Artemisinins: mechanisms of action and potential for resistance

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Abstract

Artemisinins form the most important class of antimalarial currently available, particularly because they are effective against parasites resistant to almost all the other classes. Their mechanism of action is controversial. Some aspects of this controversy are reviewed here. Whilst there is no clinical resistance yet identified to artemisinins, the potential to examine the relationship between polymorphisms in PfATP6 (a target of artemisinins) in multidrug resistant isolates of *Plasmodium falciparum*, is also discussed.
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Keywords: *Plasmodium falciparum*; Malaria; Artemisinin; Endoperoxides

1. Introduction

Some of our most important medicines come from plants, including the only two anti-malarial classes used to treat severe malaria; the cinchona alkaloids and artemisinins (Dobson, 1998). The cinchona alkaloids, like quinine, are extracted from the bark of trees originally grown in Peru and have a history that goes back more than 350 years (Greenwood, 1992). Artemisinin is extracted from a herb, *qinghao*, which in being documented in traditional Chinese pharmacopoeia for treatment of fevers over two millennia, has a longer history of use. In 1967, the Government of the People's Republic of China began a programme to identify antimalarial principles in plants used in traditional Chinese medicine (Klayman, 1985). In 1971 workers at the Pharmaceutical Institute of the Academy of Traditional Chinese Medicine succeeded in showing that extracts of qinghao killed *Plasmodium berghei* in mice (Qinghaosu and

C-ORGo., 1979; Hien and White, 1993). By 1972 they had identified the active ingredient—*qinghaosu* ("the active principle from qinghao")—which is now called artemisinin (Klayman, 1985). Artemisinin is still obtained by extraction from qinghao (*Artemisia annua*—sweet Annie, sweet wormwood), a genus of plant that also gives us absinthe (Klayman et al., 1984). Once the structure of artemisinin was solved in 1977 (Qinghaosu and C-ORGo., 1977), modifications were introduced to improve solubility in both oil and water. These derivatives (Fig. 1) now comprise our most important class of antimalarial and are having a profound impact on modern antimalarial treatment regimens.

2. Why artemisinins are important antimalarials

Artemisinins are our most important class of anti-malarial for reasons that include the following.

2.1. Pharmacokinetic properties

Artemisinins can be administered by several routes. The water soluble derivative artesunate can be given by the oral, intramuscular, intravenous, and even intrarectal routes (Krishna et al., 2001; Hien et al., 1991; Hien, 1994). These routes of administration have been studied in recent years
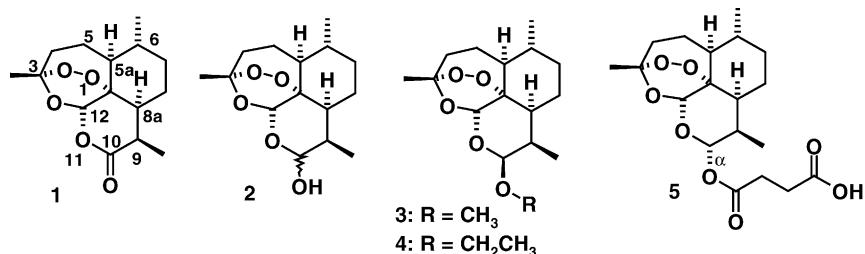
Fig. 1. *Qing hao su* or artemisinin (**1**) and derivatives dihydroartemisinin (**2**), artemether (**3**), arteether (**4**), and artesunate (**5**). The numbering scheme is that used by chemical abstracts. Artemisinin is a sesquiterpene—a natural product containing 15 carbon atoms, which like all terpenes, is biosynthesized in *A. annua* from mevalonic acid via dimethylallyl and isopentenyl pyrophosphates.

for their efficacy, toxicity and pharmacokinetics. The most clinically useful artemisinins (**2**–**5**) are metabolised to dihydroartemisinin (see Table 1 for some activities). For parent compounds such as artesunate, which have very short elimination half-times (<10 min), the metabolite is the more important in exerting antimalarial effects because the elimination half-time is somewhat longer (∼1 h). The relatively short half-time for elimination of dihydroartemisinin confers the theoretical advantage that selection for drug-resistant parasites is less likely. A potential disadvantage, however, may be a higher associated risk of recrudescence when these drugs are used in monotherapeutic regimens. Oral artesunate is rapidly absorbed in uncomplicated malaria, with good bioavailability (Alin et al., 1996).

From available data, it is unlikely that disease severity affects clearance parameters of artemisinins significantly (Krishna et al., 2001; Nealon et al., 2002; Newton et al., 2000). However, absorption may be altered by many variables including formulation of artemisinin, oil-solubility and route of administration. For example, in severe malaria, artemether absorption may be relatively slow by the intramuscular route when compared with intramuscular artesunate (Nealon et al., 2002; Murphy et al., 1997). Absorption is also quite variable (9–20-fold variation in AUC depending on dose) when artesunate is used by the intrarectal route, for example, in children who cannot take anti-malarials by mouth (Krishna et al., 2001). The use of rectal artesunate may be life-saving when there is anticipated delay between the onset of symptoms and management in hospital. In these circumstances (moderate malaria) the rectal administration of artesunate is effectively parasiticidal despite variable absorption kinetics (Krishna et al., 2001).

Artemisinins are one of the few classes of drug useful to treat severe malaria that is resistant to chloroquine. The only other antimalarial that can be used for this purpose is quinine, which has a relatively narrow therapeutic index and can cause hyperinsulinaemia and associated risk of hypoglycaemia (White et al., 1983; Agbenyega et al., 2000). This complication is not observed with artesunate therapy, which seems to be remarkably free of major toxicity. Artesunate bioavailability is acceptable even when it is given by the intramuscular route in severe malaria and there is no associated local toxicity, another complication of intramuscular quinine (Hien, 1994; Nealon et al., 2002; Kamchonwongpaisan et al., 1997; Nontprasert et al., 2002).

