Evidence that haem iron in the malaria parasite is not needed for the antimalarial effects of artemisinin

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Abstract The role of haem iron (II) and oxidative stress in the activation and antimalarial activity of artemisinin is unclear. Thus, we submitted malaria parasite to modified culture conditions: artemisinin activity increased by 20–30% under an oxygen-rich atmosphere (20% O_2 instead of "standard" 1% O_2), and by 40–50% in the presence of carboxy-haemoglobin, and 2% carbon monoxide, conditions which inhibit haem iron (II) reactivity. In all cases, parasite growth and chloroquine activity were unaffected. We conclude that in the malaria parasite artemisinin is not activated by haem iron and that free radicals are not needed for its toxicity.

Keywords: Malaria; Artemisinin; Chloroquine; Carbon monoxide; Oxidative stress; *P. falciparum*

1. Introduction

Some thirty years after the discovery of artemisinin, the active principle of the Chinese herb *Artemisia annua*, artemisinin-type compounds are the most widely used and best studied antimalarials [1,2]. They act more rapidly than any other drug and resistance has not been reported yet [2]. Whilst their activity depends on the presence of the unique 1,2,4-trioxane pharmacophore, it is widely believed that various reactive intermediates generated after cleavage of the peroxidic bond within the trioxane, rather than the intact molecule, are the actual parasiticidal species [3,4]. However, it is not clear what this presumed 'activation' consists of, or if activation is required at all prior to binding to the active site, especially after the recent report of a possible target [5].

The activation of artemisinin-type molecules is believed to be due to the reductive scission of the trioxane pharmacophore by a one-electron transfer reaction [6,7]. The electron-donor species, however, is supposed to be iron (II) haem or iron (II) non-haem, while the prevalent form of iron in the parasite is iron (III) haem, stored as insoluble haemozoin [8]. It has been shown in chemical experiments that iron (II) haem "activates" artemisinin to generate a variety of products, the majority of which appear to be inert [9]. The lethal effects on the parasite have been ascribed either to the formation of C-centred radicals that alkylate target molecules or to the generation of oxygen radicals that could damage membranes [3,4,10].

There is an apparent conflict in these hypotheses. The electron transfer, i.e., the reductive scission of artemisinin, would be favoured in a low-oxidant, reducing environment, whereas the toxic effects of artemisinin-derived free radicals would be amplified in an oxygen-rich environment; in other words, the carbon-centred free radicals would react with oxygen to produce peroxy radicals, which in turn will enhance oxidative stress.

To clarify this aspect, we set up a "live" test tube in which *P. falciparum* parasites were grown in either reducing or oxidizing atmospheres. *P. falciparum* is microaerophylic and normally grows under atmosphere containing 1% O_2 [11]. The pro-oxidant environment was obtained by culturing parasites at 20% O_2. For the reducing environment, we eliminated the O_2 carried by haemoglobin by using carbon monoxide (CO) that forms a stable complex with iron (II) haem [12]; 2% CO was also added to the final atmosphere. This model presents two main advantages: it allows a study of the activity of artemisinin in a low oxygen, highly reducing environment, with less opportunity for intercession of peroxidative reactions and provides an environment in which iron (II) haem is not available for the reductive activation of the trioxane pharmacophore. Parasite growth was monitored and drug sensitivity assays were carried out with several artemisinin derivatives and the results compared to that of chloroquine.

2. Materials and methods

2.1. Parasite cultures, reagents and drug susceptibility assays

Chloroquine-sensitive (D10) and chloroquine-resistant (CDC/Indocina III, W2) *P. falciparum* parasites were maintained at 5% haematocrit (human A^+ RBC) in RPMI 1640 medium (Gibco BRL) supplemented with 10% heat-inactivated A^+ human plasma, at 37 °C under standard conditions [11]. Chloroquine and artemisinin were from Sigma, Milan, Italy. Dihydroartemisinin and artesunate were kindly provided by WHO/TDR, Geneva, CH.

