

Second-Generation Peroxides: The OZs and Artemisone

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Abstract The emergence of multi-drug resistant strains of *Plasmodium falciparum* has rendered many affordable antimalarials, such as chloroquine, much less effective in addressing the severe health issues in sub-Saharan Africa, Southeast Asia and the Amazon region. In order to overcome the neurotoxicity of an initial series of artemisinin-derived drugs and their relatively high production costs, an intensive and all-inclusive research programme to develop new derivatives has been undertaken. Two efficient antimalarial drug candidates of different chemotype have been devised, the artemisinin derivative artemisone and 1,2,4-troxolane **OZ277**. Both are nontoxic, more potent than artemisinin and should be affordable to people of endemic regions. The same may hold for the backup candidates artemiside and **OZ439**.

1 Introduction

Great efforts have been expended over the last 30 years to discover new and efficacious endoperoxide antimalarials based on a natural product isolated from sweet wormwood – artemisinin, and related derivatives (collectively known as artemisinins) - primarily because, until very recently [1, 2], resistance to this type of drug had not been observed (Fig. 1) [3].

The main drawback of the initial series of artemisinin derivatives (Fig. 1) is their relative metabolic instability, i.e., susceptibility to hydrolysis (esters), or

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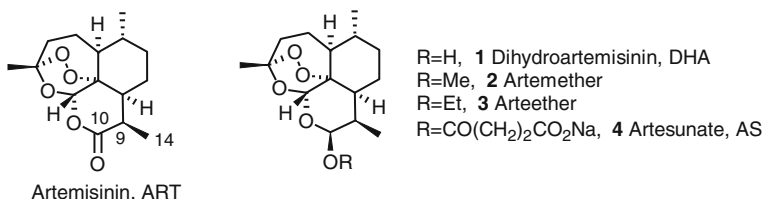


Fig. 1 Artemisinin and its early derivatives

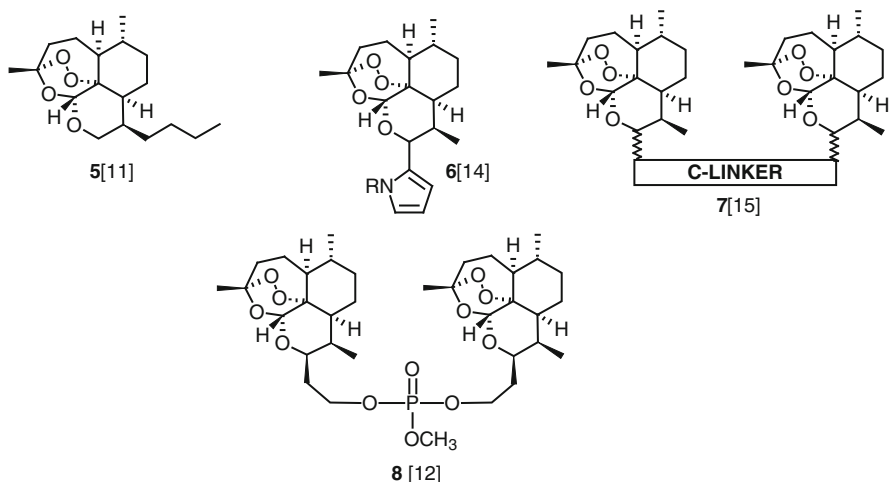


Fig. 2 Semi-synthetic artemisinins: C(14) and C(10) carba derivatives

oxidative-dealkylation (ethers) to dihydroartemisinin [4]. Dihydroartemisinin, apart from being a more potent antimalarial than artemisinin [5], was found to be neurotoxic in vitro [6, 7] and in mice [8]. However, similar neurotoxicity has not been observed in humans [9]. Although embryotoxicity has been detected within a narrow window of embryogenesis, this has not been convincingly observed in clinical trials from 1,837 pregnant women [10].

These potential toxicity issues provoked an extensive search for metabolically stable artemisinin derivatives and two major semi-synthetic artemisinin sub-classes were developed: C(10) deoxyartemisinin derivatives usually functionalized at C(9) or at C(14) (**5**) [11], and derivatives possessing a new C(10)-NR, or C(10)-C bond (**6–8**) [12–15]. The second sub-class is more diversified with artemisinin-derived dimers and artemisone (**9e**) as the most prominent members (Figs. 2 and 3).

The quest for efficacious and cheap antimalarial drugs that can be administered as a single dose, preferably orally, is not limited to natural product-derived semi-synthetic artemisinins, like artemisone, but also to other classes of peroxide antimalarials. Of these, the most prominent peroxide classes are the fully synthetic 1,2,4-trioxanes [16, 17], 1,2,4-trioxolanes (ozonides, OZs) and mixed 1,2,4,5-

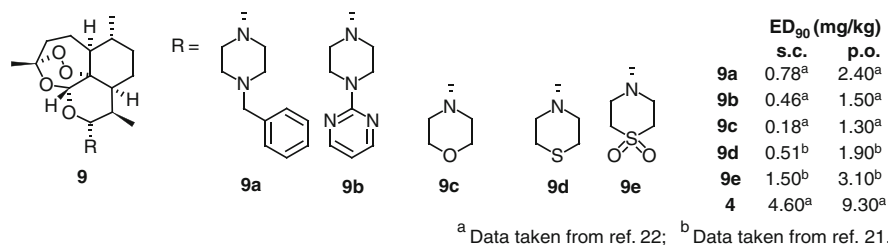


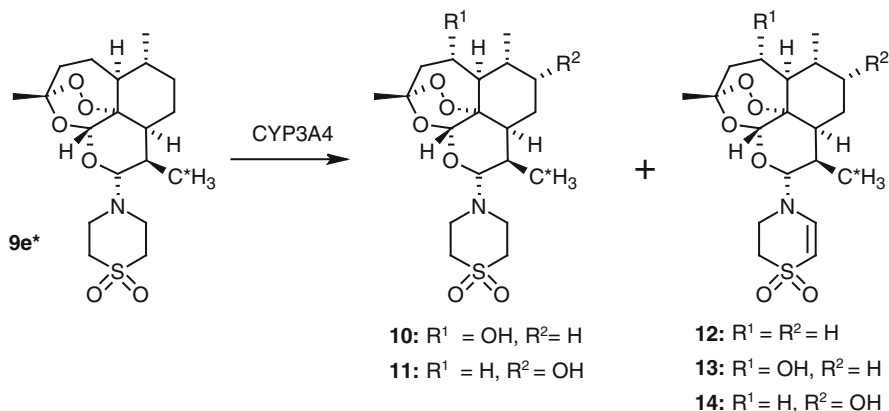
Fig. 3 Structures and antimalarial activities of derivatives **9**

tetraoxanes [18–20]. Two representatives of this class that have entered studies in humans are discussed below in detail.

