Safety assessment of peroxide antimalarials: clinical and chemical perspectives

B. Kevin Park, Paul M. O’Neill1, James L. Maggs & Munir Pirmohamed
Department of Pharmacology and Therapeutics, University of Liverpool, Ashton Street, Liverpool L69 3GE and 1Department of Chemistry
University of Liverpool, Ashton Street, Liverpool L69 3BX

Introduction

Peroxides represent a new chemical class of antimalarial drugs (Figure 1). The first generation compounds are all derivatives of artemisinin, the active principle of a Chinese herbal remedy \[1\]. Artemisinin, or qinghaosu (green plant extract), is extracted from *Artemisia annua*, a plant which grows widely in South China. The first generation compounds currently in use are derivatives of the lactol, dihydroartemisinin, which is a major metabolite for each of the derivatives, and may be the most important chemical species for antimalarial activity in *vivo*. The promise of these compounds lies in their effectiveness against chloroquine-resistant parasites and their rapid action against malaria. Moreover, they have been used for the treatment of malaria in three million patients with few reports of clinically significant toxicity \[2\].

The drawback with artemisinin and related compounds is that they suffer from poor bioavailability: the relative bioavailability of oral artemisinin (vs intramuscular) and intramuscular artether (vs oral) and the bioavailability of artesunate are all within a range of 15–30% \[3–5\]. This may be one of the reasons for the relatively high rate of recrudescence associated with peroxide monotherapy. Their complex chemical structures are not essential for pharmacological activity and there has, therefore, been a drive to design and synthesize simpler endoperoxides which have greater in *vivo* potency. To date, more than 1000 new endoperoxides belonging to several chemical classes have been prepared. A number of ‘second generation’ peroxides have been developed, including arteflene (Figure 1). This is a synthetic peroxide developed from *Artemisia annua*, the complex active constituent of the Chinese plant *Artemisia annua*, after it had been shown to have antimalarial activity \[6\]. It has been tested in clinical trials in Africa.

The ‘first generation’ artemisinin derivatives, as described above, are chemically simple esters and ethers of the parent lactol, dihydroartemisinin (Figure 1) \[7, 8\]. These compounds are generally more potent than artemisinin both in *vivo* and *in vitro*, and provide a wider choice of formulations which are more easily administered to patients in the field. Artether and arteether are more oil soluble than artemisinin and are currently undergoing clinical trials under WHO sponsorship \[8\]. Other first generation derivatives include sodium artesunate, the DHA 3-O-succinate half ester sodium salt, and sodium artelinate, the corresponding DHA 3-O-p-carboxybenzyl ether sodium salt. Both of these compounds, which are water soluble, are currently under investigation as intravenous treatments for severe malarial infections \[8\].

Given the obvious pharmacokinetic limitations of the

'first generation' analogues and the realization that the peroxide bridge is the essential pharmacophore for biological activity, medicinal chemists have developed a range of second generation derivatives. Representative compounds include the bicyclic fenozan type derivatives, synthesized by Jefford et al. \[9\], the tetroxanes synthesized by Vennerstrom \[10\] and tricyclic simplified derivatives prepared by Pouner and coworkers \[11\]. The discovery that several of the first generation artemisinin derivatives are metabolised extensively to DHA has prompted other workers to prepare C-10-carboxybenzyl ether sodium salt, and sodium artesunate, the DHA 3-O-p-carboxybenzyl ether sodium salt. Both of these compounds, which are water soluble, are currently under investigation as intravenous treatments for severe malarial infections \[8\].

The purpose of this review is to address each of these questions from chemical and clinical perspectives.

Clinical efficacy and safety

Numerous studies have shown that artemisinin compounds have several advantages over other antimalarial drugs: these include the more rapid clearance of parasites in the periphery \[2\], more rapid resolution of fever \[13, 14\], and a reduction in the transmission potential of *falciparum* malaria \[15\]. Despite these apparent advantages, no study has yet shown a beneficial effect of artemisinin compounds on survival, although this may largely be due to the fact that most studies performed so far have been under-powered to detect...
B. K. Park et al.