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For the drug susceptibility assays, compounds were dissolved in water or ethanol (<1% final concentration), diluted with medium and serial twofold dilutions made in microplates (Costar Italia, Milan). Asynchronous cultures with parasitemia of 1–1.5% and 1% final haematocrit were incubated for 72 h at 37 °C in three different atmospheres: (1 = reducing conditions) 2% CO, 5% CO_2, and 93% N_2; (2 = microaerophylic conditions) 1% O_2, 5% CO_2, and 94% N_2; (3 = pro-oxidant conditions) 20% O_2, 5% CO_2, and 75% N_2. Parasite growth was determined spectrophotometrically (OD_{650}) by measuring the activity of the parasite lactate dehydrogenase (pLDH), in control and drug-treated cultures [13]. Giemsa-stained cultures were also observed microscopically and under polarized light.

2.2. Statistical analysis

All tests were performed at least five times in triplicate and results were analysed by the Student's paired *t* test.

3. Results

Different experimental conditions were set up to evaluate the role of iron (II)-haem and oxygen tension on the growth and the sensitivity of *P. falciparum* to artemisinin derivatives. By flushing RBC with carbon monoxide (CO), we obtained a stable complexation of iron (II)-haem with CO. This was confirmed by UV–VIS spectrophotometry, which showed the shift in absorption from 414 nm of oxy-haemoglobin to 420 nm of carboxy-haemoglobin. In addition, 2% CO, and no oxygen, was part of the gas mixture used for the growth of parasites and the drug assays. The final atmospheres of the cultures contained: (1) 2% CO (reducing environment); (2) 1% O_2 (''standard'' microaerophylic environment); and (3) 20% O_2 (oxidant environment).

The viability of parasite cultures and the rate of growth did not change, nor did the production of birefringent pigment inside the parasite food vacuoles, under any of these culture conditions (Figs. 1 and 2). In particular, parasites could be cultured and were viable for more than a month under 2% CO, atmosphere (1); their spectrum of drug sensitivities did not change; and they grew normally when cultured again under oxygenated conditions [12].

The response to artemisinin and chloroquine differed when experimental conditions were changed. In the reducing 2% CO, atmosphere (1), the activity of artemisinin was significantly higher (IC_{50} lower) than in the microaerophylic atmosphere (2) of 1% O_2 ($P < 0.05$), while, like in previous experiments [12], the activity of chloroquine was unaltered (Fig. 3). On average, the activity of artemisinin was 1.86-fold increased for the D10 strain and 2.53-fold increased for the W2 strain after 72 h. For the artemisinin derivatives, artesunate and dihydroartemisinin, the IC_{50} values decreased significantly (increased activity) in atmosphere (1) (Table 1, column 1) vs. atmosphere (2) (column 2).

The antimalarial activity of artemisinin increased (IC_{50} was lower) also in the oxidant atmosphere (3) (20% O_2) compared to the microaerophylic atmosphere (2) (1% O_2) (Table 1, column 3). On average, activity increased by 1.56-fold and 1.43-fold for the D10 and W2 strain, respectively, at 20% O_2 compared to 1% O_2. Similar results were obtained with artesunate or dihydroartemisinin. For artesunate activity increased by 1.84-fold and 2.49-fold and for dihydroartemisinin by 1.53-fold and 2.00-fold for D10 and W2, respectively. The relative potency of the three drugs, dihydroartemisinin > artesunate > artemisinin was maintained under different