2 Artemisone (Artemifon)

With the aim of preventing metabolic transformation of artemisinins to dihydroartemisinin (**1**, Fig. 1) and the drive to improve pharmacokinetic characteristics, several 10-(alkylamino)artemisinins **9** were designed (Fig. 3) [21, 22]. All the tested compounds showed excellent *in vivo* activities against *P. berghei* (**9c** was the most active derivative, almost 25 times (given *s.c.*) and 7 times (given *p.o.*) more active than artesunate **4**). Unfortunately, **9c** suffers from serious neurotoxicity issues even at low doses, thus indicating, that more lipophilic artemisinins are more toxic [21, 23].

A detailed antimalarial efficacy and drug–drug interaction study of artemisone (a drug examined recently in clinical Phase II trials [24]) was performed (**9e**, Fig. 3) [25]. It was shown that the *in vitro* antimalarial activities of artemisone against 12 different *P. falciparum* strains were comparable (exhibiting a mean IC₅₀ value of 0.83 nM), independent of their drug-susceptibility profile to other antimalarial drug classes [21, 25]. During examination of the *in vitro* drug–drug interaction against drug sensitive 3D7 and multi-drug resistant K1 strains, it was noticed that artemisone showed slight antagonistic effects with chloroquine, amodiaquine, tafenoquine, atovaquone or pyrimethamine, and slight synergism with mefloquine. *In vivo* screening using the 4-day Peters test against drug-susceptible (NY), primaquine-resistant (P) and sulphadoxine/pyrimethamine-resistant (KFY) lines of *P. berghei*, chloroquine-resistant (NS) and artemisinin-resistant lines of *P. yoelii* NS and drug-susceptible *P. chabaudi* (AS) showed artemisone has superior ED₅₀ and ED₉₀ activity in comparison with artesunate (**4**, Fig. 1). Artemisone exhibited a 7-times greater activity (lower dosage) than artesunate (artemisone ED₉₀ = 12.13 mg/kg vs. artesunate ED₉₀ = 87.50 mg/kg) against the *P. yoelii* artemisinin-resistant line. The above results appear quite important in light of the recent *in vitro* isolation of artemether-resistant *P. falciparum* strains from humans [26] and



Scheme 1 Metabolic transformations of artemisone

emerging evidence for resistance *in vivo* [1, 2], suggesting more potent derivatives may be efficacious where first generation compounds are failing.

During *in vivo* drug–drug interaction examinations against the drug-susceptible *P. berghei* NY and the mefloquine-resistant *P. berghei* N1100 lines, artemisone showed synergism with mefloquine against both parasite lines. In combination with chloroquine, no interaction against drug-susceptible *P. berghei* NY parasites was detected; however, a synergistic effect against the chloroquine-resistant line, *P. yoelii* NS, was observed.

No dihydroartemisinin had been produced after 30 min, when isotopically labelled artemisone **9e*** (Scheme 1) was incubated with human liver microsomes. Only dehydrogenated **12** and mono-hydroxylated metabolites **10** and **11** and **13** and **14**, with *syn*-hydroxyl and peroxide groups were observed (Scheme 1) [21], clearly distinguishing artemisone and similar compounds from the first-generation artemisinins.¹ Incubation with microsomes and 14 recombinant CYP isoforms, together with selective inhibitors of CYP, showed that artemisone was primarily metabolised by recombinant CYP3A4. Interestingly, artemisinin induces its own metabolism [27] and is metabolised principally by CYP2B6 [28]. These results indicate that artemisone and artemisinin, in spite of being structurally similar, have a different metabolic profile in *P. falciparum*; therefore, it is possible that they can exert their antimalarial activity through similar but not identical mechanisms (*vide infra*). Isolated artemisone metabolites were tested against the *P. falciparum* K1 strain and were also found to be potent antimalarials, with **11** and **12** being the most active (**11**: IC₅₀ = 5.51 nM and **12**: IC₅₀ = 4.26 nM, artemisone: IC₅₀ = 1.99 nM) [21, 29].

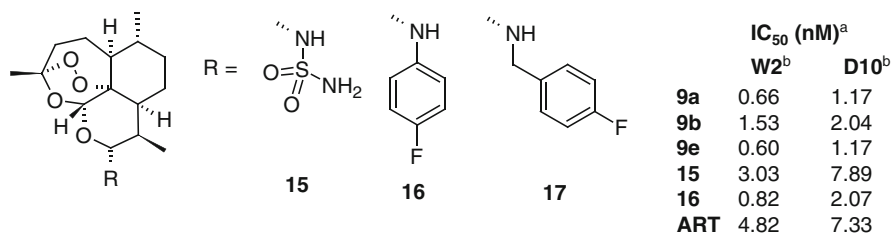
¹ However, dihydroartemisinin was detected in plasma during assessment of the safety of artemisone [27]. The concentrations were low, with geometric mean C_{max} values of 10 ng/ml after an 80 mg dose.

