (including action on the immature ring-stage forms as well as the more mature trophozoite stages) of all known antimalarial drugs, and their ability to inhibit the development of immature sexual stage parasites (gametocytes). This gametocytocidal activity acts to reduce the transmission of ART-treated *Plasmodium* parasites to their *Anopheles* mosquito vector [7]. ARTs have also shown an excellent safety profile in humans. Their major caveat, however, is their very short half-life in plasma, typically on the order of 1–3 h [8,9]. Clinically, ART monotherapy is only curative when administered for 7 days, and recrudescence (i.e. the reappearance of asexual blood stage parasites during the follow-up period) is common [10,11•]. ART derivatives have, therefore, been combined with longer lasting partner drugs, which include lumefantrine (combined with ATM and globally the most widely used ACT), amodiaquine (typically paired with artesunate), mefloquine (combined with artesunate), sulfadoxine–pyrimethamine (with artesunate), piperaquine (combined with DHA), and pyronaridine (currently being registered in combination with artesunate) (Fig. 1) [12]. These combinations typically provide radical cure with 3-day treatments and help protect against the emergence of resistance to the ART derivative [13••,14•].

Evidence for the emergence of artemisinin resistance

Preliminary indications of clinical treatment failures with ACTs came from observational studies in the early 2000s of artesunate–mefloquine use near the Thai–Cambodian border (Fig. 2) [1,15,16]. It was unclear, however, whether this resulted from resistance to artesunate or its partner drug, or other host or pharmacological factors. A subsequent report from western Cambodia by Noedl *et al.* in 2008 [17] identified two patients with clear evidence of artesunate-resistant infections. These patients recrudesced following 7 days of artesunate monotherapy (4 mg/kg per day) and showed delayed parasite clearance times (133 and 95 h, as compared with a median of 52 h for patients that were cured) despite having adequate drug levels. These times represent the interval from the start of treatment to the time the parasitemia (i.e. the percentage of parasitized erythrocytes) falls below the lower limit of parasite detection by light microscopy (approximately 0.001%). Ex-vivo drug dose–response measurements revealed that the patient isolates with prolonged parasite clearance times had DHA half maximal inhibitory concentration (IC_{50}) values that were four times the geometric mean for cured patients [18•].

Compelling evidence supporting the emergence of ART resistance came from the study published mid-2009 by Dondorp *et al.* [19], showing delayed parasite clearance rates in Pailin, western Cambodia, compared with Wang Pha, northwestern Thailand, following artesunate monotherapy or artesunate–mefloquine combination therapy. Median parasite

clearance times between Wang Pha and Pailin were prolonged from 48 to 72 h in patients treated with 2 mg/kg artesunate and from 54 to 84 h with 4 mg/kg artesunate with mefloquine. These differences could not be attributed to drug pharmacokinetics or differences in age between these two study populations. In-vitro drug susceptibilities, as measured by DHA and artesunate IC_{50} values, were comparable between these two populations. One concern with this study was the higher parasite density at baseline in the Pailin group [20]; this was rebutted by Dondorp *et al*. [19] as not affecting the core findings. It is important to stress that this indication of emerging resistance did not substantially reduce clinical efficacy, with cure rates continuing to exceed 90%.

Factors implicated in the emergence of ART resistance in western Cambodia include 30 years of ART monotherapy (now officially banned), drugs that were substandard and/or used suboptimally, and possibly parasite genetic backgrounds that favor the emergence of multi-drug resistance [21••,22,23]. Further heritability studies with isolates from Pailin calculated that 56% of the variability in parasite clearance rates could be attributed to parasite genetics, suggesting that resistance determinants could disseminate geographically under drug selection [24•]. Another factor likely to contribute is the very low rate of transmission, resulting in insufficient immunity to eliminate parasites that might have survived drug treatment, thus increasing selection pressure [25].