Figure 1  Chemical structures of ‘First’ and ‘Second’ generation peroxides. The fenozan, tetroxane and tricyclic trioxanes are totally synthetic compounds prepared from readily available starting materials. The first generation DHA derivatives and the carba derivatives (e.g. C-10-Carba) are compounds prepared directly by manipulation of the parent drug artemisinin.

Despite their extensive use, there have been very few reports of clinically significant toxicity with the peroxide exceptive against Plasmodium falciparum strains resistant to other antimalarials [17], and there have been no reports of parasite resistance [2, 7]. The artemisinin compounds have become first-line drugs in several regions in South-east Asia. The main clinical disadvantage of artemisinin and its derivatives is the high rate of recrudescence when compared with other antimalarials [2]; the reasons for this are unclear, but partly reflect the low bioavailability and short half-lives of these compounds. In addition, it has been found that artemisinin areas under the plasma concentration curves decrease during repeated drug administration, indicating that auto-induction of hepatic drug-metabolising enzymes may contribute to incomplete parasite clearance [18]. In vivo potency with respect to parasite elimination is also a problem with second generation peroxides. A comparison of single dose therapies in children with uncomplicated P. falciparum malaria in Gabon showed that arteflene was ineffective in contrast to mefloquine, which proved to be highly effective [19].

© 1998 Blackwell Science Ltd
of 82 patients receiving artesunate, and 3 of 39 patients receiving artether [2]. In a recent study, it was demonstrated that 25% of 284 adults treated with intramuscular artether for severe falciparum malaria had a QTc interval of more than 0.5 s [22]. However, it is possible that this was caused by the disease rather than the treatment [23].

Given the findings in animals (see below), the major concern with this class of compounds has been their potential to cause neurotoxicity. Clinical trials to date have not identified any cases of neurotoxicity [2] which can definitely be attributed to the artesinin compounds. However, the recent reports of a prolonged length of time to recovery from coma (when compared with patients treated with quinine) in Vietnamese [22] and Gambian [24] patients, and an increased frequency of seizures in artether-treated patients in Gabon [24], have raised concerns of the neurotoxic potential of these compounds [25]. In addition, there has been one report of the occurrence of an acute cerebellar syndrome in a 36-year-old previously healthy man after five doses of artesunate, the features of which were still present (although improved) 4 months later [26]. Clearly, one such report does not constitute proof of a causal association [27], but the possibility that repeated administration of artesinin compounds in malaria endemic areas will result in cumulative damage cannot be ruled out [2, 28], especially since these drugs are often freely available for use in tropical countries [29]. Another possibility that cannot be discounted at this stage is that some patients may have suffered subclinical neurological toxicity which would have required sophisticated neurological and neuropsychological tests for detection. The potential of the drugs to cause neurotoxicity is discussed in greater detail below.

Fear of potential toxicity has limited the use of the artesinin derivatives in pregnancy since animal studies have documented teratogenicity when the drugs are adminstered at high doses [30]. At present documentation of the safety of artesinin derivatives in the treatment of malaria in pregnant women is limited and it has therefore been recommended that these drugs should be used in pregnancy only as a last resort [31]. Thus, there is a need to develop better peroxide compounds, the ideal properties of which would be (a) a pharmacological profile which allows for shorter durations of treatment with fewer doses, (b) greater bioavailability to ensure sufficient parasite exposure, (c) a predictable pharmacokinetic profile such that the drug is retained for sufficient lengths of time to ensure that it kills the parasites, but does not accumulate to any extent to cause damage to the host, (d) low interindividual pharmacokinetic variability and (e) lack of toxicity. Rational molecular design must be based on both structure-activity and structure-toxicity/metabolism relationships.

