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Iron and heme metabolism in *Plasmodium falciparum* and the mechanism of action of artemisinins

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During the asexual blood stage of its lifecycle, the malaria parasite *Plasmodium falciparum* grows and multiplies in the hemoglobin-rich environment of the human erythrocyte. Although the parasite has evolved unique strategies to survive in this environment, its interaction with iron represents an Achilles' heel that is exploited by many antimalarial drugs. Recent work has shed new light on how the parasite deals with hemoglobin breakdown products and on the role of iron as a mediator of the action of the antimalarial drug, artemisinin.

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Introduction

Malaria is a debilitating parasitic disease caused by protozoan parasites of the genus *Plasmodium*. Every year about 200 million new infections of *P. falciparum* malaria are established, causing 655 000 deaths [1]. Mortality and morbidity are associated with the asexual blood stage, when the parasite undergoes rounds of replication in the red blood cells (RBCs) of its human host. The parasite develops through what are known as the ring, trophozoite and schizont stages (Figure 1). As it develops it consumes host RBC hemoglobin (Hb) which generates amino acid building blocks, and provides space to accommodate growth and division. This parasite-specific process of hemoglobin digestion is a point of critical vulnerability that is exploited by many antimalarial drugs, including artemisinins.

The World Health Organization currently recommends Artemisinin-based Combination Therapies (ACTs) for the treatment of uncomplicated *P. falciparum* malaria [2]. Artemisinin and its derivatives (collectively referred to as artemisinins or ARTs) clear *P. falciparum* infections rapidly, providing prompt therapy for severe infections [3]. Because current antimalarial control in endemic areas is highly dependent on ACTs, recent reports of decreased clinical efficacy of ARTs are extremely concerning [4••]. Unfortunately, efforts to monitor and overcome resistance to ARTs have been hampered by a limited understanding of the molecular basis of ART action.

ARTs (Figure 2a) are sesquiterpene lactones with a 1,2,4-trioxane core incorporating an endoperoxide linkage that is essential for activity. The mechanism of action of ARTs is still debated but a widely held view is that ARTs are pro-drugs that are activated by reductive cleavage of the endoperoxide ring (see reviews [5–7]). The resulting free radicals are thought to react with susceptible groups within a range of parasite proteins, leading to cellular damage and killing. This review focuses on work in the last few years that has increased our understanding of iron metabolism in the parasite and its relevance to understanding the mechanism of action of ARTs.

The ART activator: what is it and where does it come from?

A number of studies have demonstrated reaction of ARTs *in vitro* with both heme and ferrous iron and these are thought to be the main activators *in vivo* (for review see [5]). The intraerythrocytic parasite has access to a potentially limitless supply of heme and iron in the form of host Hb, and does indeed digest about 75% of the host RBC Hb. However, it does not appear to utilize the Hb-derived heme for incorporation into its own heme proteins (see review by Scholl *et al.* [8]). The vast majority of this potentially toxic heme is oxidized to hematin and sequestered as a crystalline form called hemozoin. Recent work has shown that Hb digestion begins when the parasites are morphologically at the ring stage of development. Live cell imaging reveals the formation of acidified compartments in the parasite cytoplasm from ~14 hours post invasion [9,10]. Electron microscopy analysis reveals that Hb digestion is initiated in small vesicles which coalesce into a single mature digestive vacuole at ~20 hours post invasion as the parasite enters the trophozoite stage of development [9,11] (Figure 1).

The unit cell of hemozoin comprises hematin dimers with reciprocal iron-carboxylate linkages [12] and such dimers have been assumed to represent the nucleation unit *in vivo*. However, the crystal structure also shows the presence of dimers stabilized by π–π interactions [13].

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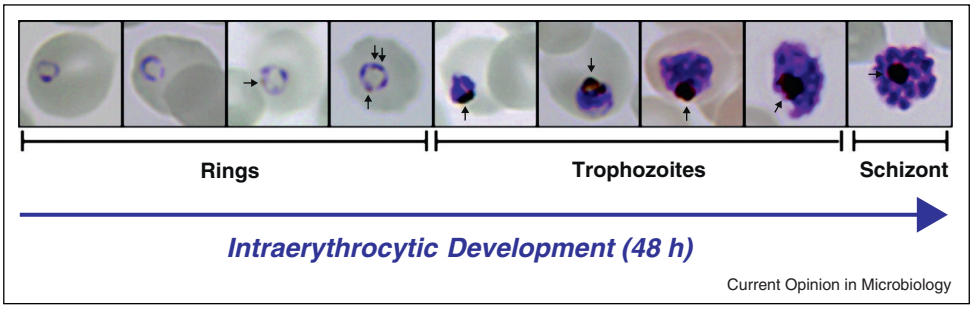
Figure 1



Stages of development of intraerythrocytic *P. falciparum* as revealed by Giemsa staining. A merozoite (the exo-erythrocytic form of the parasite) invades an RBC and adopts a ring-like morphology which persists for the first ~20 hours of development. During the trophozoite stage synthesis of parasite DNA is initiated leading to the formation of a schizont containing multiple merozoites. Detailed microscopy analyses (see [9,34] for details) reveal that Hb digestion is initiated in the mid-ring stage, leading to the formation of hemozoin (dark pigment indicated by arrows). The intraerythrocytic age of the depicted parasites is approximated by its position along the blue time arrow.