In preclinical studies, artemisone showed enhanced efficacy and improved pharmacokinetics in comparison with artesunate and did not demonstrate neurotoxicity *in vitro* and *in vivo* [21, 29], which is a characteristic of the current artemisinin derivatives used in clinical treatment [30]. Administration of artemisone as a single dose (10–80 mg) or multiple doses (40 mg or 80 mg given once daily for 3 days) was well tolerated. It appears that artemisone is devoid of time-dependent pharmacokinetics (unlike artemisinin and artemether), with comparable C_{\max} , AUC and $t_{1/2}$ values after the first and third doses following the 3-day courses of artemisone. Although not being so active *in vitro* as the parent drug, the relatively high concentrations of metabolites obtained after artemisone administration probably add to the overall parasitocidal effect of artemisone [29]. *In vivo* testing on non-immune *Aotus* monkeys infected with *P. falciparum* showed that a single dose of artemisone (10 mg/kg) in combination with single doses of mefloquine (12.5 mg/kg or 5 mg/kg) cleared parasitaemia by day 1, with complete cure for all four monkeys tested [31]. With a single dose of mefloquine (2.5 mg/kg) parasitaemia was cleared by day 1 but without cure. For 3 days of treatment with a combination of artemisone (10 mg/kg/day) and amodiaquine (20 mg/kg/day), all three monkeys tested were cured, in contrast to those that were administered with the individual drugs for 3 days. From this study, it is clear that various total dosages of artemisone (20–90 mg/kg) alone, administered over 1–3 days, were unable to cure non-immune *Aotus* monkeys infected with *P. falciparum*. However, cure can be achieved when artemisone is combined with a single, sub-curative dose of mefloquine, or with a 3-day treatment course of amodiaquine (or clindamycin).

Efficacy of several artemisinin derivatives was examined for the treatment of murine cerebral malaria, CM, ECM [32]. It was shown that artemisone and artemiside (**9d**, Fig. 3) were more effective in the treatment of ICR and C57BL mice in comparison with dihydroartemisinin or artesunate. In all experiments performed on *P. berghei*-infected mice, treatment with artemisone led to complete cure (at least parasitaemia was reduced to an undetectable level). The observed recrudescence parasites were successfully eradicated by repeating the treatment with artemisone without selecting for parasite resistance. In addition, it was shown that complete cure of infected mice could be achieved even when treatment with artemisone was commenced at late stages of pathogenesis, 6 days post-infection. These results opened a considerable time window for adequate treatment, since human malaria is diagnosed after clinical symptoms are apparent. Following WHO's instructions for artemisinin combination therapy (ACT), the efficacy of artemisone–CQ combination was examined and it was shown that this combination was more successful than single therapy of both drugs individually. Combination of these two drugs prevented recrudescence and cured all mice (2×5 mg/kg/day artemisone + 15 mg/kg CQ). In this study, artemiside appeared to be even more successful than artemisone, but this derivative has to be submitted to more detailed preclinical toxicological evaluation. However, the preliminary results suggest that artemiside may represent a new option for antimalarial therapy based on artemisinin derivatives [32].

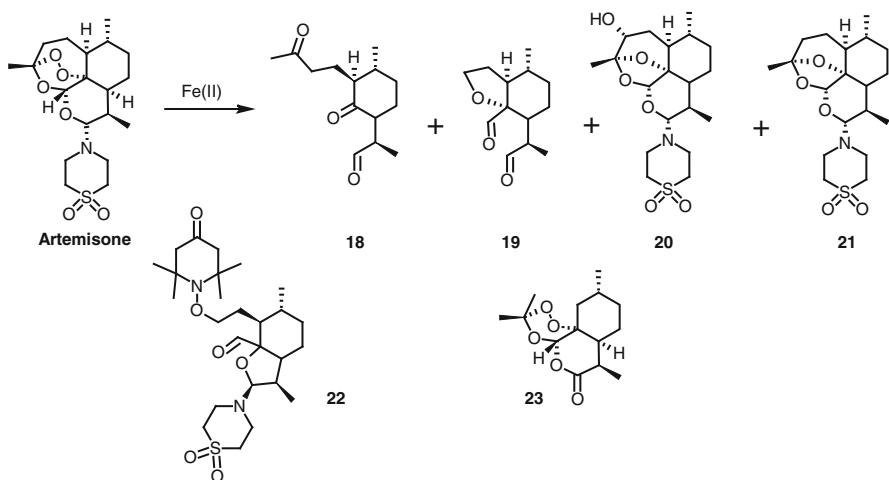
2.1 Possible Mechanisms: Fe(II) Interaction and the Peroxide Bond

In an effort to define more clearly a reasonable mechanism for the action of artemisone and related derivatives, experiments with various Fe(II) salts were performed [33]. It was found that artemisone, unlike other examined aminoartemisinin derivatives **9a**, **9b** (Fig. 3) and **15–17** (Fig. 4), reacts with Fe(II) salts under aqueous conditions to afford the products **18–20**, which are structurally similar to the ones obtained from artemisinin and dihydroartemisinin. The relative ratio of products **18–20** (Scheme 2) depends on the employed salts and solvent mixtures. A reasonable mechanism was proposed for Fe(II)-induced generation of the products, including artemisone \rightarrow **18**, **19** formation via iminium cations. Evidence that a primary C-radical is generated during Fe(II) peroxide scission came from the isolation of adduct **22** (Scheme 2), arising from reaction in the presence of the radical trapper 4-oxo-TEMPO. However, the yield of this



^a Data taken from ref. 33; ^b W2 is *P. f.* CQR strain, D10 is *P. f.* CQS strain.

Fig. 4 Semi-synthetic artemisinins: C(10) aza derivatives



Scheme 2 Products **18–21** obtained after reaction of artemisone with various Fe(II) salts

product is low (10%) and the authors argued that the role of Fe(II) is during decomposition rather than activation of artemisone [33].