Adding to the concerns of diminished ART efficacy, the Thai–Cambodian border has historically been a focal point for multidrug resistance in *P. falciparum* [26•]. Parasites from this region were among the first to develop resistance to chloroquine, sulfadoxine–pyrimethamine, and mefloquine, and in the case of the two former drugs this resistance subsequently spread to Africa [27–30]. Accordingly, intense efforts are being pursued throughout the Greater Mekong Subregion (GMS), composed of Cambodia, Laos, Myanmar, Thailand, Vietnam, and the Yunnan Province of China, to monitor for the emergence and spread of ART resistance [31••]. Data from a longitudinal study of over 3200 patients along the Thai–Myanmar border showed continued efficacy of a 3-day course of artesunate–mefloquine (over the period 1995–2007), with 96% of patients being cleared of parasitemia on day 3 [32]. Nevertheless, the study observed a small but significant increase in parasite clearance times, associated with a small increase in treatment failure rates (i.e. parasite recrudescence during a 42-day follow-up period). These increases were not accompanied by significant reductions in artesunate sensitivity *in vitro*. Slower parasite clearance was also associated with increased gametocyte carriage, thereby increasing the risk of transmission of drug-tolerant parasites. To date, ARTs remain highly effective in Vietnam [33], where clinical outcomes are being closely monitored.

Active surveillance for the emergence of ART resistance is also being pursued globally, including efforts outlined in the WHO Global Plan for Artemisinin Resistance Containment (http://www.who.int/malaria/publications/atoz/9789241500838/en/index.html) and activities coordinated by the WorldWide Antimalarial Resistance Network [34•]. A recent report by Stepniewska *et al.* [11•] collected parasite clearance data from over 18 000 patients, in 25 different countries ranging from low to high transmission. This meta-analysis confirmed the earlier suggestion from western Cambodia [19] that elevated parasite clearance time represents the most robust marker of ART resistance. The study reported that parasite clearance rate is largely determined by parasite density on admission and found that the proportion of patients with positive blood smears on day 3 could be used as a simple measure of artemisinin susceptibility *in vivo*. The report concluded that ART resistance in a given area is highly unlikely if at least 97% of patients presenting with a parasite density of less than 100 000 infected erythrocytes per microliter and given a 3-day course of ACTs are smear-negative on day 3. Of note, emerging resistance to ARTs appears to be largely restricted to the GMS, and no definitive evidence has yet been reported from Africa [35].

The intense logistical requirements to clinically monitor ART resistance have led several teams to explore in-vitro assays as a surrogate of resistance. Indeed, reduced in-vitro susceptibility has been closely associated with an increased risk of treatment failure for all other classes of antimalarial drugs [36]. Evidence in favor of the applicability of in-vitro studies to ART resistance comes from the work by Noedl *et al*. [17], cited above, showing elevated DHA IC_{50} values in patients that failed artesunate monotherapy. Longitudinal studies conducted by the Cambodian National Malaria Control Program also showed increased geometric means of artesunate IC_{50} values in isolates from western Cambodia compared with eastern Cambodia. IC_{50} values were significantly higher in patients that failed artesunate–mefloquine therapy [37•]. Furthermore, this study reported a recent increase in artesunate IC_{50} values in genetically distinct isolates from eastern Cambodia. Other studies from northwestern Thailand reported an approximately 10-fold decrease in artesunate in-vitro susceptibility over a 10-year period; however, no clinical assessments were conducted [38]. Nonetheless, the work from Pailin provides compelling evidence that the clinical surrogate marker of parasitemia on day 3 (after initiation of treatment) did not translate into reduced ART susceptibility *in vitro* [21••]. Further studies are required to assess to what extent in-vitro assays can predict the emergence of ART resistance.