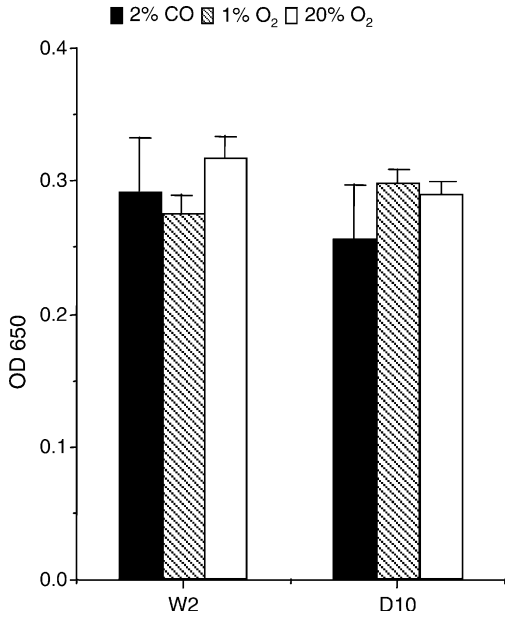

Fig. 1. Growth rate of *P. falciparum* parasites, D10 (CQ-S) and W2 (CQ-R) strains, cultured under atmosphere (1), 2% CO (full bars); atmosphere (2), 1% O_2 (striped bars); atmosphere (3) 20% O_2 (empty bars). Parasite growth was checked after 72 h of culture using the pLDH method. Data are means ± SD of 15 different experiments.

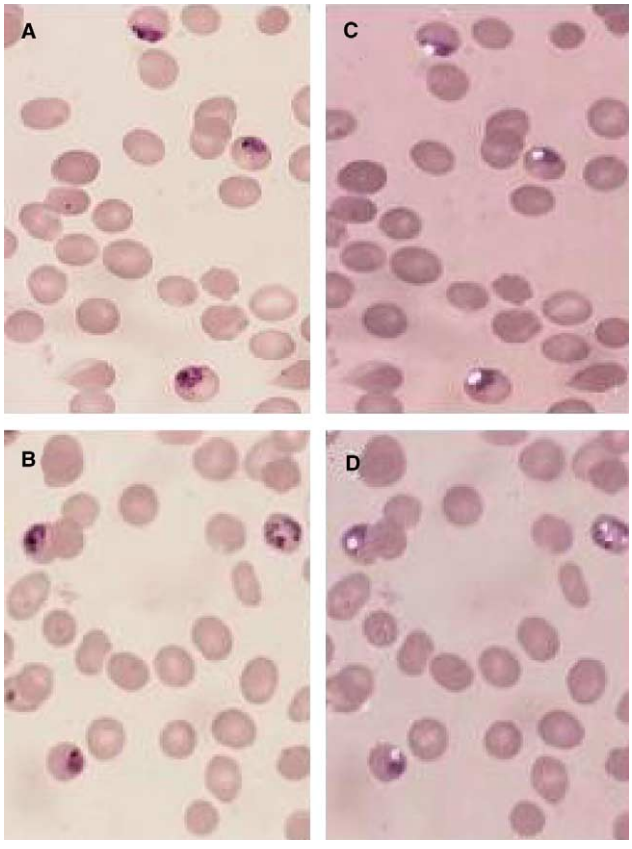

Fig. 2. Wright–Giemsa stained slides of *P. falciparum* parasites cultured under 1% O_2 and oxy-haemoglobin (panels A and C) or 2% CO and carboxy haemoglobin (panels B and D). (A, B) Normal light; (C, D) polarized light to visualize birefringent pigment.