Others have shown that artemisone readily reacts with haem *in vitro* producing alkylated products [34] very similar to artemisinins [35]. The ability of artemisinin itself to alkylate haem has been confirmed *in vitro* [36, 37] and *in vivo* [38]. These data indicate that Fe(II) species are able to activate artemisinins, including artemisone, in their antimalarial action. However, it should be noted that the conditions used to determine alkylation by artemisinins have been questioned, in particular the use of dimethyl sulphoxide that produces a reducing environment [39, 40], and, currently, there is no evidence that artemisone alkylates haem *in vivo*. In addition, the theory of Fe(II)-initiated activation of artemisinins could not explain the pronounced antimalarial activity of 5-nor-4,5-seco-artemisinin **23** [41–43], which is clearly less capable of forming C radicals upon peroxide bridge cleavage by Fe(II). Another inconsistency in applying the general Fe(II) mechanism (*vide supra*) to artemisinin and artemisone, and their derivatives, arises from their activities in the presence of desferrioxamine B (DFO). DFO acts as a free radical scavenger (for hydroxyl and peroxy radicals) and as a Fe(III) chelator and reduces artemisinin inhibition of PfATP6 (a proposed site of action; see below) [44]. In contrast, artemisone, which exhibits approximately the same reactivity with aqueous Fe(II) as artemisinin, retains inhibition potency towards PfATP6 in the presence of DFO [33]. This detail strongly indicates that although the compounds are of similar structure, they might not exert their antimalarial activity by sharing the same mechanism of action.

In order to understand better the manner in which artemisinins exert their antimalarial activity, extensive research was launched bearing in mind that cleavage of the peroxide bridge via an iron-dependent mechanism (as the sole mechanism) does not provide complete answers to observed peroxide drug behaviour. Based on the finding that the *in vitro* antimalarial activity of artemisinin was significantly increased under a 2% carbon monoxide atmosphere (by 40–50%) and under a 20% oxygen atmosphere (by 20–30%) [45], a detailed study of the interaction of artemisone and related derivatives with various forms of haemoglobin (Hb), haem, as well as an analysis of their antimalarial activity was performed [39]. In contrast to artemisinin, artemisone does not react with Hb–Fe(II) and oxyHb–Fe(II) to produce metHb–Fe(III); in addition, it was shown that both artemisinin and artemisone (and **9a**, **9b**, **16**) are inactive towards Hb–Fe(II) in a 2% carbon monoxide atmosphere. On the other hand, both compounds induced the oxidation of the haemoglobin catabolic product, haem–Fe(II), to produce haem–Fe(III). In addition, on exposure of haem–Fe(II) and Hb–Fe(II) to carbon monoxide, stable complexes were formed, which are also inactive for reactions with peroxide antimalarials. However, in a biologically relevant experiment, both compounds showed increased antimalarial activity against *P. falciparum* W2 strain under a 2% carbon monoxide atmosphere, while the activity of chloroquine remained unchanged. The authors suggested [39] that peroxide antimalarials behave as reactive oxygen species (ROS), or that they produce ROS via Haber–Weiss chemistry. Furthermore, it was concluded that passivation of haem–Fe(II) by its conversion

into the CO complex results in decreased decomposition of artemisinins (artemiseone included), thus making them more available for reaction with their actual target. The authors proposed that Fe(II), regardless of its origin (Hb-Fe(II) or haem-Fe(II)), is not the sole initiator of O-O scission, and suggested that another mechanism be sought [39].

2.2 Possible Mechanisms: Primary Targets, Accumulation and Co-Factors

The discovery that artemisinins target SERCA orthologues in *P. falciparum* (PfATP6) and *P. vivax* (PvSERCA) [44] shed new light and encouraged research towards the same and other novel targets for peroxide antimalarials. Thus, it was found that artemiseone is more potent against plasmodial SERCA orthologues [$K_i = 1.7 \pm 0.6$ nM (PfATP6) and $K_i = 0.072 \pm 0.012$ nM (PvSERCA)], as compared with values for artemisinin [$K_i = 169 \pm 31$ nM (PfATP6) and $K_i = 7.7 \pm 4.9$ nM (PvSERCA)] [46].

In order to better understand the mechanism of the accumulation into infected and uninfected erythrocytes, radiolabelled artemiseone **9e*** was submitted to uptake assays [47]. It was found that artemiseone was accumulated in infected erythrocytes at significantly higher levels than in uninfected ones, by a saturable, competitive, time- and temperature-dependent mechanism. Most radioactivity was detected in pellet fractions which predominantly contain proteins suggesting that artemiseone is probably associated with proteins [48]. The distribution of artemiseone is dependent on the stage of parasite maturation: after incubation with immature parasites, which are highly susceptible to action of artemisinins *in vitro*, as well as *in vivo*, most artemiseone was found in a Triton-soluble fraction, which contains proteins, DNA and RNA. In mature parasites most artemiseone was found in the Triton-insoluble fraction, which predominantly contains haemozoin. This finding suggests that mature infected erythrocytes can act by removing the artemisinins. This was further supported by the observation that mature infected erythrocytes take up artemiseone much faster than do the more sensitive ring-stage-infected erythrocytes.

Methylene blue (MB) was the first synthetic drug ever used in humans, and it was Paul Erlich who cured two patients from malaria using MB in 1891 [49]. The discovery that artemisinin and artemether exhibit synergic effects with methylene blue [50], unlike chloroaminoquinolines, initiated systematic examination of reactivity relationships between the drugs with the aim to possibly correlate their mechanism of antimalarial action. Subsequent research by the same group [51] revealed MB as a redox-cycling agent that produces H_2O_2 at the expense of O_2 and of NADPH in each cycle (Fig. 5). Thus, MB consumes NADPH and O_2 needed for the pathogen's metabolism, with probably serious consequences to the NADPH/NADP ratio.