Recent advances in defining artemisinin mode of action and mechanisms of resistance

The mechanism by which ARTs exert their antimalarial action has long remained enigmatic (Fig. 3) [39•,40•,41–45,46••,47•–49•]. Investigations with ART derivatives have defined a critical requirement for the endoperoxide moiety that is generally believed to produce active compound upon interaction with intracellular iron. The likely source of this iron is in Fe-protoporphyrin IX that is released as a result of proteolytic degradation of host hemoglobin, which is imported into the acidic digestive vacuole of the intra-erythrocytic parasite (Fig. 3) [50]. Several mechanisms of ART action have been proposed, including oxidative damage to parasite membranes or inactivation of parasite proteins [51,52]. One candidate parasite protein that has received considerable scrutiny is the calcium-dependent endoplasmic reticulum-resident ATPase PfATP6, which can be inhibited by ART when expressed in *Xenopus laevis* frog oocytes. Mutations were identified that abolished this inhibition or that were associated with reduced ART susceptibility in *P. falciparum* field isolates [41]. A recent study, however, found no significant increase in IC_{50} values for ART derivatives in recombinant *P. falciparum* cell lines expressing mutant vs. wild-type *pfatp6*, although a subset of dose–response assays was suggestive of reduced susceptibility in mutant parasites [53].

Multiple investigations have focused on *pfmdr1*, which encodes an ATP-binding cassette (ABC) transporter resident on the digestive vacuole and can vary via point mutations or copy number [42]. Field studies and laboratory investigations demonstrate that *pfmdr1* gene amplification is associated with an increased risk of parasite recrudescence following mefloquine or artesunate–mefloquine treatment, and in-vitro resistance to mefloquine accompanied by reduced susceptibility to ART [36,54,55]. Related studies have found that in some strains of *P. falciparum*, selection for resistance to ART or its derivative artelinic acid was accompanied by amplification of *pfmdr1* [56,57]. Removal of drug pressure frequently resulted in deamplification of this gene and attenuation of the resistance phenotype. Genetic studies of drug-pressured rodent *Plasmodium chabaudi* parasites also identified amplification of the *mdr1* ortholog, which was associated with rodent parasite resistance to ART and the partner drugs mefloquine and lumefantrine [58]. The *pfmdr1* polymorphisms have also been associated with diminished ART susceptibility: in a recent study of field isolates from the Thai–Myanmar border, novel polymorphisms in this gene (and the related ABC transporter gene *pfmrp1*) were associated with reduced in-vitro

susceptibility to ART and DHA [59]. The *pfmdr1* gene may, therefore, play a role in reducing parasite susceptibility to some ACTs. However, this is not thought to constitute a major determinant of delayed parasite clearance, as evidenced by recent genotyping of *pfmdr1*, *pfatp6*, and *ubp-1* (a gene associated with resistance from ART selection studies in *P. chabaudi* [44,60]). This genotyping study found no association between these genes and *P. falciparum* clearance rates in patients from western Cambodia and northwestern Thailand [61•].

Genome-wide studies are also being actively pursued to identify candidate determinants of ART susceptibility, including linkage analysis of a *P. falciparum* genetic cross that identified several regions of the genome (including *pfmdr1*) that were associated with decreased ART susceptibility [62]. Recently, drug susceptibility profiles for over 2800 compounds in 61 *P. falciparum* lines were used to map response phenotypes to genetic loci [63••]. This identified 15 genes associated with *P. falciparum* responses to ART and mefloquine, many of which had not been previously identified, making these important candidates to examine in patient isolates showing delayed responses to ACTs. Gene disruption studies have also implicated PfMRP1, located on the asexual blood stage parasite's plasma membrane, as a minor modulator of ART potency [43]. As an alternative approach, field isolates from Pailin and neighboring sites in the GMS were briefly cultured *ex vivo* and RNA was prepared from parasites harvested regularly throughout a 48-h period (corresponding to one intra-erythrocytic developmental cycle). Transcriptional analysis of time-matched samples revealed altered regulation profiles for several hundred genes in the isolates with delayed parasite clearance times [49•,64•]. Those isolates revealed downregulation of several metabolic and cellular pathways (including glycolysis, redox regulation, and glutathione synthesis) early in the cycle, and a lesser degree of upregulation of pathways including protein synthesis in later stages. These complex studies involved a small sample size and could only be performed once as isolates were cultured directly from patient blood. Additional experiments are required to provide further evidence that these broad transcriptional changes represent an underlying molecular basis of reduced ART susceptibility. Preliminary transcriptome studies were also applied to a *P. falciparum* line selected for tolerance to very high concentrations of ART following continuous exposure to increasing ART doses over a 3-year period [47•]. These studies reported a number of differentially expressed genes in the resistant line compared with its parental control, including genes encoding a heat shock protein and a cell cycle regulator. However, only two time points were selected over a single 48-h cycle, precluding accurate matching of time points. The selection of ART-tolerant lines in several laboratories and their ongoing molecular characterization should provide important new insights into general mechanisms of ART resistance or tolerance in *P. falciparum* parasites in the near future.