2.2. Pharmacodynamic properties

Artemisinins act more rapidly than other types of antimalarial, both in killing parasites and in inhibiting their major metabolic processes, such as glycolysis, nucleic acid and protein synthesis (ter Kuile et al., 1993). Artemisinins also attack the broadest age range of parasites, from the tiniest rings that have recently invaded erythrocytes to more mature stages of parasites such as developing trophozoites and schizonts (ter Kuile et al., 1993; Angus et al., 1997). Their relatively broad stage-specificity of action extends to an ability to impede the development of gametocytes (Price et al., 1996). This contrasts with some other widely used antimalarial classes such as the 4-aminoquinolines or antifolates, which do not have the potential to interrupt transmission of malaria. These favourable properties also have some correlates when artemisinins are used to treat patients. For example, removal of ring stage parasites from within red cells can be inferred in patients who are treated with artemisinins, but not other antimalarials (Angus et al., 1997). This property reduces circulating parasitaemia more rapidly than other antimalarials, sometimes by up to 2–3 log orders of magnitude (White, 1999). In areas of low malaria transmission, gametocytocidal activity may reduce transmission and contribute to malaria-control programmes (Price et al., 1996), although similar benefits have not been shown when artemisinins are used in areas of higher malaria transmission (von Seidlein et al., 2003). Furthermore, artemisinins inhibit the ability of

Table 1
In vitro activities (IC$_{50}$ ng mL^{-1}) of artemisinins against *P. falciparum*

Compound	D-6	D-6	W-2	W-2
Artemisinin (**1**)	6.41	2.33	10.53	1.21
DHA (**2**)	1.79	0.11	1.83	0.04
Artemether (**3**)	3.34		4.19	
Arteether (**4**)	2.94	0.87	4.07	0.16
Artesunate (**5**)	1.66		2.18	
10-Deoxoartemisinin (**9**)		0.58		0.15

maturing parasites to make the red cell surface sticky (cytoadherence to endothelial cells) much more effectively than most other antimalarials (Udomsangpetch et al., 1996). As cytoadherence is a recognised virulence determinant, inhibiting it more efficiently may confer a comparative advantage over other classes of drug.

2.3. Activity against multidrug resistant parasites

Malarial parasites become resistant to different antimalarials with frequencies that vary depending upon the class of antimalarial. For example, resistance to atovaquone which arose because of a mutation in the target mitochondrial enzyme cytochrome *bc1* occurs at a relatively high frequency that affects about 30% of individuals treated with atovaquone monotherapy (Korsinczky et al., 2000; Winstanley et al., 2002). In contrast, it took 15 years for aminoquinolines to begin losing efficacy, and quinine is still useful despite more than 350 years of continuous use. Parasites in some parts of the world are resistant to separate drug classes such as the antifolates and 4-aminoquinolines. Also, some parasites may have an increased capacity to develop resistance compared with others (Rathod et al., 1997). The phenomenon of drug resistance is most common in southeast Asia, and has been studied intensively on the borders of Thailand, where mefloquine-resistant parasites emerged after only a few years of monotherapy (Nosten et al., 1991). When artemisinins are combined with drugs that no longer work well enough to cure >95% of patients, then failing efficacy can be restored in some areas and for particular combinations. Artesunate added to mefloquine consistently cures >95% of patients, where mefloquine alone only worked for about 50–60% of cases (Nosten et al., 1991; Nosten et al., 2000).

These findings (and the other described potential benefits) have given rise to the idea that effective treatment of patients should combine antimalarials, and that such combinations should always include an artemisinin derivative (White, 1999a,b; White, 1998). What advantages does using an artemisinin confer? First, it does raise the cure rates when combined with antimalarials that are taken from different classes (Olliaro and Taylor, 2004). However, a key determinant of cure rates is the underlying efficacy of the partner to an artemisinin, when it is used as monotherapy in a particular geographic area. For example, amodiaquine given with artesunate can cure >90% or ∼70% of individuals, depending on where it is used (Adjuik et al., 2002). A significant contribution to this geographic variation will be parasites' susceptibility to artemisinin (allied to other factors such as age-related antimalarial immunity) (Adjuik et al., 2004).

Artemisinin containing combination therapies will undoubtedly increase in their usage and perhaps in their public health importance with time as resistance becomes more widespread to a wider range of single anti-malarial drugs. Their value has been discussed in greater detail in a recent review (Kremsner and Krishna, 2004).

Lack of cure after mefloquine treatment of patients in Thailand has been linked to increased copy number of *pfmdr1* in resistant parasites (Cowman et al., 1994; Price et al., 1999; Price et al., 2004). Interestingly, there is also an association with increased IC_{50} for artesunate or artemisinin and increase in *pfmdr1* copy number in these isolates. However, this increase in IC_{50} detected by in vitro assays on parasites in culture does not translate to impairment in clinical efficacy when artemisinins are used, because the increased IC_{50} is still within a narrow (and therapeutic) range. A new amino acid polymorphism in a receptor for artemisinins (PfATP6, see below) has been identified in parasites in the region, which contains some of the most highly drug resistance parasites worldwide. However, there is no association between this polymorphism and IC_{50} values to artemisinins (Price et al., 2004).

3. How may artemisinins work?

Artemisinins act via mechanisms that are distinct from other antimalarial classes, including those that inhibit well defined targets such as enzymes of folate biosynthesis, the DOXP reductase pathway or the cytochrome electron transport system. The peroxide within the 1,2,4-trioxane system of artemisinins is essential for antimalarial activity. Thus, artemisinins lacking a peroxidic oxygen atom such as the desoxy compounds **6** and **7** (Fig. 2), (Avery et al., 1993) and the 1-carba analogue **8** in which one of the peroxide oxygen atoms is replaced by carbon (Ye and Wu, 1989; Ye et al., 1991) are also devoid of activity. However, 10-deoxo-10-dihydroartemisinin derivative **9** in which the peroxide is intact retains antimalarial activity, and in fact is more active than artemisinin against the malaria parasite in vitro (Ye et al., 1991; Jung et al., 1990). The peroxide structure, has therefore, become a focus for considerable chemical analysis aimed at trying to understand how artemisinins work.