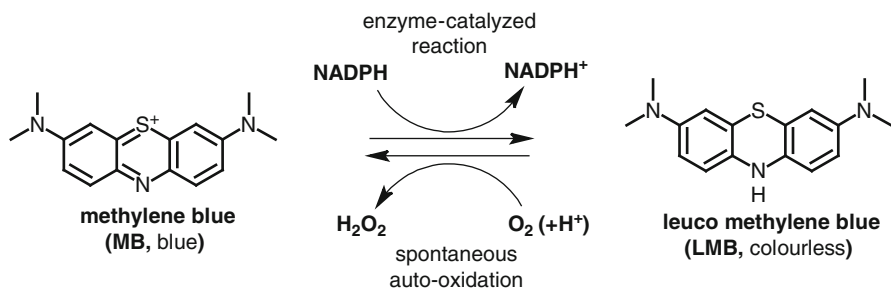
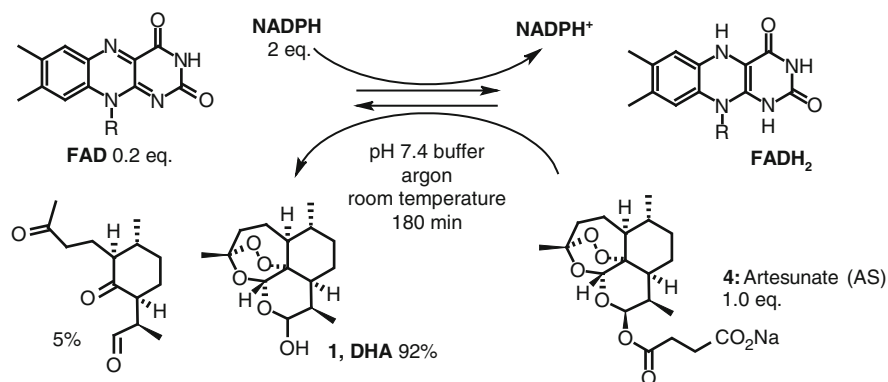


Fig. 5 Redox-cycling interconversion between methylene blue (MB) and leuco methylene blue (LMB)



Scheme 3 Biomimetic catalytic system under physiological conditions: works with other flavins, all examined artemisinins, tetraoxane and trioxolane antimalarials

The results of subsequent research revealed that artemisone, other artemisinins [52] and other peroxide antimalarials like ozonides and tetraoxanes [53] are active in the presence of MB-ascorbic acid, MB-*N*-benzyl-1,4-dihyronicotinamide (BNAH), riboflavin-BNAH and riboflavin-NADPH systems and yield identical products to those that were isolated from the reaction of the same antimalarials with Fe(II). According to this proposal, antimalarial peroxides act as oxidants re-oxidising LMB and FADH₂, so contributing to depletion of NADPH (Scheme 3).

The observed results suggest that peroxide antimalarials disrupt a highly sensitive redox balance established in the parasite and thereby cause its death. The results also correlate well with the observed SAR of artemisinins, i.e., artemisinins that exhibited low antimalarial activity, like 9-epiartemisinin [54, 55], also show low reactivity under the applied reaction conditions. In addition, high antimalarial activity of 5-nor-4,5-seco-artemisinin **23** can easily be comprehended using the new mechanism proposal. The proposed explanation involves iron-free reactions; however, Fe(III) rapidly oxidises FADH₂, and thus contributes to redox cycling without interfering with artemisinins.

3 1,2,4-Trioxolanes (Ozonides)

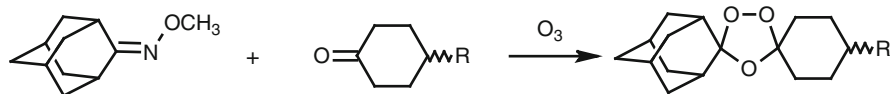
1,2,4-Trioxolanes are a very well-known class of organic compounds. They are intermediates in the transformation of olefins into carbonyls during ozonolysis. It was an unexpected and surprising discovery [56] that ozonides are relatively stable and that many of them express excellent activity against malaria parasites, as do the structurally similar 1,2,4-trioxanes.

3.1 Development of 1,2,4-Trioxolanes: OZ209, OZ277 and OZ339

Preparation of 1,2,4-trioxolanes is relatively simple and relies on the Griesbaum co-ozonolysis of suitable methyl oximes and ketones (Scheme 4) [57–59]. This method provides a highly applicable synthetic approach to tetrasubstituted 1,2,4-trioxolanes (ozonides), otherwise accessible only with great difficulty by ozonolysis of the corresponding alkenes, or by other means.

The synthesis of the final OZ antimalarial drug significantly benefits from the stability of the ozonide peroxide bridge to reduction and alkylation conditions, as well as to other standard reaction conditions. This enables transformation of the initial co-ozonolysis products into a vast array of unsymmetrically substituted ozonides ($n > 500!$) [60]. Usually, the final 1,2,4-trioxolane antimalarials are prepared in 1–4 steps, depending on the modifications required [57–59, 61–63], affording the final products in yields up to 75%. The vast majority of these antimalarials are achiral, which greatly facilitates production in the developmental step. A substantial improvement in drug design in the antimalarial field was the development of the 4-substituted cyclohexyl-adamantyl ozonide (adamantane-2-spiro-3'-1',2',4'-trioxaspiro[4.5]decane) chemotype. The advantage of OZ antimalarials is the use of the adamantane moiety that has lipophilic functionality, allowing the opposite part of the molecule to be fine-tuned using a number of polar functional groups, preferably basic in nature [56, 58–62]. Many of the OZ compounds obtained in this way were active in all stages of development of the malaria parasite and are more active than artemether and artesunate, both *in vivo* and *in vitro*. All these findings, gathered during much experimentation, contributed to the discovery of amines **OZ209** (**24** as mesylate, Fig. 6) and **OZ277** (**25** as tosylate, Fig. 6) as the best drug candidates.