In summary, molecular studies on the basis of ART resistance have identified some genes associated with reduced ART susceptibility *in vitro* (most notably *pfmdr1*), but researchers have yet to define common genetic determinants that can account for prolonged parasite clearance times in drug-treated patients. This gap in knowledge can be addressed through expanded genome-wide association studies with large numbers of clinically characterized field isolates, transcriptome and metabolomic studies on highly synchronized cultured parasites, and confirmation of candidates using transfection and allelic exchange techniques. Adding to the complexity of finding these determinants is the difficulty in defining markers of clinical or in-vitro resistance. It is conceivable that single determinants of resistance will not be found, and that clinical resistance involves multi-factorial molecular processes that achieve little more than to permit parasites to survive the initial onslaught of ART action and to resume growth once the levels of these very short-lived drugs have dropped below therapeutic concentrations. One mechanism by which *P. falciparum* might survive is a recently described dormancy trait whereby small numbers of parasites can abruptly arrest

their intra-erythrocytic ring-stage development after a single exposure to DHA and remain dormant for several days to weeks before resuming normal growth rates [48•]. This is consistent with a similar quiescence trait observed in ART-pressured ring-stage parasites selected for tolerance to high concentrations of drug [47•]. Recent mechanistic investigations provide evidence that parasites might achieve a state of dormancy by reducing the rate of hemoglobin degradation in ring-stage parasites. This would effectively reduce the amount of hemoglobin products available to potentiate ART activity and enable stalled ring-stage parasites to survive short-lasting drug exposure [46••]. This dormancy–recovery phenomenon is not thought to contribute to the initial slowing of parasite clearance following treatment with ACTs or artesunate monotherapy, but might help explain the high rates of recrudescence observed following ART monotherapy [65]. Mathematical modeling of parasite responses to treatments conducted in Cambodia and Thailand suggest that prolonged parasite clearance times are likely to reflect reduced susceptibility of ring-stage parasites, via cellular mechanisms yet to be elucidated [66•].

Conclusion

The evidence for the emergence of resistance to ARTs in South-east Asia is compelling and demands aggressive intervention measures. Modeling studies using parasite responses to ACT treatment in western Cambodia argue that as intervention measures decrease the local parasite pool, the relative proportion of resistant parasites will increase [6]. These authors concluded that the spread of resistance can only be halted by eliminating malaria in this region. Proposals to counter resistance include mass drug administration, mass screen and treat campaigns including the use of rapid diagnostic tests, the use of newer ACTs such as DHA–piperaquine or artesunate–pyronaridine that have favorable efficacy and compliance characteristics, and improved case detection and treatment [67•–69•,70]. Vector control measures are also critical and need to include careful monitoring for the emergence and spread of insecticide resistance [71••]. Additional efforts include the Affordable Medicines Facility-malaria (AMFm), an innovative financing mechanism to expand access to affordable ACTs [72,73•]. Defining the molecular basis of resistance will also be critical to effectively monitor for the spread of resistance. With precious few drugs available to replace ARTs should they fail, the importance of countering resistance cannot be underestimated.