These π–π dimers are also present in solution [14] and have been proposed as an alternative nucleation unit [15]. There is also on-going debate about whether crystallization occurs in the aqueous phase [16•] or a lipid phase [17] and whether specific proteins help coordinate Hb digestion and hemozoin formation [18]. Although most of the host-derived hematin is sequestered as hemozoin, earlier work demonstrated the presence of detectable levels of hematin in the infected RBC that was not associated with Hb or hemozoin (see review by Scholl *et al.* [8]). The presence of hematin within the reducing environment of the cytoplasm would produce a pool of heme capable of activating ARTs (Figure 2b).

Recent work confirmed that infected RBCs contain a larger labile iron pool than uninfected RBCs and showed that this pool increases during asexual development [19]. Surprisingly, the source of the parasite iron is still unclear. Early studies concluded that neither extracellular iron nor the labile iron pool of the host RBC are required for parasite survival (see [8] for review). However, recent work shows that the labile iron pool can be modulated by iron in the medium [19].

The degradation of Hb-derived heme could provide an additional source of iron for the parasite. Earlier studies indicated that iron might be released during degradation of hematin by hydrogen peroxide in the digestive vacuole or by reaction with reduced glutathione in the parasite cytoplasm (see review by Scholl *et al.* [8]). A putative heme oxygenase was recently identified in the parasite [20,21]; however, no heme oxygenase activity has been detected in infected RBCs and the designation of these enzymes as heme oxygenases has been questioned [22].

Hb in the reduced state can also activate ART and lead to the production of heme-ART adducts [23,24]. This reaction is relatively slow and is likely to reflect Hb that is destabilized by limited proteolysis [25,26]. It is possible that such an 'unstable' Hb is produced in acidic digestive compartments and is capable of activating ART.

What is the ART activator *in vivo* ?

To date, only a few studies have provided direct insights into the nature of the activator *in vivo*. A recent study examined the role of hemoglobin degradation in the action of ART against trophozoites subjected to short drug pulses [27••]. Almost complete ablation of ART activity was observed in the presence of hemoglobinase inhibitors providing the first conclusive evidence for a critical role of a Hb degradation product in the mechanism of action of ARTs. A previous study failed to detect this antagonism [28] as it examined the interaction over the complete lifecycle, introducing complications due to the stage-dependence of action of both the ART (see below) and the protease inhibitor. For example, protease inhibitors can be potent inhibitors of schizont rupture and of merozoite invasion of RBCs [29] as well as inhibiting Hb digestion.

The question remains: Is the *in vivo* activator heme or free ferrous iron; or perhaps unstable Hb? The suggestion that ferrous iron is the activator is supported by studies showing that iron chelators, such as desferroxamine (DFO), antagonize the activity of ARTs [30,31]. Iron chelators have been shown to reduce the potency of an artesunate pulse against trophozoites by fivefold [32]; however, this is much less dramatic than the antagonism observed with hemoglobinase inhibitors (100-fold reduction in dihydroartemisinin potency [27••]). Moreover a recent report showed that ART was able to alkylate heme in mice infected with an ART-susceptible strain of *P. yoelii* but not in an ART resistant strain [33]. These results suggest that both iron and heme-mediated