In comparison with other OZ compounds, trioxolanes **OZ209** and **OZ277** showed superior pharmacokinetic results, such as prolonged half-life and enhanced



Scheme 4 Griesbaum co-ozonolysis: Ozonides

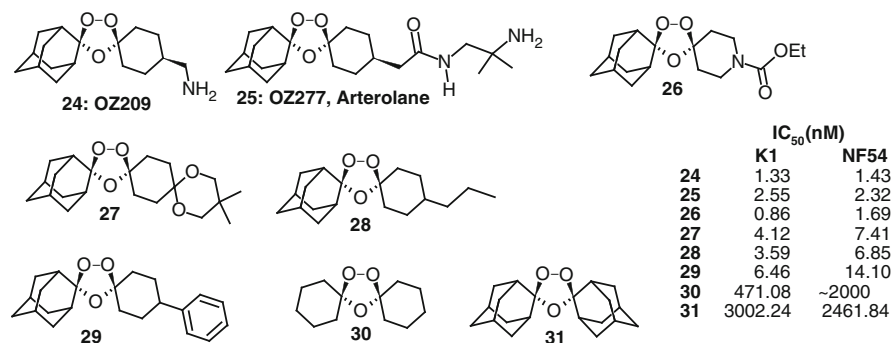


Fig. 6 1,2,4-Trioxolanes **OZ209** and **OZ277**, and selected congeners. Compound **OZ277** has been advanced to Phase III clinical trials (vide infra)

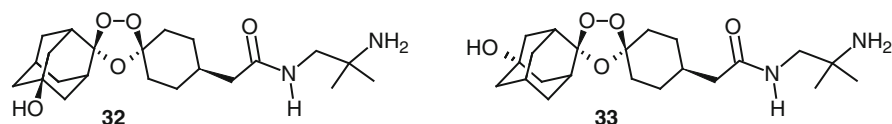


Fig. 7 Microsomal metabolites of ozonide **OZ277**

bioavailability after a single oral dose. Compound **OZ209** had somewhat better antimalarial results and a lower recrudescence level. However, **OZ277** was chosen as the development candidate, primarily because of its improved toxicological profile and reduced concentrations in brain tissue after oral dosing [56]. For example, 2 h after dosing, both **OZ209** and **OZ277** were distributed throughout the liver, kidney, lung and heart, while after 18 h, **OZ277** was detected only in the lungs and in several-fold lower concentrations than **OZ209**.

Unlike **OZ209**, which was quantified in brain tissue after both 2 h and 18 h, trioxolane **OZ277** could not be quantified in this organ at all. In view of potential neurotoxicity issues, these findings were taken as a considerable advantage of **OZ277** over **OZ209**. Trioxolane **OZ277** appeared quite stable to metabolic transformation ($t_{1/2} = 17$ h, *p.o.* in healthy rats) [56]. The metabolic profile of **OZ277** was studied with human liver microsomes and only two, monohydroxylated derivatives at the adamantane angular positions (**32** and **33**, Fig. 7), were identified as major metabolites, thus confirming the stability of the trioxolane moiety to metabolic transformation. Interestingly, both metabolites were inactive against the *P. falciparum* K1 strain (IC₅₀ > 100 ng/ml), thus demonstrating the indispensability of the unsubstituted spiro-adamantane moiety to the antimalarial activity of **OZ277** (IC₅₀ (K1) = 1.0 ng/ml) [64]. Unlike the artemisone products (Scheme 1), OZ metabolic derivatives **32** and **33** very probably lower the overall **OZ277** antimalarial activity. The other derivatives **26–29** (Fig. 5) afforded further insight into SAR in the context of the physico-chemical, biopharmaceutical and toxicological profiles of trioxolanes [61].

Recently, the same authors revealed data for a series of OZ compounds with weak base functional groups, which were responsible for a high antimalarial efficacy in *P. berghei*-infected mice [65]. Their antimalarial efficacy and ADME profiles are equal or superior to **OZ277**. One of the most promising is **OZ339** (as tosylate salt). The two trioxolanes, **OZ339** and **OZ277** are evaluated in Table 1, with artesunate added for comparison. Despite the obvious difference in in vitro activity, both ozonides eradicate parasitaemia below the detectable level 1 day after administration (99.9%, 1×10 mg/kg, and 3×3 mg/kg). The drug candidate **OZ277** is a powerful fast-acting antimalarial with a 67% cure record at a 3×10 mg/kg dosage (mice) [56]. However, at a 3×3 mg/kg dosage, the same compound cured no mice, while trioxolane **OZ339** cured 3/5 mice with an excellent survival time of 27 days (**OZ277** had a 2.4 times lower survival time). These good pharmacokinetic characteristics are additionally enhanced by the favourable bio-availability data for **OZ339** (78%, Table 1). In all experiments, artesunate showed inferior activity. Inhibition assays revealed that **OZ339**, like **OZ277**, did not inhibit CYP3A4, CYP2C9 and 2D6CYP450 at concentrations up to 50 μ M. Finally, preliminary toxicological experiments indicated that **OZ339** was minimally toxic (liver) and, similar to **OZ277**, demonstrated no detectable signs of neurotoxicity.

As mentioned above, the tolerance of the 1,2,4-trioxolane moiety to diverse reaction conditions [57] and resistance to metabolic transformation [64] enabled the synthesis of a significant number of derivatives and many of them showed very good antimalarial activity, e.g., derivatives **34–38** (Fig. 8) [66], and derivatives which contain aliphatic and aromatic amino functional groups or azole heterocycles as substituents (**39–45**) (Fig. 8) [62].

The lack of activity of trioxolane **46** [62], and the isolation of inactive hydroxylated **OZ277** metabolites [64], point to the essential contribution of an unsubstituted spiro-adamantane system to the antimalarial properties of this class of compounds.

Many of the examined derivatives exhibited excellent in vitro results, but failed during in vivo tests, toxicity trials or metabolic stability and bioavailability tests. More lipophilic trioxolanes tend to have better oral activities and are metabolically less stable than their more polar counterparts. Such behaviour is consistent with results obtained for other classes of synthetic peroxides. Trioxolanes with a wide range of neutral and basic groups had good antimalarial profiles, unlike derivatives with acidic groups. Based on the collected extensive screening results, the authors concluded that in vitro activities of 1,2,4-trioxolanes are not (always) a reliable predictor of in vivo potency [66]. Rather, their experiments in *P. berghei*-infected mice confirmed that in vivo results were essential for compound differentiation and selection for further metabolic and pharmacokinetic profiling [65].