Acknowledgments

The authors thank Dr Pascal Ringwald, from the Global Malaria Program at the WHO, for kindly providing figures. D.A.F. gratefully acknowledges funding from the National Institutes of Health (R01 AI079709) and the Medicines for Malaria Venture. C.O.B. gratefully acknowledges funding from the Howard Hughes Medical Institute through their Medical Fellows Program.

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 615).

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Key points

- Artemisinin-based combination therapies have been adopted worldwide as official first-line policy for the treatment of *Plasmodium falciparum* malaria. These combine a fast-acting but short-lived artemisinin derivative with one of several longer-lasting partner drugs.
- Evidence of emerging resistance to artemisinin derivatives has been gathered from clinical studies in western Cambodia, a known source of multidrug-resistant *P. falciparum.*
- Resistance manifests as prolonged parasite clearance time, as evidenced by microscopically detectable blood stage infections on day 3 after initiation of treatment.
- Intense efforts are being pursued worldwide to confirm, characterize, and contain resistance to artemisinins. The urgency of these efforts is increased by the paucity of alternative antimalarials should artemisinins fail.
- Molecular markers have yet to be defined. Nevertheless, evidence is mounting that the ability of early ring-stage intra-erythrocytic parasites to resist artemisinin action, possibly including mechanisms of quiescence or dormancy, might contribute to prolonged clearance times and/or parasite recrudescence after treatment.

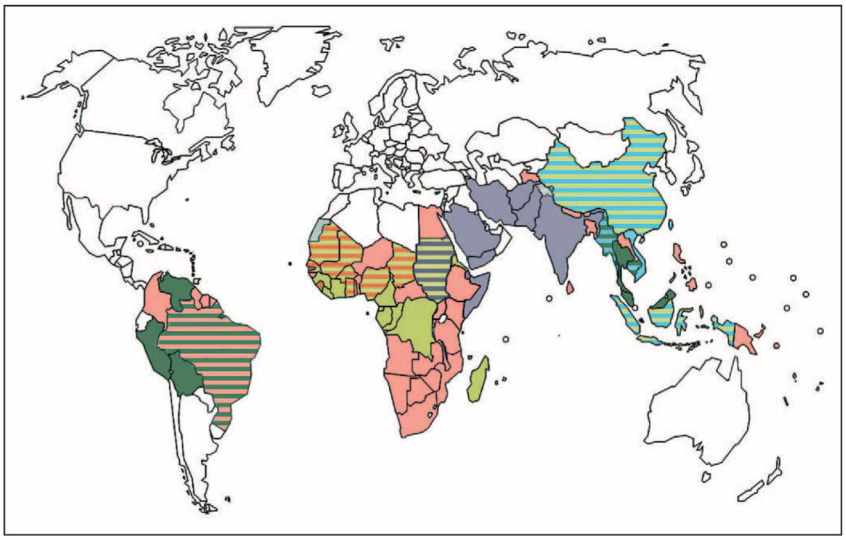

Figure 1. Current global distribution of artemisinin-based combination therapies as the first-line treatment of uncomplicated falciparum malaria

This distribution was collated from maps of country-wide artemisinin-based combination therapy use, as published in [1]. ●, Artemether–lumefantrine; ●, dihydroartemisinin–piperaquine; ●, artesunate–amodiaquine; ●, artesunate–mefloquine; ●, artesunate–sulphadoxine/pyrimethamine.