Mechanism of action of peroxide antimalariais: role of parasite bioactivation

The essential chemical features required for pharmacological activity are simply a peroxide moiety, which is sterically hindered, and in the case of many second generation peroxides a lipophilic aromatic ring system (Figures 1 and 2). Artesinin contains a peroxide bridge within a more complex 1,2,4-trioxane structure. A comparison with the second generation peroxides indicates that the 1,2,4-trioxane structure per se is not essential for pharmacological activity in the parasite, although it has not yet been established whether the overall structure provides (a) ‘protection’ for the host by restricting the intracellular (e.g. in the nervous system) bioactivation of the peroxide, and (b) alternative routes of metabolism in man (i.e. direct detoxication). Artether undergoes several hydroxylations in vivo, most of which lead to a decrease in pharmacological activity [32]. Dihydroartesinin undergoes extensive glucuronidation [33], which again results in loss of activity [34].

The parasite digests haemoglobin to obtain amino acids but releases haem, a potentially toxic species (Figure 3). Haem is detoxified in the parasite by the formation of a nontoxic but not entirely unreactive polymer, the malaria pigment, haemozoin. Free haem catalyses the conversion of peroxides into free radicals and other electrophilic intermediates which alkylate haem and specific parasite proteins, and thereby it is supposed, kill the parasite [35, 36]. As well as the formation of carbon-centred radicals there is also the formation of a high valent iron-oxo species (FeV=O) (Figure 4).

In vitro studies have shown that all endoperoxide antimalarial compounds share common targets with respect to protein alkylation [35]. It has been demonstrated that dihydroartesinin associates with parasite membranes, haemozoin and mitochondria; damage occurs in the mitochondria, rough endoplasmic reticulum and the nuclear envelope, and there are also changes in the nucleus, ribosomes and food vacuole [36].

Antioxidants such as catalase, dihydroartether and α-tocopherol have been shown to antagonise the antimalarial action of artesinin [37]. The role of iron was defined in experiments which showed that iron chelators antagonise the antimalarial activity of an artesinin derivative, artether [38].

Toxicology in animal models

Undoubtedly the major concern raised by preclinical studies in animals is that of the neurotoxicity which has been observed in rats, dogs [39, 40] and more recently in monkeys [41]. It seems to be dose-dependent and has occurred at relatively high doses (20–50 mg kg−1 day−1); the currently recommended dose for both artether and artesunate in man is 10 mg kg−1 day−1. However, relative systemic exposure to drug in man and in the animal models has not been determined. A progressive syndrome of clinical neurological deficits with cardio-respiratory collapse (with prolongation of the QTc interval) and death was noted in dogs dosed for 8 days with artether (20 mg kg−1). Prominent neuropathologic lesions were limited to the pons and the medulla [39]. In the rat, brain stem histologic lesions have been seen in the absence of neurologic signs or deficits in behavioural performance [1]. Toxicity has been confirmed in vivo in Neus2a neuroblastoma cells; these cells took up much less dihydroartesinin than P. falciparum-infected red blood cells, suggesting that the compounds may show selective toxicity for the parasite in vivo. As pointed out earlier, despite many years of use there is little evidence...
Figure 2 Structural features required for activity. In both peroxide drugs, C-centred radicals can be formed following iron (II)-mediated one-electron reduction by haem and subsequent rearrangement of the initially formed oxyl radical (see Figure 5 for more detail). In arteflene, the crucial endoperoxide pharmacophore is linked to a lipophilic bistrifluorinated ring system as shown.

Figure 3 General scheme for the mechanism of action of antimalarial peroxides. The critical step for activity of the drug is believed to be the formation of a C-centred radical that is proposed to kill the parasite by alkylation.