Trioxolane **OZ277** alkylates haem (Fig. 9) [67], and its in vitro activity against *P. falciparum* is antagonised by DFO [68]. In vitro, artesunate and **OZ277** act antagonistically against *P. falciparum*. These findings, together with only weak interaction with the proposed artemisinin target PfATP6 [44], unlike artemisone [33], suggest that interaction with food vacuole-generated haem is probably how trioxolanes are activated. Further support can be found in the fact that the

Table 1 Comparative data for fast-acting antimalarials: **33** (OZ339), **24** (OZ277) and sodium **4** (AS)

Compound	IC ₅₀ (ng/mL)		In vivo activity (3 × 3 mg/kg, p.o.) ^a				V _d (i.v., L/kg)	Bioavailability (p.o., %)
	KI	NF54	Activity (1 × 10 mg/kg, p.o.) ^b	ER ^c	Survival (days)	Cure ^d (%)		
34 (OZ339) ^{e,f}	0.35	0.39	>99.9	0.45	27.0	60	19	78
25 (OZ277) ^{e,f}	1.0	0.91	99.9	0.32	11.4	0	16	26
4 (AS) ^f	1.3	1.6	67	0.43	9.2	0	40 (DHA) ^g	N.D.

N.D., not dosed

^aGroups of five *P. berghei*-infected NMRI mice were treated orally on days +1, +2, and +3 (3 × 3 mg/kg). Activity measured on day +4

^bGroups of five *P. berghei*-infected NMRI mice were treated orally on day +1 (1 × 10 mg/kg). Activity measured on day +2

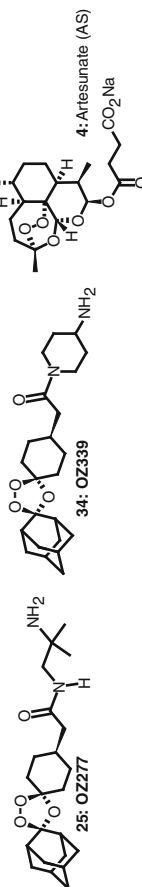
^cPredicted hepatic extraction ratios (ERs) using human microsomes

^dPercentage of mice alive on day 30 with no evidence of blood parasites

^eTosylate salt

^fTaken from [61]

^gTaken from [58, 59]



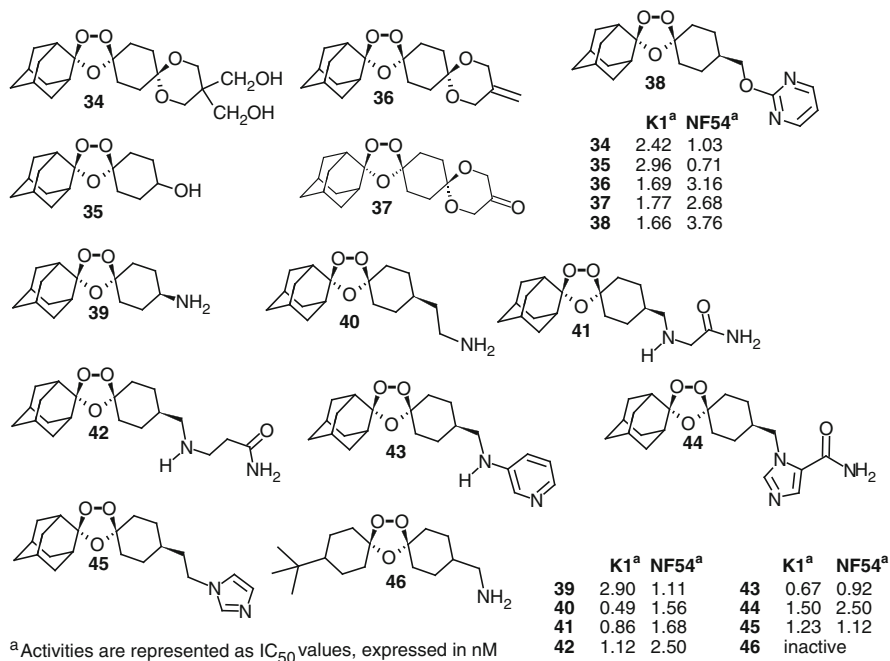


Fig. 8 Structures of ozonides 33–45 with their in vitro antimalarial activity

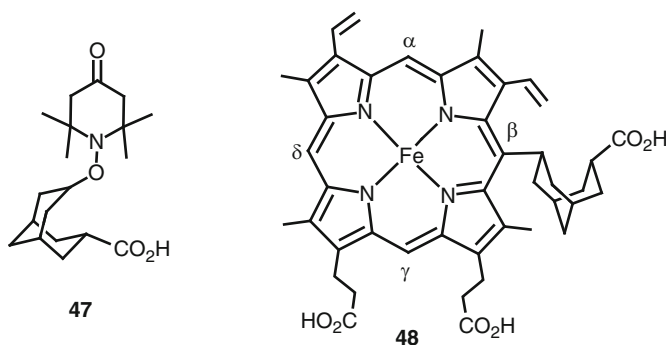
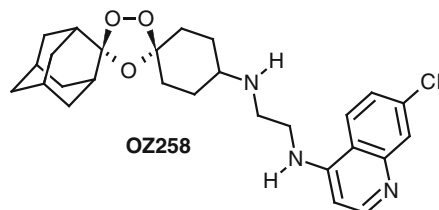


Fig. 9 Structures of adducts of the secondary radical derived from OZ277 with 4-oxo-TEMPO and haem

stereochemistry of a given compound has little effect on the in vitro potency of trioxolane antimalarials, thereby strongly pointing to the interaction of an antimalarial peroxide (chiral or achiral) with an achiral target (haem). The selectivity of trioxolanes towards infected and not-healthy erythrocytes may be explained based on their reactivity towards free haem and stability in the presence of oxy- and deoxyHb [69].

Fig. 10 Chimaeric trioxolane **OZ258**



IC ₅₀ (nM)		% Activity (10 mg/kg)	
K1	NF54	p.o.	s.c.
11.98	10.74	75	99.95

Based on the concept that compounds with two integrated pharmacophores might have enhanced activity [70], the chimaeric trioxolane **OZ258** was prepared (Fig. 10) [58, 59]. Although it is very active in vitro against the K1 and NF54 strains of *P. falciparum* and in vivo against the ANKA strain of *P. berghei*, **OZ258** did not achieve the synergic effect of two pharmacophores, especially when compared with trioxolanes **39** and **43**. The same holds for other chloroaminoquinoline and acridine chimaeras [71].

