***Review***

**Artemisinin Inspired Synthetic Endoperoxide Drug Candidates: Design, Synthesis and Mechanism of Action Studies**

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Abstract

Artemisinin combination therapies (ACTs) have been used as the first-line treatments against P. falciparum malaria for decades. Recent advances in chemical proteomics into semi-synthetic ARTs, has shed light on their promiscuous mechanism of action via previous heme-mediated bioactivation of the endoperoxide bond. Alarmingly, the rise of resistance to ART in South East Asia and the synthetic limitations of the ART scaffold have pushed the course for the necessity of entirely synthetic endoperoxide-based antimalarials. Several classes of synthetic endoperoxide antimalarials have been described in literature utilizing various endoperoxide warheads, including 1,2-dioxanes, 1,2,4-trioxanes, 1,2,4-trioxolanes, and 1,2,4,5-tetraoxanes, where two of these classes have been explored extensively. 1,2,4-trioxolane (Arterolane, Artefenomel) and 1,2,4,5-tetraoxane (N205, E209) based antimalarials appear to still be in active development – the most recent publication pertaining to the development of Arteflene, Fenozan-50F, DU1301 or PA1103/SAR116242 was published in 2008. This review summarises the synthesis, biological evaluation, and mechanistic studies of the most developed synthetic endoperoxide antimalarials, providing an update on those classes still in active development.

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# Abbreviations

ABPP, based protein-profiling probes; 1-ABT, 1-aminobenzotriazole; ACPR, adequate clinical and parasitological response; ACT, artemisinin combination therapy; ART, artemisinin; Asn, Asparagine; Asp, Aspartic Acid; AUC, area under the curve; CD50, median convulsive dose to reduce parasitemia by 50%; CQ, chloroquine; Cyp450, Cys, Cysteine; cytochrome P450; DHA, dihydroartemisinin; DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid sodium; DMF, dimethylformamide; DMPK, drug metabolism and pharmacokinetic; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; ED50, dose required to reduce parasitemia by 50%; ED90, dose required to reduce parasitemia by 90%; EDC.HCl, 1‐ethyl‐3‐(3‐dimethylaminopropyl) carbodiimide hydrochloride; EPR, Electron paramagnetic resonance; FaSSIF, fasted state simulated intestinal fluid; FeSSIF, fed state simulated intestinal fluid; Fe(II)-PPIX, Fe(II)-protoporphyrin IX; HDA, 4-hydroxy deoxoartemisinin; hERG, human ether à-go-go-related gene; GD50, dose required to reduce parasite gametocytes by 50%; GD90, dose required to reduce parasite gametocytes by 90%; HPLC, High performance liquid chromatography; IC50, half maximal (50%) inhibitory concentration of a substance; IM, intramuscular; IV, intravenous; LC-MS, liquid chromatography–mass spectrometry; Lys, Lysine; *m*‐CPBA, meta‐chloroperoxybenzoic acid; MMV, medicines for malaria venture; MS, liquid chromatography–mass spectrometry; PC50, 50% parasite clearance; *Pf*K13, *Plasmodium falciparum* Kelch 13; *Pf*TCTP, *P. falciparum* translationally controlled tumor protein; Phe, Phenylalanine; P.O., Oral administration; PK, pharmacokinetics; RCT, ring-contracted tetrahydrofuran; ROS, reactive oxygen species; SAR, structure‐activity relationship; SERCA, sarco/endoplasmic reticulum Ca2+ ATPase; TCP1, Blood-Stage killers of candidates against malaria; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy; Thr, Threonine; TPP, Tetraphenylporphyrin; WHO, World Health Organization.

# Introduction



**Figure 1:** Structural representation of Artemisinin and its semi-synthetic derivatives with clinical use as antimalarials.

The earliest literature reference to malaria was in the ancient Chinese medical text the “Nei Ching” fabled to be written by the semi-mythical Emperor Huangdi in 2600 BC – though it is more likely to have been written much later around 300 BC by a combination of writers.1,2 In this work, malaria is described as a progressive flu-like disease with references to splenomegaly and seasonal differences in symptoms.3 As malaria has been present throughout human history it is no surprise that over 1200 plant species have been utilised in traditional medicine to treat malaria – including the leaves *of Artemisia annua* or sweet wormwood.4 The use of sweet wormwood for the treatment of malaria comes from traditional Chinese medicine as first described in “A Handbook of Formulas for Emergencies” by Ge Hong (283-363CE) which states “Have a handful of herb of sweet wormwood and soak it in two sheng (200 mL) of water. Squeeze it to get the juice and drink.”5 Owing to the efficacy of sweet wormwood in treating malaria significant interest was taken in determining the active component.