Figure 4 Chemical details of the iron-mediated bioactivation reactions of artemisinin that are believed to be critical for antimalarial activity. At the top of the scheme, the initially formed oxyl radical rearranges by a 1,5-H shift to produce a C-centred radical that is then ultimately transformed into the hydroxy deoxy derivative (HODEOXART) as shown. Note that this pathway may also produce a high valent Fe(IV)=O species which is proposed to oxidatively damage cellular macromolecules [52]. At the bottom of the scheme, association of Fe(II) with O1 and scission of the alkyl bond produces a primary radical, which in the absence of a protein or porphyrin acceptor is further transformed into the THF-acetate derivative as shown.
of neurotoxicity in man. An opportunistic postmortem examination of the brain stem of a patient who took single-dose artemesin showed no abnormalities [42]. Artemisinin derivatives have been shown to be toxic to neutrophilic cells in vitro, as measured by either radiolabelled precursor incorporation or neurite outgrowth. Toxicity is specific to neuronal cells and does not affect glial cells [43]. Structure-toxicity relationships indicate that the chemisis of the –ring is an important determinant of in vitro toxicity, with dihydroartemisinin being considerably more toxic than other derivatives [43, 44]. However, whether this toxicity derives from an inherently more reactive peroxide group or the formation of neurotoxic ketones through the rapid isomerization of dihydroartemisinin that occurs in the presence of iron (II), has not been defined (Maggs et al. unpublished). Addition of haemin to in vitro systems can markedly potentiate the neurotoxicity of peroxides indicating a role for iron-catalysed radical formation [45]. Arteflene (Figure 1) is a synthetic peroxide which, as mentioned earlier, was brought forward for preclinical and clinical evaluation from a series of yinghaosu A derivatives [6]. The compound was selected on the basis of its greater chemical stability than artemisinin and thus its anticipated improved pharmacokinetic properties. Arteflene was found to be negative in a battery of mutagenicity tests and did not produce any neurotoxicity when given in high doses to dogs (up to 700 mg kg⁻¹). However, reproductive studies revealed clear embryotoxic effects (37–57% intrauterine deaths) in rats at 20 mg kg⁻¹ and a slight effect in rabbits at 40 mg kg⁻¹. Autopsy examination of the foetus showed the presence of blood vessel anomalies and fused sternebrae in the 20 mg kg⁻¹ dose group [30]. The embryotoxicity was comparable with the effects seen with artemisinin. Pregnant rats given extremely high doses (up to 3000 mg kg⁻¹) of artemisinin within 6 days of gestation had normal foetuses; however, the drug caused foetal death and resorptions in rodents, even at relatively low doses when given after the sixth day of gestation [46, 47]. There was no evidence of teratogenicity in the offspring that survived organogenesis. No such abnormalities have been reported in man, but the safety data are extremely limited [30]. While such effects may not be considered a handicap for possible use in the treatment of malaria [6], such findings must necessarily preclude the use of these compounds for prophylaxis in women unless specific contraceptive precautions are undertaken.

The acute toxicity of artemisinin in mice which involves the gastrointestinal tract is antagonised by an iron chelator [38], indicating a role for iron-catalysed bioactivation of the peroxide group. This may reflect the availability of free iron in the gut. However, the role for peroxide bioactivation in particular, and metabolism in general, in chronic toxicity (neurotoxicity and reproductive toxicity) is not known. Since neurotoxicity is not observed with arteflene [6], this would suggest that the toxicity when observed after administration of artemisinin and its derivatives is not simply a function of the presence of a peroxide group. In vitro studies with neutrophilic cells have shown that iron-catalysed activation of artemether significantly enhances the neurotoxicity of artemether with concomitant formation of a rearrangement product, artemether tetrahydrofuran acetate [48]. The antiparoxysmal anticonvulsant and glutathione afforded protection against the toxicity. Although it is thought that products of iron-catalysed bioactivation are responsible for both the parasite and mammalian toxicity of peroxides, it is also possible that the radicals might damage iron-containing proteins and thereby release iron which could be a cause of oxidative damage [49]. Iron is thought to be a crucial factor in reactive oxygen metabolite-induced cytotoxicity. In order to induce lipid peroxidation, superoxide and/or hydrogen peroxide must react with iron to generate hydroxyl radicals via the iron-catalysed modified Haber-Weiss reaction [50, 51]:

\[
\begin{align*}
    & \text{Fe}^{2+} + \text{O}_2 \rightarrow \text{Fe}^{3+} + \text{O}_2^- \\
    & \text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{OH}^- + \text{OH}^+ \text{ (Fenton reaction)}
\end{align*}
\]

Thus, it is possible that the disposition of iron at both the cellular and molecular levels might be intimately involved in the bioactivation of peroxides and their subsequent biological effects.