3.1. Peroxides as "prodrugs"—putative action via reactive oxygen species

Simple peroxides such as *tert*-butyl hydroperoxide rapidly kill intraerythrocytic malarial parasites (*Plasmodium vinckei*) in mice and induce haemolysis, whereas there is no effect on erythrocytes in unparasitized mice (Clark et al., 1984). As peroxides are generally reactive entities, a link has been drawn between the mechanism of action of artemisinins and the generation of reactive oxygen species (ROS)—hydroxyl, alkoxyl, (protonated) superoxide or peroxyl radicals—within the parasitized erythrocyte. Recent studies suggest that haemolysis of infected erythrocytes caused by solutes such as sorbitol entering through novel permeation pathways may arise through oxidant stress of proteins also present in uninfected erythrocytes, and can be induced by exposure to *tert*-butyl hydroperoxide (Huber et al., 2002). Formation of ROS can be enhanced through the Fe^{2+}-dependent Fenton
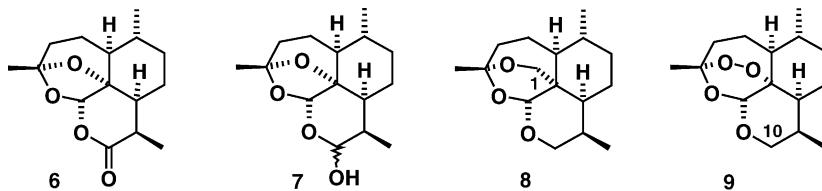
Fig. 2. The desoxy compounds **6** and **7** probably arise via non-metabolic breakdown of artemisinin mediated by reducing agents such as endogenous thiols. 1-Carba-10-deoxoartemisinin (**8**) has no antimalarial activity. In contrast, the 10-deoxo compound (**9**) with intact peroxide group is a more active antimalarial than artemisinin.

process, although intracellular antioxidant reserves (such as glutathione) can still be depleted directly by *tert*-butyl hydroperoxide, which will generate oxidized glutathione (GSSG) according to a well-established heterolytic process even without the presence of free Fe^{2+} (Halliwell and Gutteridge, 1999a). Conversely, desferrioxamine inhibits the parasiticidal activity of peroxides, presumably by scavenging the intracellular Fe^{2+} iron required for the Fenton process through its redox equilibration with Fe^{3+} and formation of an extremely stable Fe^{3+} complex. In line with the antiparasitic activity of *tert*-butyl hydroperoxide, other structurally simple peroxides were also found to possess moderate activities in vitro but were inactive in vivo (Vennerstrom et al., 1989). The general interpretation for parasite death and hemolysis based on these observations is that these are mediated by reactive oxygen species whose presence, greatly enhanced by the exogenous peroxide, eventually overwhelms the parasite anti-oxidant defense systems (Hunt and Stocker, 1990). A similar mode of action has been assumed for the artemisinins (Postma et al., 1996), although the exceptionally high in vitro activities of these compounds towards the malaria parasite argue against what is a relatively indiscriminate process.

3.2. Artemisinins as "prodrugs"—activation via C-centred radicals or formation of stable, antimalarial-active end products

Many workers consider that the artemisinins per se are not antimalarial, but rather act as 'prodrugs', requiring generation of antimalarially-active intermediates after encounter with ferrous iron. Translating the idea that Fe^{2+} is important in activating peroxides to artemisinin activation raises the question as to where Fe^{2+} is found in an infected erythrocyte. An obvious source of Fe^{2+} was considered to be the food vacuole of the parasite, where digestion of haemoglobin takes place and free iron is detoxified by deposition in haemozoin, an insoluble dimer of haem which forms hydrogen-bonded aggregates.

Accordingly, a large amount of work has been carried out on iron-mediated decomposition of artemisinins, and of totally synthetic trioxane analogues, either by using 'free' Fe^{2+}, or haem-Fe^{2+}. The purported aim has been to seek to demonstrate a connection between the structures of putative unstable intermediates such as carbon-centred radicals, or of stable end products, formed from these intermediates and an-

timalarial activity. The reactive intermediates are held to react with 'sensitive' or 'vital' biomolecules in the parasite, or the end products themselves are claimed to possess antimalarial activity in their own right. In other words, the artemisinins are considered to exert parasiticidal activity by an apparently 'random process', as opposed to high affinity binding of the intact artemisinin to an active site, prior to any 'activation'.