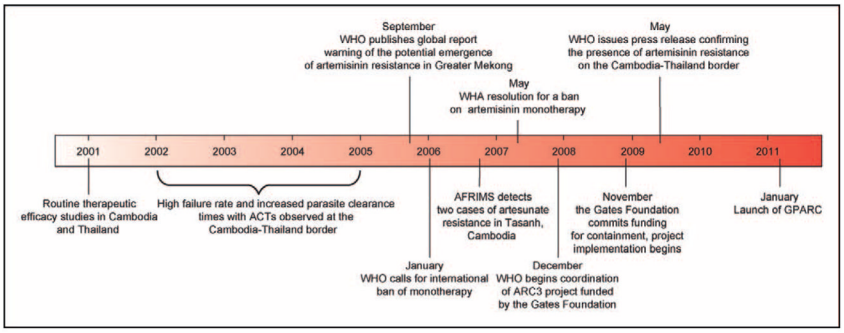

Figure 2. Schematic representation of recent events relating to the emergence of and response to artemisinin resistance

AFRIMS, Armed Forces Research Institute of Medical Sciences; ARC3, artemisinin resistance project: pilot studies to confirm, characterize and plan for containment; the Gates Foundation, Bill & Melinda Gates Foundation; GPARC, Global Plan for Artemisinin Resistance Containment; WHA, World Health Assembly; WHO, World Health Organization. Reproduced with permission from [1].

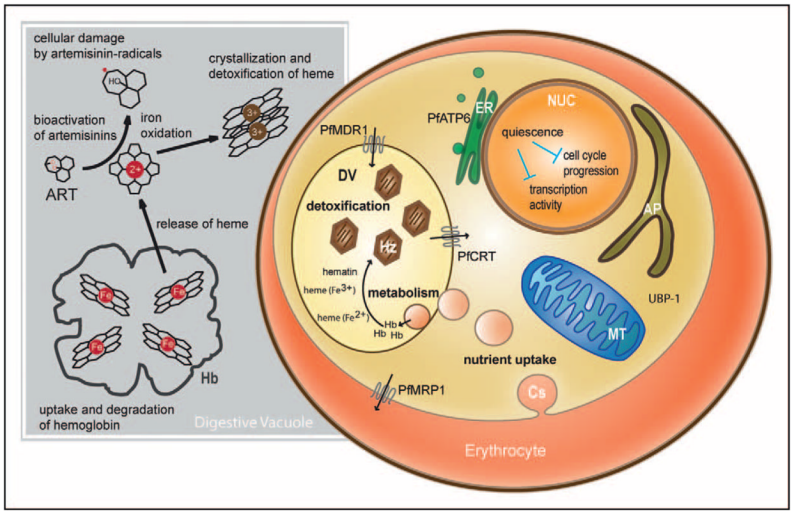

Figure 3. Depiction of an intra-erythrocytic *Plasmodium falciparum* parasite showing proteins and biological processes implicated in artemisinin action

Several parasite proteins have been implicated in decreased susceptibility to artemisinins (ARTs), including PfATP6 (proposed to be in the endoplasmic reticulum [41]), PfMDR1 on the digestive vacuole [42], PfMRP1 on the parasite plasma membrane [43], and UBP-1 whose ortholog in *Plasmodium chabaudi* is associated with ART resistance [44]. The digestive vacuole protein PfCRT is also indicated as mutations that confer chloroquine resistance have been shown to significantly increase susceptibility to ARTs [45]. Host hemoglobin is delivered via cytostomes to the digestive vacuole, wherein it is proteolytically degraded. This liberates iron-heme (Fe-protoporphyrin IX) moieties, with subsequent oxidation of iron. Iron-heme is detoxified via its incorporation into hemozoin crystals. Iron-heme is thought to activate ARTs via interaction with the endoperoxide bridge, with the resulting ART radicals causing cellular damage [46••]. Investigations of field isolates and drug-pressured laboratory lines have implicated quiescence or dormancy of early ring-stage parasites in resistance to ART action [47•–49•]. ART, artemisinin; AP, apicoplast; Cs, cytostome; DV, digestive vacuole; ER, endoplasmic reticulum; Hb, hemoglobin; Hz, hemozoin; MT, mitochondria; NUC, nucleus.