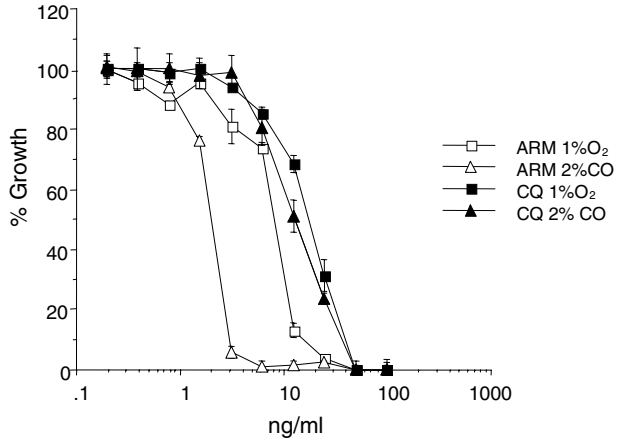

Fig. 3. Antimalarial activity of artemisinin (ARM) and chloroquine (CQ) against *P. falciparum* strain D10. The assay was done for 72 h under atmosphere (1), 2% CO or atmosphere (2), 1% O_2. Parasite growth was evaluated using the pLDH method. The data are the results of a representative experiment in triplicate, out of six conducted under the same conditions. Artemisinin in atmosphere 1 vs. atmosphere 2, $P < 0.05$; Chloroquine in atmosphere 1 vs. atmosphere 2, not significant.

conditions. For all drugs, activity ranked 2% CO > 20% O_2 > 1% O_2 (Table 1).

4. Discussion

The 'activation' theory for artemisinin antimalarials is largely based upon data obtained from experiments using chemical reagents in conditions which may not reflect the complexity of the living parasites. In the present paper, we addressed the question using whole parasite cultures and adapting simple methodologies to evaluate the putative roles of iron (II) haem and oxygen in determining drug action. We reasoned that working on the whole parasite, as opposed to mere chemical or biochemical assays, would offer new biological insights for further, more advanced, investigations.

Two questions were addressed: (1) Is iron (II) haem needed for the activation of artemisinin type antimalarials? (2) Do the artemisinins kill the parasite via enhanced oxidative stress?

Our data strongly indicate that haem iron (II) is not essential and that oxidative stress is likely to play a marginal role in the mechanism of action of artemisinins, as we already reported for chloroquine [12]. The experimental model adopted was appropriate for the studies: parasite viability was unaffected by carbon monoxide or different oxygen tensions and the relative potencies of artemisinin compounds remained the same.

In the presence of CO-haemoglobin and 2% CO in the atmosphere, the activity of artemisinin and its derivatives increased significantly in both chloroquine-sensitive and -resistant strains, while no change was observed for chloroquine [12].

This is the first demonstration in parasites that iron (II) haem is not necessary for the antimalarial activity of artemisinin, since in the presence of CO, iron (II) is no longer available for the reductive scission of the trioxane pharmacophore. These data in parasites are in good agreement with the observation that the chemical reactivity of artemisinins with iron (II) haem does not correlate with their biological activity [14]. In a previous paper, we also showed that artemisinin and derivatives do not inhibit beta haematin formation, thus providing evidence against these molecules binding to iron (III) haem [15].

If haem iron is not the main activator of artemisinins, we have to postulate the existence, in the parasite, of an alternative one-electron transfer reaction, which is CO-insensitive and which can activate these molecules. It may be located either in the parasite cytoplasm or in the food vacuole, as suggested by the sub-cellular distribution of artemisinins in infected RBC [5,16]. Alternatively, activation may not be required at all for the parasiticidal activity. Due to the propensity of the molecule to accept one electron, artemisinin could act as a competitive inhibitor of an electron transfer system crucial for the parasite. Artemisinin would be intrinsically active, independently of generation of scission products.

More intriguing are the results of the effect of oxy-haemoglobin and oxygen tension on artemisinin activity. When oxy-haemoglobin is used, iron (II) haem may be present as a transient intermediate. It will undergo rapid oxidation by oxygen to iron (III) haematin, which transforms to haemozoin. The iron (II) haem may also react with artemisinin, forming artemisinin haem-adducts [3,4,14]. As indicated above, the radicals leading to the haem adducts may escape from the haem environment and cause damage via alkylation of yet undefined biomolecules [3,17], or generate either hydroperoxides or oxygen radicals toxic for the parasites [4].