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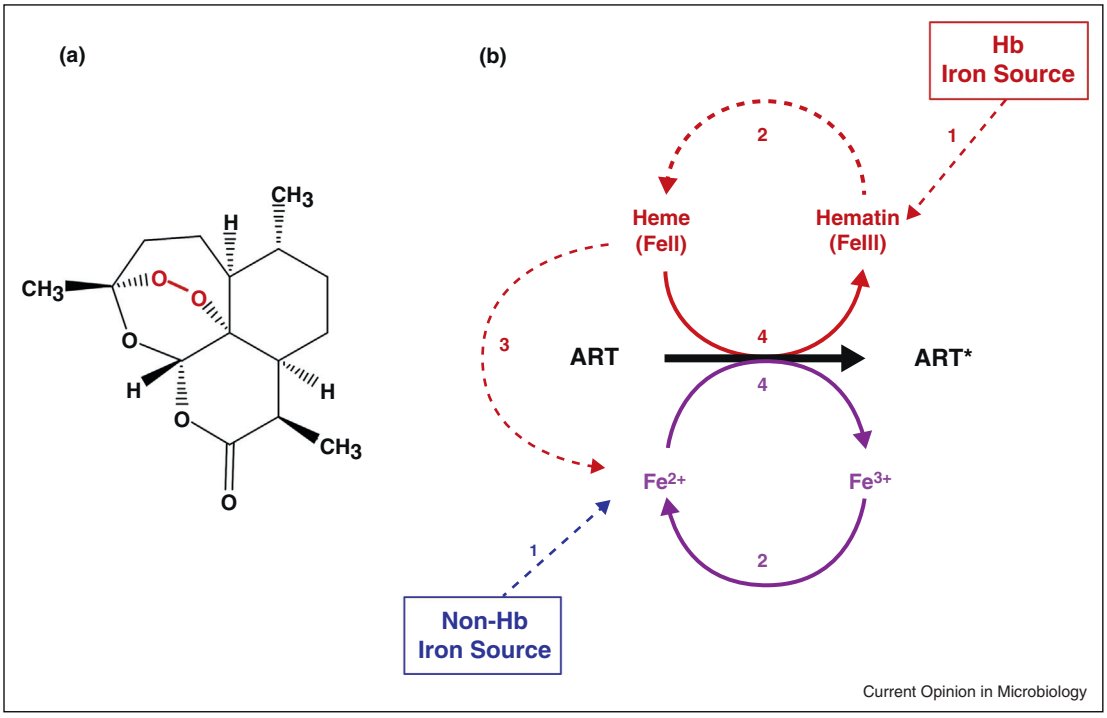
Figure 2



Structure of artemisinin **(a)** and potential *in vivo* pathways for its activation **(b)**. Artemisinin and its derivatives contain an endoperoxide bridge (a, red) that is necessary for activity. ARTs may be activated by reduced iron (Fe^{2+}) or heme (4) to produce activated ART (ART*). The activator pool could be derived from Hb and non-Hb dependant pathways as indicated by the dashed lines in b. Hematin is produced during the degradation of Hb (1, red) within acidic compartments. This can diffuse into the parasite cytoplasm where it would be reduced to produce heme (2, red). Some may be degraded to Fe^{2+} (3, red) to augment the parasite's labile iron pool produced by conventional means (1, blue). For most of the ring stage, a smaller flux through different pathways may be sufficient to generate a low level of ART*. In late rings/trophozoites, the levels of heme and Fe^{2+} are enhanced by Hb degradation, driving efficient ART activation (and hence potency). A saturable effective dose means that ART* production is not first order with respect to ART concentration. The rate of ART activation could be limited by the rate of production of activators (1) and/or by rate of redox cycling (2) that is required to reduce/regenerate the activators.

activation occur but that the potent ART activity against trophozoites likely reflects heme-mediated activation.

If Hb degradation is needed for potent ART action it might be predicted that ring stage parasites (particularly those less than 14 hours post-invasion) would exhibit much lower sensitivity than trophozoites. A recent study used tightly synchronized parasites exposed to short (physiologically relevant) drug pulses [34••]. Under these conditions large stage-dependent differences in ART response were observed that reflected a pronounced lag time in the drug response of rings. Ring stage parasites exhibited up to 100-fold lower sensitivity to short drug pulses than trophozoites. Most previous studies had failed to detect this difference in ring stage sensitivity because they used longer periods of exposure to ARTs, which masks the difference in sensitivity. This stage dependence mostly correlated with the level of Hb digestion, with reduced ART activity when Hb digestion is low. However, this study also reported the surprising finding that there is a short period of hypersensitivity soon after invasion.

An alternative ART activation pathway in ring stage parasites?

A detailed analysis of ART action showed that the rate of parasite killing following exposure to ARTs reflects an underlying saturable process [34••]. This indicates that parasites experience an 'effective' drug dose that is different from the applied dose of ART. One interpretation is that the effective dose corresponds to the dose of activated ART. The saturable nature of ART activation in trophozoites may reflect the fact that the rate of production of the activator is ultimately limited by redox cycling (i.e. reduction to regenerate the active form of the activator) and/or the rate of its production from Hb (i.e. heme or ferrous iron derived from Hb digestion (Figure 2)). Interestingly, only a threefold to fourfold reduction in the rate of production of ART activator is needed to explain the dramatic decrease in ring stage sensitivity to short drug pulses. Thus ART activation may still occur at some level in rings. Given that Hb digestion does not appear to occur in the first half of the ring stage there may be alternative pathways for ART activation. Of particular interest is the hypersensitivity to ARTs of a

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population of very early parasites (2–4 hours post-invasion) [34••]. This suggests the presence of a highly potent activator at this stage, and/or a particularly sensitive downstream target.