With the intention of finding a solution to the massive deaths caused by Malaria during the Vietnam War, the People’s Republic of China recruited a large number of Chinese scientists to work towards a project codenamed Project 523 which, in part, involved the screening of traditional Chinese herbs for antimalarial effect.6,7 As part of this, the antimalarial activity of various extracts of sweet wormwood leaves was investigated by Tu Youyou, finding that the initial ether-extract of *Artemisia annua* was shown to be 100% effective at treating parasitaemia in rodents. Subsequent work led to the isolation of the active compound artemisinin (ART, **1, Figure 1**) for which Tu Youyou was awarded the Nobel prize for medicine in 2015.6,8 Since its isolation, ART has been a key molecule in the treatment of malaria and the first antimalarial of the endoperoxide class, owing to the presence of a peroxide containing 1,2,4-trioxane ring presumed to act as the warhead of the molecule.9 However, issues with solubility and extensive first pass metabolism make artemisinin somewhat unsuitable as a therapeutic medicine. First pass metabolism of ART within the liver resulted in the formation of inactive metabolites lacking the required endoperoxide warhead.10 This led to a demand for artemisinin derivatives with a better solubility profile and increased metabolic stability.

The first generation semi-synthetic ARTs dihydroartemisinin (DHA, **2, Figure 1**), artemether, artether and artesunate were designed and synthesised by Chinese scientists while working on Project 523.7 DHA was first synthesised by Tu’s group as the lactone reduced analogue of ART.11 DHA was found to retain potent antimalarial activity and displayed improved aqueous solubility however plasma stability remained an issue. DHA has been marketed as Artenimol and is recommended for use in combination with piperaquine by the WHO.6,12 In 1976 a number of ether and ester derivatives of DHA were synthesised by other scientists working on Project 523 and screened against *P. falciparum* yielding the ART ethers Artemether (**3**) and Artether (**4**),11 Artemether and Artether are methyl and ethyl ether derivatives of DHA respectively displaying greater plasma stability than the parent drug.13 Artemether is recommended for treatment of uncomplicated *P. falciparum* malaria in combination with lumefantrine and, in cases of *P. vivax* infection, this is followed by treatment with primaquine to prevent relapse.14–17 Artemether/Lumefantrine was granted international licensing approval in 1999, marketed as Coartem by Novartis, and was granted FDA approval in 2009, defining the first WHO backed fixed dose Artemisinin-based combination therapy.18 However, poor solubility of the ART-ethers necessitates administration by intramuscular (IM) injection or oral routes (po) which is not ideal for the rapid systemic exposure required for clearing parasite burden in severe malaria.13 Artesunate (**5**) is the succinic ester of DHA displaying greatly improved aqueous solubility which enables administration by intravenous (IV) infusion allowing for effective treatment of severe malaria.19

The current recommendation of WHO for the treatment of uncomplicated *P. falciparum* malaria is using ART combination therapy (ACT) which involves the co-administration of a semi-synthetic ART derivative with a second longer acting drug. The ART derivative acts as a fast killer to rapidly reduce initial parasite burden while the long acting drug maintains a plasma concentration above the minimum parasiticidal concentration allowing the eradication of parasites in as few doses as possible20

Though effective, ACTs are threatened by the growing prevalence of ART resistance first reported on the Thai-Cambodian border in 2009.21 Since, resistance has spread throughout South East Asia with most resistant cases occurring on the Thai-Cambodian Border.22 Clinically, ART resistance is defined as a delayed parasite clearance in response to artesunate monotherapy or an ACT.23 Partly to blame for acquired ART resistance is the prevalence of inadequate treatment due to substandard medicines, incorrect dosing, and poor compliance. Poverty in areas with ART resistance result in patients being unable to afford full courses of treatment or they may reserve remaining doses after clinical symptoms have alleviated.24