Biomimetic Fe(II) catalysed reactions and biomarkers of bioactivation

As mentioned earlier, the malaria parasite *Plasmodium falciparum*, in the intracellular stage of its life cycle, digests haemoglobin, which results in the generation of ferryltropoloporphyrin IX (haem). Since ‘free haem’ is toxic to the parasite, it is normally removed by polymerization to haemoxyn, an insoluble pigment. A number of model studies utilizing iron (II) salts and haem iron (II) have been reported for artesinale [36] with several intermediates being proposed as the ultimate cytotoxic species (Figure 4). Association of either of the peroxide-oxigenyl in the trioxane skeleton produces oxyl radicals that can either participate in a 1,5-H-shift or carbon C-C bond scission to produce a carbon-centred radical species. Also implicated in the mechanism of action of artesinale is the formation of a high-valent iron-oxo species produced by -scission of the C-C radical intermediate (X) as shown [52] (Figure 4).

More recently, we have proposed a mechanism of action for artesinale based on similar mechanistic studies using Fe(II) salts [53]. Arteflene, on reaction with FeCl₂·4H₂O, yielded two products, the two-electron reduction diol product and the enone, a surrogate marker for the formation of the cyclohexyl radical (Figure 5).

The formation of the THF acetate from artemisinin and the enone products from artesinale may be effective surrogate markers of bioactivation. However, further work is required to determine whether the generation of these entities in a mammalian system leads to radical-induced toxicity. In theory, such radicals might cause toxicity which would be partly determined by the availability of iron (II) and its tissue distribution.

Two distinct (but not exclusive) mechanisms for the iron-mediated activation of peroxides in mammalian tissues are envisaged. Firstly, simple non-enzymic reactions, involving free iron and/or iron bound to protein, although the chemical reactivity of peroxides with iron associated with,
for example, transferrin has not been defined. Secondly, reactions catalysed by haemthiolate enzymes, and specifically by the microsomal cytochrome P450 enzymes [54] and the prostacyclin and thromboxane synthases [55]. The synthases, in particular, catalyse isomerizations of endogenous prostaglandin H2 endoperoxide which are analogous in detail to the iron-mediated isomerizations of artemisinin.

**Disposition of peroxides in relation to human safety**

The main safety concern for present and future peroxide antimalarials is whether the peroxide pharmacophore can act as a toxicophore and if so, under what circumstances could this occur. At present, little is known about the metabolic fate of peroxides. Important questions which need to be addressed are:-

- does the peroxide bridge undergo bioactivation in vivo in mammalian cells?
- what are the biological consequences of bioactivation?
- is the development of peroxides with longer half-lives, for more sustained antimalarial activity, likely to be associated with a greater risk of toxicity?
- can prolonged exposure to free radicals exhaust the scavenging or repair systems resulting in host cell damage and if so, can drugs with longer half-lives be (clinically) more toxic?
- In order to address these issues, it is necessary to define all the major and minor routes of metabolism. Of particular relevance are metabolites (including rearrangement products and fragments) which indicate bioactivation of the peroxide group, and also metabolites which signal the formation of potentially toxic species such as aldehydes and enones. However, peroxide antimalarials were introduced into clinical use without any formal knowledge of either disposition or metabolism [56] because of the lack of suitable analytical techniques. Thus, assessment of the risk to man of findings in animals by normal pharmacokinetic analysis was precluded. Furthermore, the importance of development age and disease with respect to toxicological risk had not been defined.