One possibility is that ART activation occurs via both Hb-dependant and independent pathways, with the Hb-dependant pathway dominating in trophozoites but other pathways becoming important in rings. For example, activation may occur through the labile iron pool in ring stages but be augmented by increased iron and/or heme levels during stages where Hb uptake and digestion are most active. Alternatively, other modes of action/activation have been proposed which may also play an important role in ring stage ART activity. One study has suggested that iron-mediated activation in the mitochondria can lead to specific inhibition of the electron transport chain and mitochondrial depolarization [35]. The sarco/endoplasmic reticulum Ca^{2+}-ATPase (PfSERCA) has also been considered as potential direct target [28,36] although its role in ART mechanism of action has been questioned [37,38]. Other studies suggest metal-independent ART activity resulting from direct interaction with cofactors involved in maintaining redox homeostasis [39].

Reduced ring stage sensitivity may underlie delayed parasite clearance following ART treatment

There is particular interest in understanding the molecular basis of the decreased clinical efficacy of ARTs in some regions of South East Asia [4••,40]. Decreased efficacy is linked to delayed parasite clearance in treated patients and the behavior of ring stage parasites appears to be of particular importance. Intra-host modeling suggests that delayed parasite clearance may result from decreased drug sensitivity of this stage [41••]. Indeed it might be anticipated that further extension of the lag time for drug action could enable the already poorly susceptible ring stage parasites to survive until the concentration of the short-lived ARTs drops below lethal levels [34••]. Thus it is of particular interest that reduced sensitivity to ARTs has been observed in ring stages of *P. falciparum* freshly isolated from patients in western Cambodia [42••]. The molecular changes that affect the responses of ring stage parasites from this region remain to be identified but it would be useful to examine processes that affect reactive iron levels.

What are the optimal characteristics of novel endoperoxides?

The major disadvantage of ARTs is that they have very short half-lives *in vivo* (~0.5–2 hours), and cannot be used as monotherapies for uncomplicated malaria due to frequent recrudescence of infections after a standard 3-day treatment [43]. Modeling of the response of ring stage parasites to ARTs indicates that relatively small changes in the parasite's drug response are sufficient to lead to infections exhibiting delayed clearance times [34••]. However, the same modeling indicates that relatively small improvements in drug stability can have profound effects on drug efficacy (e.g. a 50% decrease in parasite survival for every 0.3 hours increase in drug half-life). Alternative endoperoxide antimalarials, including 10-alkylamino derivatives, such as artemisone [44] and fully synthetic ozonide compounds, OZ277 and OZ439 [45••,46], are currently in development. While artemisone has a similar *in vivo* half-life to currently employed ARTs [47], the half-life of OZ277 is twofold higher [48] and that of OZ439 is substantially higher (~20 hours) [45••]. Other things being equal, longer-lived endoperoxides might be expected to provide significantly enhanced efficacy against ART resistant parasites; however, this needs to be confirmed.

New ART-like compounds are routinely screened for iron reactivity; however, no direct correlation between iron-reactivity and parasiticidal activity has been observed [49,50]. Indeed, it has been suggested that one reason for the short *in vivo* half lives of endoperoxide antimalarials is premature opening of the endoperoxide ring when the drug is located away from the site of action in the parasite. Thus there is likely to be a compromise between the parasite-specific reactivity leading to parasite death and extra-parasitic reactivity leading to drug deactivation. As an example, OZ439 is 50-fold more stable than OZ277 to iron-mediated degradation *in vitro*, which may provide enhanced stability against blood-mediated degradation, yet retains comparable reactivity with heme, which may be sufficient for activation and subsequent parasite killing [45••,51]. Resolving the nature of the *in vivo* activator(s) and its associated stage specificity is important both for understanding the molecular basis of ART action and for guiding the development of longer-lived and more effective endoperoxide antimalarials.

Conclusion

It is now clear that the potent activity of ARTs against mature blood stage trophozoites is dependent on Hb uptake and digestion. However, a number of questions remain unanswered: What is the nature of the Hb-derived activator? Does this activator play a role in the action of ARTs against ring stages or do ARTs act via a different mechanism in these stages? What is the basis of the hypersensitivity of very early rings? And how do these processes determine rates of parasite clearance following ART treatment *in vivo*? The recent demonstrations that the response of ring stage parasites is the key to understanding delayed parasite clearance times in ART resistant parasites make it critical to address these questions.

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