3.2.1. Activation by 'free' ferrous iron

In considering 'free' Fe^{2+}-decomposition of artemisinins first, we note that reductive cleavage of a peroxides by ferrous iron—the Fenton reaction—to generate O-centred, or alkoxyl radicals, and then C-centred radicals, and finally neutral products—is well understood, having been thoroughly examined before the advent of the artemisinins (for reviews see: Davies, 1961; Saito and Nittala, 1983; Sosnovsky and Rawlinson, 1971a,b; Matsugo and Saito, 1992a; Clennan and Foote, 1992). The detailed course of the decomposition of artemisinin and synthetic trioxane analogues with Fe^{2+} has been subject to an intensive examination and large amount of discussion, some of it much too speculative, in the literature (Posner and Oh, 1992; Posner et al., 1995; O'Neill et al., 2001; Hindley et al., 2002; Haynes and Vonwiller, 1996a; Wu et al., 1998; Li and Wu, 2003). The essential aspects in the case of artemisinin are illustrated in Fig. 3. Cleavage of the peroxide generates two different alkoxyl radicals, labelled the 'O-2' and 'O-1' radicals. The radical centre in the former is adjacent to an oxygen atom, and so is predisposed to undergo 'α-cleavage' (cleavage of the C3–C4 bond) to generate the 'seco' C-4 radical **10**. This decomposes via extrusion of Fe^{2+} to give the furan **12**, which has no antimalarial activity (Wu et al., 1998; Li and Wu, 2003). The O-1 radical abstracts the proximate α-disposed hydrogen atom from C-4 to generate the C-4 radical **11**. This is converted into the 4-hydroxo-desoxoartemisinin **14**, which also has no antimalarial activity, via the unstable epoxide **13**. Although this last compound was claimed to be a 'potent alkylating agent' and 'cytotoxic', and also to possess the remarkable ability to generate antimalarial hypervalent iron species (Posner et al., 1995; Posner and O'Neill, 2004), these claims have been refuted (Wu et al., 1998; Robert et al., 2002).

It is important to note that the overall conversion of artemisinin into products **12** and **14** does not involve any overall change in oxidation states of iron or of artemisinin, that is, these are isomerization reactions catalysed by Fe^{2+} which
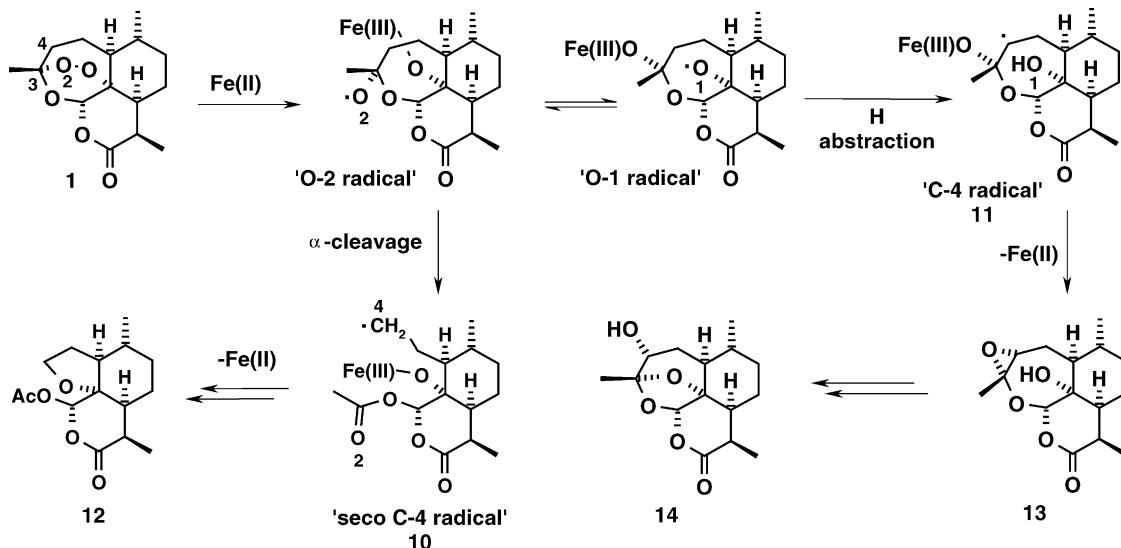
Fig. 3. Pathways proposed for activation of artemisinin by ferrous iron leading to the seco C-4 radical **10** and C-4 radical **11**, and the end-products **12** and **14**. Neither **12** or **14** possesses antimalarial activity, and thus antimalarial activity is claimed to reside in the radicals **10** and **11** by their reacting with 'sensitive biomolecules' in the parasite.

may also be effected in part by simply heating artemisinin to relatively high temperatures (190 °C for 10 min) in the absence of iron (Lin et al., 1985).

The C-centred radicals have been held to be important for antimalarial activity by their reacting with 'sensitive biomolecules'. Both types of radicals can be intercepted with spin-trapping agents, which provide longer-lived radicals capable of detection by ESR spectroscopy (O'Neill et al., 2001; Wu et al., 1998; Butler et al., 1998). When the Fe^{2+}-mediated decomposition of artemisinin or other artemisinin derivatives is run in the presence of cysteine or glutathione under aqueous conditions, the adducts **15–17** (Fig. 4), among others, are isolated in low yields; these are presumed to arise via entrapment of the C-4 radicals in rather complicated pathways held to involve ferrous-cysteine complexes in the case of cysteine (Wu et al., 1999; Wang and Wu, 2000; Wu et al., 2001; Wu, 2002; Wu et al., 2003). However, the trapping of thiols appears to be quite general—in the presence

of 2-naphthalenethiol, the adduct **18** (Fig. 4) may be isolated (Haynes et al., 2004). Unfortunately, there is no evidence that discrete radicals are involved in thiol entrapment, and the reactions are reminiscent of those examined much earlier involving ligand exchange from a redox-active metal ($Fe^{2/3+}$ and/or Cu^+/Cu^{2+}) to a cationic centre generated by oxidation of a C-centred radical proximate to the metal (Porter, 1992; Matsugo and Saito, 1992b).

3.2.2. Activation by haem ferrous iron

In turning to 'activation' of artemisinin by haem, we note that from the early trophozoite stage, the malaria parasite catabolises haemoglobin as a source of amino acids in an acidic, oxygen-rich lysosome-like digestive vacuole (Sullivan, 2002).