Recently, specific and sensitive h.p.l.c and LC-MS methods [3, 33, 57] have been developed for the analysis of peroxides in plasma, and for the characterization and quantification of metabolites. Following intravenous administration, artesunate is rapidly (t1/2 ~ 3 min) hydrolysed to dihydroartemisinin which in turn has a relatively short half-life of only 40 min [4]. In both the rat [33] and man (Maggs et al. unpublished), the principal pathway of metabolism for dihydroartemisinin (Figure 6) is glucuronidation. It is possible that the rate of glucuronidation may serve to limit the therapeutic effectiveness of the drug with respect to disease recrudescence, as glucuronides are inactive [34], but it may also preclude the neurotoxicity observed with high doses of first generation peroxides in the rat and dog by preventing isomerization of DHA to ketone intermediates which are potentially neurotoxic (although so far only observed in in vitro systems). There is no evidence for detoxication of the peroxide group per se, by reduction, for example, as has been described for prostaglandin peroxides [58].

A key question is whether bioactivation of peroxides catalysed by iron occurs in vivo during therapeutic administration. The body takes great care to safely sequester iron and copper in non or poorly reactive forms [59]; the body store is divided between essential iron-containing compounds and excess iron which is held in storage [60]. Iron disposition is extremely tightly controlled, with only 10% of total stores being excreted per year. The major form of essential iron is haemoglobin, although there are a variety of haem-dependent enzymes including the cytochrome
Figure 6 Chemical mechanisms for the formation of metabolites derived from dihydroartemisinin (DHA). DHA can undergo either direct glucuronidation or Fe(II)/Fe(III)-catalysed reactions, as shown, to give deoxydihydroartemisinin (DEOXDHA) and a ring contracted derivative (DHATHF).

P450 family. Ferritin is the intracellular storage protein, and may contain 4000 atoms of iron (III) per ferritin molecule. Aggregated ferritin (haemosiderin) constitutes about one third of normal stores. The two predominant sites of iron storage are hepatocytes and the reticuloendothelial system. Internal distribution and disposition of iron is facilitated by the plasma protein transferrin, which has two binding sites for ferric iron. Iron is delivered to intracellular sites via specific transferrin receptors and subsequent endocytosis; iron is then released in the endosome.

Iron can catalyse the rapid bioactivation of all peroxide antimalarials to chemically reactive radical intermediates [36]. However, this ability depends upon the regeneration by using specific metabolites as biomarkers. Parallel in vitro studies can be performed to define the role of disposition in toxicity and the chemical species which is responsible. Such information would allow extrapolation of the toxicities observed in animals to man.

Conclusions
Malaria remains a major health problem world-wide, with more than 200 million people infected at any time and at least one million deaths annually. Chloroquine has been a cheap and effective treatment for malaria for several decades but there is now widespread resistance to this and newer antimalarials such as halofantrine and mefloquine.

Peroxides are an important new chemical class of antimalarials which are at present undergoing extensive clinical evaluation and further chemical development. No major forms of human toxicity have been detected for the peroxides, although assessment of long-term human safety is limited by a situation in which the clinical use of the drug is not accompanied by the standard practice of postmarketing surveillance. The concern over long-term
Figure 7 Careful risk-benefit analysis of the peroxide antimalarials will be essential in order to maximize their ability to eliminate parasites, while at the same time minimize their potential for toxicity, particularly with regard to neurotoxicity and embryotoxicity.

toxicity in man is exacerbated by the practice of self-medication and uncontrolled drug use in many parts of the world. Laboratory studies have identified a number of potential hazards associated with long-term use, the most notable being reproductive toxicity and neurotoxicity. To define the human risk posed by peroxides, it is still necessary to define the chemical entities and chemical reactions which initiate the pathological processes, and then determine the propensity of individual peroxide drugs to undergo such bioactivation in vulnerable patient groups, and the fates, by the use of chemical and molecular analysis. In order to develop new, longer-acting peroxide antimalarials, it will be necessary to define molecular characteristics which determine parasite-selective bioactivation and those which preclude the risk of serious human toxicity (Figure 7).

BKP is a Wellcome Principal Research Fellow. The authors also wish to acknowledge the support of Roche Pharmaceuticals (PMG).

References

28. Gachot B, Elsasserer M, Dupont B. Artemesine and


(Rceived 8 July 1998, accepted 13 July 1998)