We infer from our results that the increased activity seen in 2% CO may be due to the fact that the cleavage of artemisinin by iron (II) haem is a competitive process, which actually leads

Table 1
Antimalarial activity of artemisinin derivatives against D10 (CQ-S) and W2 (CQ-R) strains of *P. falciparum* cultured under different atmospheres

	Drugs	2% CO atmosphere 1	1% O_2 atmosphere 2	20% O_2 atmosphere 3
D10 IC_{50} (ng/ml)	Artemisinin	4.44 ± 2.67^a	8.27 ± 2.41	5.27 ± 3.22^c
	Artesunate	1.05 ± 0.69^a	2.37 ± 1.56	1.29 ± 0.41^c
	Dihydroartemisinin	0.56 ± 0.40^b	1.04 ± 0.69	0.68 ± 2.26^c
W2 IC_{50} (ng/ml)	Artemisinin	1.39 ± 0.82^a	3.51 ± 1.41	2.47 ± 1.19^c
	Artesunate	0.58 ± 0.22^b	1.32 ± 0.56	0.53 ± 0.05^c
	Dihydroartemisinin	0.22 ± 0.07^a	0.52 ± 0.23	0.26 ± 0.06^d

Data are means ± SD of five different experiments in triplicate.
[a] Atmosphere 1 vs. atmosphere 2, $P < 0.01$.
[b] Atmosphere 1 vs. atmosphere 2, $P < 0.05$.
[c] Atmosphere 3 vs. atmosphere 2, $P < 0.05$.
[d] Atmosphere 3 vs. atmosphere 2, $P < 0.01$.

for the most part to inert by-products, and which is unrelated to antimalarial activity [9]. That is to say that, in the standard microaerophylic culture conditions, a competitive degradation of the drug occurs, which leaves only a relatively small proportion of it available to exert its antimalarial effect. There are biological observations in support of this hypothesis: unlike other antimalarials, artemisinins are effective against the ring stage of intraerythrocytic parasites, during which very little haemoglobin is ingested and little, if any, iron (II) haem is available. Interestingly, Skinner et al. [18] unlike others [19] reported that the relative potency of artemisinin on later trophozoites is lower. The increasing levels of iron (II) haem during trophozoite growth may be the reasons for that.

The scene changes again when the oxygen pressure is increased to 20%. Under this atmosphere, we observed a 20–30% improvement in artemisinin activity over 1% oxygen. This was not unexpected, since previous data showed that 17% O_2 in the atmosphere induced a 29% increase of artemisinin activity [10]. These results may be attributed to the generation of increased oxidative stress [4].

However, we favour an alternative hypothesis which we consider more consistent with our overall results: under 20% oxygen, iron (II) haem is preferentially and more rapidly oxidized to iron (III) haem than in 1% oxygen. Thus, less iron (II) haem reacts with artemisinin and more (bioactive) artemisinin is available to exert its parasitocidal activity. Yet a certain level of cleavage must still take place at 20% oxygen, because artemisinin in this case is on an average 10% less active than under CO.

We should mention here that it is indeed possible that artemisinin acts via a different mechanism to oxidative stress. A new target, the Ca^{2+}-dependent *Plasmodium falciparum* ATPase (PfATP6), an enzyme located in the membrane of the parasite endoplasmic reticulum, was recently reported [5]. Although it was stated in that work that iron was required for activation, our data indicate that iron (II) haem may not be required at all.

What is the real situation in vivo? We would like to point out that, while the conditions used for the CO model have clearly no bearing with the reality of parasite infection in humans, they serve to establish an interesting experimental system to assess the role of iron (II) haem and oxidative stress in artemisinin activity. Also, this model confirms that *P. falciparum* is strongly microaerophylic, probably even anaerobic in certain stages of its life cycle. In a microaerophylic environment, the hypothesis that oxygen radicals and oxidative stress are involved in artemisinin activity, as outlined above, should be reconsidered. This leaves open the question of the existence of different, yet to be identified target(s) for these drugs as indeed suggested by the significant antitumour activity demonstrated for several artemisinin derivatives [8,20].

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