The catabolism releases ferriprotoporphyrin IX (haem), and the haem undergoes a non-enzymatic dimerization to form haemozoin, the insoluble, highly crystalline malaria
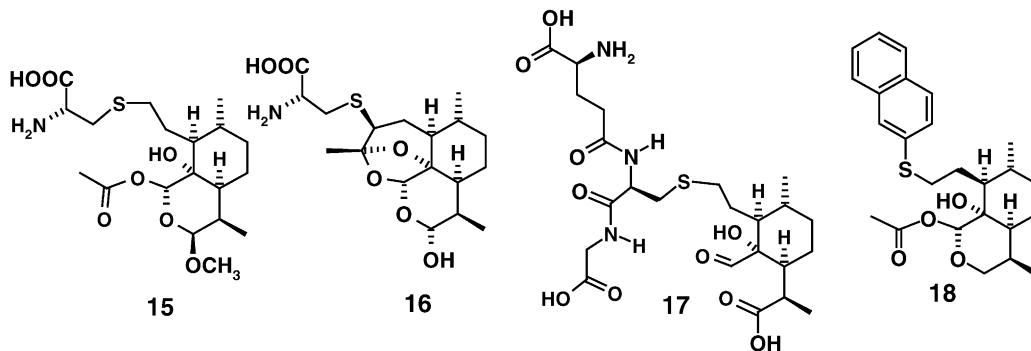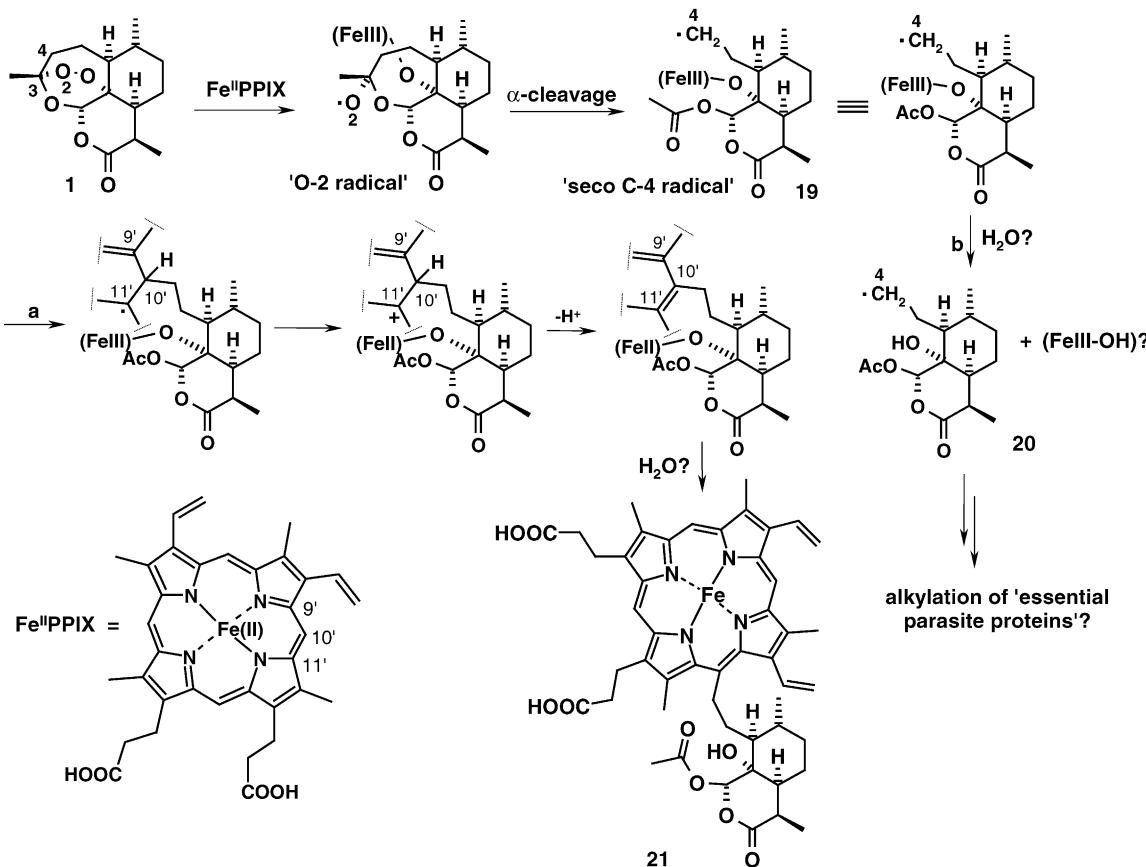

Fig. 4. Adducts formed from artemisinin, artemether or 10-deoxoartemisinin and cysteine, glutathione or 2-naphthalenethiol in the presence of Fe^{2+} under aqueous conditions. **15** and **16** are isolated from artemether (**3**) in 1–3% yield, **17** is isolated from artemisinin (**1**) in 1% yield, and **18** is isolated from 10-deoxoartemisinin (**9**) in about 15% yield.

pigment (Pagola et al., 2000). Virtually all of the haem is converted into haemozoin, and very little is degraded by other pathways (Egan et al., 2002). Of the total iron present in *Plasmodium falciparum* trophozoites, approximately 92% is located within the food vacuole, and of this, approximately 88% is in the form of haemozoin. Indeed, haemozoin is the only detectable iron species in trophozoites, and the haemozoin loading in the food vacuole is very high. The progenitor of haemozoin, namely haem, has been studied as an 'activator' of the artemisinins. [14C]-Artemisinin was found to interact with haem, either within the parasite, or in vitro in a medium containing ferriprotoporphyrin IX chloride at pH 7.5–7.8 (Meshnick et al., 1991). Convincing evidence for formation of a haem–artemisinin adduct was presented. It was established that haem has to be in the ferrous state in order to react with artemisinin, although the claim (Zhang et al., 1992) that the ferrous haem could induce catalytic decomposition of artemisinin could not be verified (Haynes and Vonwiller, 1996b). The haem–artemisinin adduct was found not to be toxic to the malaria parasite. One study also found that a substantial amount of [14C]-artemisinin incubated with malaria parasites became associated with the dimer (Hong et al., 1998); the nature of this interaction involving hemozoin has not been examined further.