Very often, promising peroxide drugs eradicate parasitaemia quickly, which is crucial for rapid treatment of life-threatening cerebral malaria, and this property is inherently protective against the development of resistance. Since the drugs are typically administered for only a few days and they have short half-lives, the recrudescence of malaria parasites occurs frequently (artemisone cf. [24, 32]; **OZ277** cf. [58]). In an attempt to overcome this problem, artemisinin-based combination therapies (ACTs) are recommended (as indicated for artemisone, see above) by the WHO. The WHO currently distributes, under a no-loss and no-profit agreement with Novartis, the fixed-dose ACT drug Coartem[®] (artemether 20 mg/lumefantrine 120 mg) [72]. The drug has been recently approved by the FDA for the treatment of acute, uncomplicated malaria infections [73]. Although each ACT is specific [73, 74], the following concept can be applied to all: antimalarial peroxides eliminate most of the infection and the remaining parasites are then exposed to high concentrations of the slow-acting partner drugs; because of the rapid reduction in parasites, the selective pressure for the emergence of mutant parasites is greatly reduced. In accord, **OZ277** (RBX-11160) entered Phase III clinical trials in combination with piperazine (arterolane maleate + piperazine phosphate) [75].

3.2 The Second Generation of 1,2,4-Trioxolane Drug Candidates: **OZ439**

In Phase I clinical trials, the half-life of **OZ277** in healthy volunteers was only about two- to threefold longer than that of dihydroartemisinin. **OZ277**'s possible first-generation ozonide alternative, **OZ339**, only has a slightly higher $t_{1/2}$ value

Table 2 Comparative data for first and second generation of ozonide antimalarials: **25** (**OZ277**) vs. **49** (**OZ439**)

Compound	IC ₅₀ (ng/mL)	Postinfection in vivo activity ^a kg, <i>p.o.</i> ^b			In vivo prophylactic activity (1 × 30 mg/ kg, <i>p.o.</i>) ^b			V _d (<i>i.v.</i> , L/kg)	Bioavailability (<i>p.o.</i> , %)	
		NF54	1 × 30 mg/kg, <i>p.o.</i>	% Cure ^c	Activity (%)	Survival (days)	Cure ^c (%)			<i>t</i> _{1/2} (<i>p.o.</i> , min)
25 (OZ277) ^{d,e}	0.71	0.63	99.9	0	0	7	0	55	4	13
49 (OZ439) ^e	1.6	1.9	>99.9	100	99.8	>30	100	1380	15	76
4 (AS) ^e	1.2	1.5	92	0	21	7	0	40 (DHA) ^f	3.0 (DHA)	N.D.

N.D., not dosed

^aGroups of *P. berghei*-infected ANKA mice (*n* = 5) were treated orally on day +1 (1 × 30 mg/kg). Activity measured on day +3

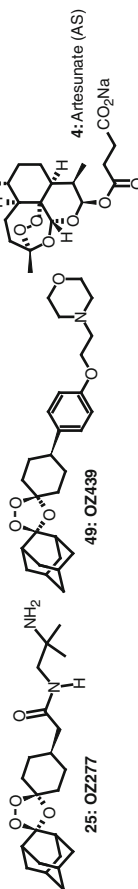
^bGroups of *P. berghei*-infected ANKA mice (*n* = 5) were treated orally on day -1 (1 × 30 mg/kg). Activity measured on day +3

^cPercentage of mice alive on day 30 with no evidence of blood parasites

^dTosylate salt

^eTaken from [76]

^fIntravenous, taken from [58, 59]



(Table 1); so the search for an ozonide with significantly increased half-life continued.

As a result, screening of the second generation of ozonide antimalarials has been completed, recently [76]. Of the several very active OZ compounds of undisclosed structure it appears that the most promising antimalarial candidate is **OZ439** (Table 2) [76]. Initial results indicate that this compound provides single-dose oral cure in a murine malaria model at 20 mg/kg, a situation not known for any of the peroxide antimalarials except for artemininc acid at >7 times higher dose [76]. The second-generation ozonide **OZ439** completed Phase I studies and is currently undergoing Phase IIa clinical trials. In accord with other ozonide antimalarials, **OZ439** is considered to be an Fe(II)-initiated pro-drug. However, it is >50-fold more stable to Fe(II)-mediated degradation compared with **OZ277** [76]. Consistent with proposed Fe(II) degradation of ozonides [67] is the significantly enhanced stability (15–20 times) of **OZ439** over **OZ277** in healthy and infected human and rat blood. This prolonged blood stability and improved pharmacokinetic characteristics (Table 2) led to the positioning of **OZ439** as the current major OZ drug candidate – with respect to post-infection cure (3×5 mg/kg/day and 20 mg/kg single dose), and exclusive prophylactic characteristics (Table 2). The absence of metabolic products significantly contributes to overall activity (prophylactic and post-infection) of **OZ439** relative to other peroxide antimalarial drugs [76].

To conclude, as a consequence of intensive and comprehensive research, efficient antimalarial drug candidates of different chemotype have been devised: artemisone and 1,2,4-trioxolane **OZ277**. They are nontoxic, effective at small doses and very probably inexpensive to produce² [77]. The same may hold for a prospective backup candidate artemiside and the newest breakthrough drug candidate **OZ439**. It would be of benefit if their combination partner would cure malaria through different mechanisms, since resistance is then less likely to occur.

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²The annual demand for artemisinin as a starting material for transformation into semi-synthetic products amounts to ca. 114 tonnes for Coartem[®] production only. Since synthesis of artemisinin is uneconomical currently, Novartis initiated an increase of the agricultural cultivation of *Artemisia annua* in Kenya, Tanzania and Uganda and extraction of artemisinin therefrom, in addition to Chinese supplies of artemisinin.

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