The molecular mechanism of ART resistance has been attributed to a mutation in the K13 propeller gene in *P. falciparum*. While the function of PfK13 has not yet been identified, there are homologies with the human Keap1 protein, specifically the C-Terminal domain and the 6-Kelch propeller.25–27 Keap1 is responsible for ubiquitination to trigger subsequent proteasomal degradation; this process is implicated in response to oxidative stress and PfK13 dependent ubiquitination has since been observed. It is believed that mutation in K13 infers an increased tolerance to oxidative stress, altered cell cycle and resistance by parasite quiescence.28



**Figure 2:** Structure representation of the semi-synthetic ART Artemisone developed by Haynes

Resistance to ART and limited opportunities for optimisation due to the constraints of the ART scaffold are both limitations that cannot be easily overcome by the development of more semi-synthetic ARTs. The development of Artemisone (**6, Figure 2**) by Haynes provides an example of a recently developed semi-synthetic ART attempting to improve bioavailability and neurotoxicity in comparison to artesunate, where this is a cause for concern.29,30 Artemisone is a thiomorpholine 1,1-dioxide derived C10 amine analogue of ART which possesses low lipophilicity, and low cyto- and neurotoxicity. Artemisone was found to outperform Artesunate *in vitro,* and though *in vivo* efficacy was 4.8- and 2.4 fold lower than Artesunate by subcutaneous and oral routes, respectively, the ED90 values were low enough to suggest utility as an antimalarial drug.29,31 Results of a phase I clinical trial of Artemisone were published in 2008, revealing a short half-life of 2.79 hours, similar to other semi-synthetic ARTs.32 A Phase II clinical trial on the efficacy of Artemisone in the treatment of uncomplicated malaria in Western Cambodia was planned, however approval for recruitment was not given.33

This has paved the way to the development of fully synthetic endoperoxide antimalarials – this class of antimalarial maintains the endoperoxide warhead responsible for rapid clearance of parasitaemia whilst the lack of semi-synthetic chemistry constraints allows greater control over physicochemical properties. Over three decades of development a variety of classes of endoperoxide antimalarials emerged, defined by their use of one of many peroxide-containing saturated heterocycles as analogous warheads to the 1,2,5-trioxane in ART. This review summarises the synthesis, biological evaluation and mechanistic studies of the most developed endoperoxide antimalarials, also providing an update on those classes still in active development. Prior to examining the lead synthetic analogues, the following section deals with proposals for the mechanism of action of the ARTs. This work has inspired additional research into mechanism of action of the synthetic endoperoxide antimalarials with a focus on comparisons and contrasts in reactivity and plausible endoperoxide parasite targets.

# The Mechanism of Artemisinin and its Derivatives

***Bioactivation by ferrous iron.*** Endoperoxide-containing antimalarials share a common and unusual mechanism of action, which remains under scrutiny, despite more than four decades of investigation by different research groups worldwide. Although many targets have been explored34, it is generally considered that endoperoxides do not have a specific biomolecular target. The data available suggests that the mechanism of action of these molecules requires initial bioactivation of the peroxide pharmacophore of artemisinins or synthetic endoperoxides by ferrous (Fe2+),34–45 leading to highly reactive carbon-centred radicals via reductive cleavage of the peroxide bond.41,46–48

In early investigations performed by Meshnick and co-workers, labeling studies using 14C artemisinin and derivatives in *Plasmodium falciparum* cultures indicated that there was a higher preference for the infected erythrocytes compared to the non-infected cells.49 Subsequently, the same group demonstrated that heme could catalyse the reductive decomposition of artemisinin and its derivatives, also showing that the process was antagonized by iron chelators, both *in vitro* and *in vivo*.50 Meshnick *et al.*49 suggested that the activation of artemisinin was triggered by Fe(II) to generate highly reactive species. Eventually, these radicals could then induce parasite death by alkylation of the parasitic proteins and peroxidation of lipids of the membranes.51–54 Since these initial discoveries, two different hypotheses have been proposed to explain artemisinin's bioactivation and its derivatives:

**a) reductive scission**: Due to the asymmetric peroxide bond in artemisinin **1**,the oxygen atoms can interact with Fe(II) ions in two different pathways: 1) Association of ferrous iron or heme with peroxide oxygen **O1** gives an oxy radical that rearranges through C-C bond scission (β-scission) to provide a primary carbon-centred radical **7** (**Scheme 1, a**). This carbon radical could react with heme, forming a heme-peroxide adduct, thus stopping β-hematin (hemozoin) formation55; or promote protein alkylation. Detection of ring-contracted tetrahydrofuran (RCT) product **8** supports the hypothesis of the generation of the radical species. Alternatively, the reaction of ferrous iron or heme with peroxide oxygen **O2** provides an oxy radical species that, through a 1,5-hydrogen shift, generates a secondary carbon-centred radical **9**. This secondary *C*-centred radical could promote a nearby substrate's alkylation, or it could rearrange to form the hydroxydeoxoartemisinin product (HDA **10**, **Scheme 1, a**).13,34,36,56–59

**b) peroxide ring-opening**: alternatively, ferrous ion may act as a Lewis acid instead of a radical activator, which induces ring-opening by protonation of the peroxide bond.60 The endoperoxide bridge undergoes (Lewis) acid-catalysed heterolytic cleavage, generating an unsaturated hydroperoxide **11.** Subsequent Fenton like degradation of the hydroperoxide by reductive cleavage with ferrous iron or other reducing agents produces a hydroxyl radical (•OH), a short-lived but very reactive species that can promote oxidation of target amino acid residues (**Scheme 1, b**).13,34,36,56,57 Evidence to support this model was provided by Haynes and co-workers,61, which arises from the discovery that artemisinin has been shown to facilitate *N*-oxidation of tertiary alkylamines via the intermediacy of such an hydroperoxide form.



**Scheme 1:** Proposed mechanisms of bioactivation and action of artemisinin: a) Reductive scission; b) Peroxide ring-opening.

***Activated endoperoxides promote nonspecific alkylation of proteins.*** In the 1990s, Meshnick and co-workers reported the first studies of alkylation of parasite proteins by antimalarial endoperoxides. They have demonstrated that six plasmodial proteins were radioactively labeled by artemisinin derivatives.62–64 Other parasite proteins that were proposed as targets of artemisinin and related endoperoxide compounds include the translationally controlled tumor protein (*Pf*TCTP)65 and the parasite’s sarcoendoplasmic reticulum calcium-dependent ATPase (also known as P*f*ATP6 or SERCA).66,67

Chemical proteomics has emerged as a powerful affinity-based approach for elucidating proteome-wide interactions of antimalarial peroxide-containing molecules in living cells. In three studies recently undertaken, alkylated *P. falciparum* proteins were identified using activity-based probes of artemisinin (**12-16, Figure 3**).47,68–70



**Figure 3:** Peroxide-based probes with clickable alkyne and azide groups: (a) Artemisinin derivatives and their controls (**12** from Wang *et al*.47 and **13**-**16** from Ismail *et al*.68);

Infected *P. falciparum* erythrocytes were treated with the endoperoxide probes. Following incubation, if probes were active against the parasite, they would promote activation and lead to parasite proteins' covalent binding. The probes consisted of an antimalarially active endoperoxide tagged with biotin or a fluorescent label, via a click reaction, and the alkylated proteins could be identified by mass spectrometry techniques (LC–MS/MS) or processed in 1D-Gel analysis.47,68–70

Wang *et al.*47 used a non-optimised artemisinin-alkyne activity-based (**12, Figure 3**) protein-profiling probe associated with 124 artemisinin-alkylated proteins through click-chemistry procedures. The addition of free-iron chelator deferoxamine, in the presence of hemin or heme, was found to decrease the alkylation of a few protein targets slightly, but not all of them. This observation shows that besides the role of heme in the bioactivation of endoperoxides, free ferrous iron may also have a role in this process. Ismail *et al.*69 optimised the endoperoxide-ABPPs strategy, using artemisinin probes and also their deoxo-analogs, as controls (**13-16**,Figure 3). Copper(I)-catalyzed and bioorthogonal copper-free click chemistry were employed to identify the protein-ART adducts, where the group identified 59 ART-labeled proteins.