In chemical studies, a covalent haem–artemisinin adduct was isolated in the high overall yield of 80–85% from reduction of ferriprotoporphyrin IX dimethyl ester under highly reducing conditions (Robert et al., 2001). However, the relevance to formation of haem–artemisinin adducts in vivo remains to be established. Mass spectra of the adducts isolated from incubation of artemisinin with *Plasmodium*-infected erythrocytes correspond approximately with the sum of molecular weights of the reactants (artemisinin and haem) (Meshnick et al., 1991) but little else is known of their structures. Several pathways that link the Fe^{2+}-mediated decomposition of artemisinin to alkylation of haem have been proposed (Robert et al., 2002). This type of haem alkylation was proposed to be important for antimalarial activity of the artemisinins in general. The proposal has been used as a basis to rationalize the relationship between antimalarial activity of synthetic trioxanes and stereochemistry of substituents on the periphery of the trioxanes, and to design a hybrid class of putative antimalarials referred to as trioxaquines.

If indeed the artemisinins function as antimalarials according to the haem activation idea, this requires juxtaposition of several events (Fig. 5):

Fig. 5. Proposed activation pathway for artemisinin by ferrous haem (Fe(II) protoporphyrin IX) leading to the seco C-4 radical **19** and alkylated adduct **21** (pathway a), or by presumed loss of the seco C-4 radical **20** from the haem (pathway b) to alkylate 'essential parasite protein' ((FeII)- and (FeIII)-: ferrous and ferric haem residue).

i. encounter of artemisinin with the reduced (Fe^{2+}) haem in the food vacuole, whose intercession between breakdown of haemoglobin and formation of hemozoin has never been unequivocally demonstrated, and must be transient at best, given the lack of a reducing environment within the food vacuole;

ii. binding of the peroxide to the haem iron atom—in the Fe^{2+} state—in order to induce inner sphere electron transfer;

iii. generation of the seco C-4 radical **19**;

iv. dissociation of the oxygen ligand of the seco C-4 radical from the Fe^{3+} in the haem;

v. escape of the resulting 'free' seco C-4 radical **20** from the environment of the ferric haem;

vi. migration and encounter of the free seco C-4 radical with an 'essential parasite protein'.

The inner sphere electron transfer involves the iron atom being coordinated to the oxygen atom throughout the decomposition process. Therefore, ligand exchange with a putative external ligand (water?) is required to release the radical from the haem (pathway b, Fig. 5) more rapidly than the very fast intramolecular reaction between the radical and the haem. Not surprisingly, it has not proved possible to trap the seco-C4 radical **20** with cysteine, when this is used to reduce ferric haem to the ferrous haem required for generation of the radical.

Alternative explanations put forward were that either the haem–artemisinin adduct itself will inhibit formation of hemozoin (Robert and Meunier, 1997; Robert and Meunier, 1998), or that the C-4 radical alkylates the histidine-rich protein (HRP) presumed to catalyze formation of hemozoin (Sullivan, 2002; Papalexis et al., 2001). Both events will allow build up of haem monomer, which is toxic to the parasite by virtue of pro-oxidant activity and/or interaction with essential proteins in the food vacuole.

3.3. Problems with the 'free' ferrous iron and ferrous haem-activation and reactive intermediates hypotheses

The fitness of the C-centred radicals derived from the artemisinins and synthetic trioxanes as parasiticidal agents must be questioned for the following reasons:

i. These radicals, substantially less reactive than O-centred radicals, react at a diffusion controlled rate with oxygen, and are reduced by thiols. They are normally oxidized by high-valent metal ions, such as Fe^{3+}, to carbocations. The C-centred radicals formed from unsaturated fatty acids and lipids through abstraction of hydrogen atoms by ROS, or from xenobiotics, for example carbon tetrachloride, by reduction or other metabolic pathways, react with oxygen to form peroxyl radicals (Halliwell and Gutteridge, 1999b). These initiate chain processes resulting in eventual formation of breakdown products such as malondialdehyde from lipid peroxidation. Further, the environment within a parasitized erythrocyte,

with the greatly enhanced recycling of ROS and greatly enhanced levels of lipid peroxidation, is not a reducing one at all. Therefore, to expect that a carbon-centred radical will survive such a hostile environment for it to be 'cytotoxic' in its own right, in other words, to migrate from its point of formation to react with 'sensitive' or 'vital' biomolecules in the parasite, is physically unrealistic.

ii. The C-centred radical hypothesis is difficult to countenance in the face of antimalarial data for secoartemisinin and carba analogues of artemisinin (Fig. 6). Whilst compound **22** possesses approximately 10% of the activity of artemisinin, compound **23** is equipotent with artemisinin. Compound **24** possesses approximately the same activity as compound **22**. In all cases, cleavage of the peroxide by Fe^{2+} will result in alkoxyl radicals which are no longer constrained to be in the proximity of carbon atoms bearing abstractable hydrogen atoms (cf. Fig. 3) (Avery et al., 1990; Avery et al., 1994). The carba analogues **24** and **25** of artemisinin (**1**) and 10-deoxo-10-dihydroartemisinin (**9**) possess approximately 3 and 24% of the antimalarial activity of artemisinin, yet these are clearly capable of generating C-centred radicals (Avery et al., 1996a). Indeed, as the compound **25** reacts cleanly with Fe^{2+} to generate products, which apparently arise via a C-4 radical, the expectation of antimalarial activity is the reverse in fact of what is observed. These splendid examples provide strong argument for the irrelevance of the C-centred radical idea as a basis for antimalarial activity.

iii. It is established that dihydroartemisinin derivatives bearing various amino groups at C-10 react sluggishly with Fe^{2+} in 1:1 acetonitrile-water, even though these are extremely active antimalarials (Fig. 5) (Haynes et al., 2004). Further, it has been shown that 10-deoxo-10-dihydroartemisinin (**9**), an active antimalarial, is completely decomposed by Fe^{2+} under the foregoing conditions. In other words, there is no correlation with the ability of artemisinin derivatives to react with Fe^{2+} to generate the putative C-centred radicals, and antimalarial activity.

iv. Other non-peroxidic starting materials, which can generate C-centred radicals on treatment with Fe^{2+}, namely oxaziridines, are either inactive, or possess in vitro antimalarial activities in the $\mu g\,mL^{-1}$ range (Ningsanont et al., 2003).

There are also problems and inconsistencies that cannot be easily accommodated by the haem activation hypothesis.

i. The haem–artemisinin adduct generated within infected erythrocytes has no antimalarial activity in vitro, and the currently-used artemisinin derivatives do not inhibit formation of hemozoin in parasites in infected erythrocytes (Asawamahasakda et al., 1994). Aside from the difficulty of alkylation of the protein by the radical, it is uncertain if HRP plays any essential role in the dimerization of the haem.
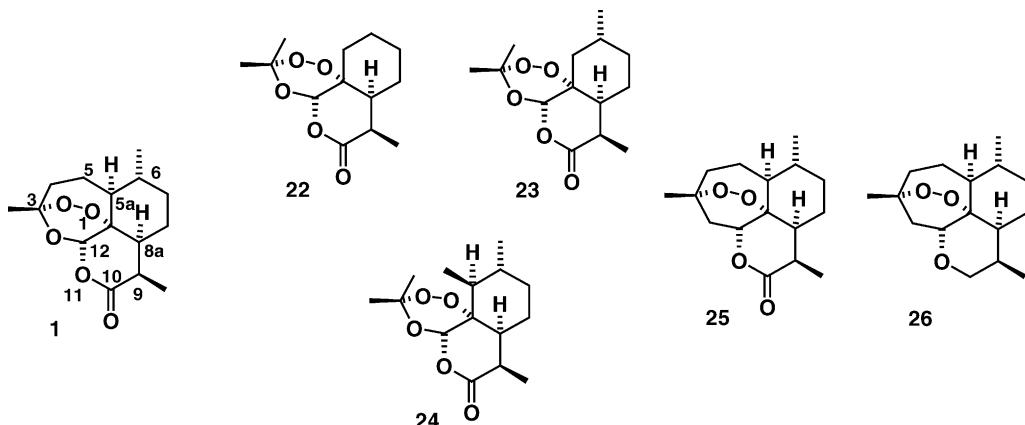
Fig. 6. Totally synthetic 4,5-secoartemisinin derivatives possess antimalarial activity, but are less likely to generate carbon-centred radicals once the peroxide bridge is cleaved. Relative to artemisinin **1** [IC_{50}: 100% W2, D6], compound **22** 6% W2, 20% D6; compound **23** 75% W2, 108% D6; compound **24** 14% W2; 7% D6. Conformational effects in the seco compounds, which will be conformationally more mobile than artemisinin, are likely to influence activity. The carba-analogues **25** and **26** relative to artemisinin **1** [IC_{50}: 100% W2, D6], compound **25** 3.8% W2, 2.5% D6; compound **26** 16% W2, 32% D6. These data indicate that the presence of the third non-peroxide oxygen atom is required for optimal activity.

ii. There are artemisinin derivatives that are chemically unable to react with haem, but are very potent antimalarials (Haynes et al., 2004). 10-Deoxoartemsinin **9** is an example that is more stable than artemisinin and dihydroartemisinin under aqueous conditions, and therefore, less likely to bind to haem. 10-Deoxoartemisinin does not interfere at all with hemozoin formation, which conclusively shows that inhibition of β-hematin in the haem polymerisation inhibitory activity assay does not relate to antimalarial activity, and that furthermore, binding to F(III)PPIX is unnecessary for antimalarial activity (Haynes et al., 2003). Whilst these in vitro assays do not necessarily represent what is happening in vivo, they do suggest that alternatives should be sought to the idea that artemisinins act by inhibiting haem polymerisation, as 10-deoxoartemisinin cannot react with reduced haem. Also, the K_d for haem–artemisinin interactions is outside of the nanomolar range ($IC_{50} > 10\,\mu M$ for artemisinin) (Pandey et al., 1999).

iii. We have noted previously that artemisinin derivatives do not inhibit, but rather are potent inducers of, Phase I-metabolizing P-450 (CYP) enzymes, in which the prosthetic haem shuttles though the ferrous and ferric states, and hydroxylates the periphery of the artemisinin derivatives on the same side as the peroxide without interfering with it (Haynes, 2001).

iv. There has been an implicit assumption that the target organelle of the artemisinins is the food vacuole. In contrast to the study quoted above, the localisation of artemisinin in living parasites is not associated with the food vacuole (Eckstein-Ludwig et al., 2003), and in one study, only 13–15% of radiolabelled artemisinin is found in the haemozoin fraction after parasites are exposed to this tracer (Asawamahasakda et al., 1994) Artemisinins act against tiny ring stages of parasite development, both in vitro (ter Kuile et al., 1993) and in vivo (Angus et al., 1997). Yet these stages do not contain haemozoin, once again suggesting that the food vacuole is not critical for antimalarial activity of artemisinins.

A fundamental point here is that even if chemical reactions can be shown to occur in vitro, this still requires a demonstration of the relevance of these reactions in vivo. Further, the prodrug hypothesis would require that stable end-products resulting from decomposition of an antimalarial-active peroxide in a laboratory study be demonstrated to possess antimalarial activity. Unfortunately, very little work to this effect has been carried out, and unsupported statements asserting antimalarial activity of this or that ketone, epoxide, or other 'electrophilic' product continue to appear.

3.4. Labelling of parasite proteins by artemisinins

A decade ago, Meshnick and colleagues demonstrated that parasite proteins could be labelled and extracted after exposure to radiolabelled artemisinins (arteether and DHA) (Asawamahasakda et al., 1994). Most radiolabel taken up ($\sim$60%) was found in an SDS-soluble fraction, and in trophozoite stages of parasite development. Four major protein bands were labelled (25, 50, 65 and >200 kDa), although competition for this labelling with unlabelled artemisinin was not carried out to control for specificity of this interaction. Subsequent identification of one, probably covalently labelled band, has been achieved and the protein found to be the Translationally Controlled Tumour Protein (TCTP) orthologue of mammalian and other cells (Bhisutthibhan et al., 1998). The function of TCTP in parasites is unknown, and the importance of binding of artemisinins to this protein in their mechanism of action has yet to be defined.

4. An alternative hypothesis

4.1. Binding of the intact molecule to an active site.

Some time ago, it was proposed that artemisinins may be 'activated' for antimalarial activity after complexation, or binding of the intact molecule, into an active site (Haynes and Vonwiller, 1996a; Haynes et al., 1999; Olliaro et al., 2001a,b). One crucial reason for this is that in vitro anti-malarial activity is sensitive to steric effects which cannot be accommodated within a carbon centred radical scenario. Replacement of the methyl group at C-3 of artemisinin (**1**) and 10-deoxodihydro-artemsinin (**9**) by much larger groups, for example, phenylethyl, and including groups which are held to stabilize radicals, in artemisinin results in diminution in activity (Avery et al., 1996b). Groups at C-4, C-5 or C-12 in artemisinin analogues which are disposed on the same side as the peroxide markedly attenuate activity. In artemisinin itself, inversion of configuration at C-9 such that the methyl group is now on the same face of the molecule as the peroxide also attenuates its activity. (A comprehensive summary of structure–activity relationships, including comparison of antimalarial activities of artemisinin derivatives and analogues relative to artemisinin appears in Avery et al., 2002.) A relatively more subtle, but crucial aspect is displayed by the secoartemisinin analogues, as described above (Fig. 6). As pointed out above, compound **23** is approximately equipotent as an antimalarial with artemisinin. In lacking the carbocyclic system in artemisinin, all compounds are likely to be conformationally more mobile than artemisinin, but variation in the degree of methyl substituents will further affect conformational mobility in each of compounds **22**–**24** (Avery et al., 1990; Avery et al., 1994). This will be reflected in the difference in antimalarial activities of these compounds.

The nature of the 'activation' process which ensues upon complexation was held to be fundamentally different to the random activation hypotheses described above. The carba analogues **25** of artemisinin (Fig. 6) have a 3D structure superimposable upon that of artemisinin, yet it possesses 3% of the activity of artemisinin (Avery et al., 1996a). In order to account for this, it was argued earlier that the third non-peroxidic oxygen atom was required to stabilise a cationic intermediate produced by ring opening of the intact peroxide, once it is activated by acting as a hydrogen bond acceptor, or by electrostatic interaction with an electrophilic site at the receptor site (Haynes et al., 1999). However, it may match better with a putative hydrophilic part of the binding pocket, or act as a hydrogen-bond acceptor required to align the molecule in the binding pocket.

4.2. Proposal for the active site

More recently, an alternative hypothesis for the mode of action of artemisinins has been proposed, based on structural similarities between the sesquiterpene moieties of thapsigargin and in artemisinins. Thapsigargin (another plant product
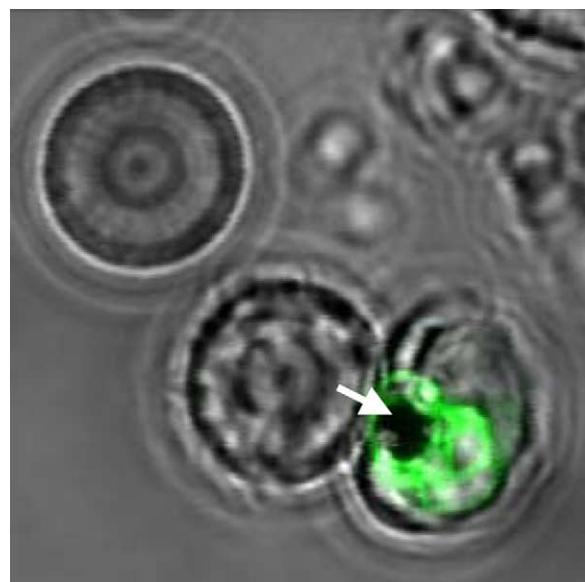

Fig. 7. Localisation of PfATP6 by labelling with BODIPY-thapsigargin. The arrow indicates the food vacuole.

from *Thapsia garganica*) is an extremely potent inhibitor of Ca^{2+}-transporting ATPases (sarcoplasmic reticulum Ca^{2+}-transporting ATPases or SERCAs) from a wide variety of organisms. It was suggested that artemisinins may act in a similar way, but more specifically to inhibit the SERCA of malarial (PfATP6) but not mammalian pumps (Eckstein-Ludwig et al., 2003). Evidence in favour of this hypothesis includes demonstrated specificity for inhibition of the SERCA of *P. falciparum*, excellent correlation between assays for inhibiting PfATP6 and killing of parasites, competition between thapsigargin and artemisinins, and also appropriate Fe^{2+}-dependency for inhibition of PfATP6. The localisation of PfATP6 was assayed by visualizing fluorescent thapsigargin in living parasites (Fig. 7). Whilst there are many aspects of this hypothesis that are urgently being examined, it provides an alternative that is amenable to testing that relies both on chemical and molecular biological expertise. Identification of PfATP6 as a target for artemisinins will also permit assessment how changes in this sequence can contribute to drug resistance in parasites that have been exposed to artemisinins in vivo.

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