Additionally, Li *et al.*71 have investigated the artemisinin-binding site within *Pf*TCTP, through chemical proteomics. Using artemisinin-based probes (using the ART-probe **13,** Figure 3), the group was able to identify the artemisinin-alkylated peptide fragments within *Pf*TCTP, in which the ART-probe modified multiple nearby amino acid residues, such as Asn, Asp, Lys, Phe, Thr, and Cys. Interestingly, these results emphasized the role of the Cys19 residue as an electron donor during endoperoxide bioactivation and of both Phe12 and Cys19 as important residues on the interaction of artemisinin within *Pf*TCTP.

All these studies allowed to detect parasite proteins implicated in a wide range of cellular processes, including hemoglobin digestion, ribosomal proteins, and transport proteins, metabolic processes, and antioxidant defenses. Expectedly, the peroxide bond was observed to be indispensable for protein alkylation, whereas heme was shown to have a more important role in bioactivation than free ferrous iron. A drawback of these studies is that they only assessed covalent protein alkylation, while non-proteinaceous alkylation targets such as heme, lipid peroxidation, and mechanistic pathways concerning non-covalent interactions with the parasite proteins, should not be dismissed. Another downside is that there wasn’t enough correlation in the alkylated proteins identified between all findings,47,68–70, which can be explained by the differences in the conditions used in each study. However, these results can also suggest that activated endoperoxides unsystematically and non-specifically alkylate parasite proteins, thus inhibiting essential proteins involved in key parasite pathways, in a multiple target approach.

# Arteflene- The first Synthetic Clinical Candidate Endoperoxide



**Figure 4:** Structure representations of the natural product Yingzhaosu A **18,** ofthe synthetic endoperoxide antimalarial Arteflene **17** andof its active 8-hydroxy metabolite Ro 47-6936 **19**,

Arteflene (**17, Figure 4**) is a synthetic 1,2-dioxane containing antimalarial reported by F. Hoffmann-La Roche in 1994.72 Itwas selected during a project focusing on the synthesis of a series of simplified analogs of Yingzhaosu A (**18**) – a natural product endoperoxide isolated from the Chinese herb Yingzhao. Yingzhaosu A possesses potent antimalarial activity however, due to lack of abundance and a lengthy total synthesis from R-(-)-carvone, it is not suitable for use as a therapeutic.73,74 Arteflene was synthesised from R-carvone (**20**, scheme 2) with an initial acid catalysed epoxidation of the alkene followed by opening to the diol. The diol was cleaved to the ketone **21** followed by conversion to **22** by Wittig olefination. The 1-2-dioxane moiety was installed by singlet oxygen ene reaction followed by intramolecular cyclisation by Michael addition to produce the trioxane **23**. Ozonolysis of the alkene provided a handle for Wittig olefination to yield Arteflene.72



**Scheme 2**: Synthetic route to the trioxane antimalarial Arteflene (**17**)

Arteflene possesses a modest *P. falciparum* IC50 of 70 nM, 4-fold less potent than Yingzhaosu A, however it was found to have a potent ED50 of 10 mg/kg in the *P. berghei* mouse model, comparable with Artemether and Artesunate. It was noted that Arteflene displayed a rapid and long-lasting suppressive effect on parasitaemia in the *P. berghei* mouse model.72 Additionally, it was seen that arteflene possessed a poor pharmacokinetic (PK) profile, with high metabolic clearance, poor oral bioavailability, and a short half-life of 1.4 – 4.7 hours.75 The counterintuitive *in vivo* activity can be explained by the rapid formation of an active 8-hydroxy metabolite (Ro 47-6936, **19, Figure 4**) upon extensive first pass metabolism of Arteflene. The *in vitro* activity of Ro 47-6936 was found to be just 4-fold lower than the parent drug and was therefore determined to be likely to contribute to the high *in vivo* activity after oral administration.75

Phase I clinical trials in healthy volunteers were reported in 1994, assessing for tolerability and PK.76 Six groups of 9 volunteers were administered either Arteflene in single ascending doses or a placebo. No adverse events were reported with no clinical changes identified, suggesting a good safety profile for Arteflene. 76 Additionally, the PK profile was found to scale linearly with dose, however due to the metabolic instability the elimination half-life was found to be relatively short, ranging between 2 to 4 hours. 76 Additionally, Cmax for Arteflene was found to be reached quickly at 1.7 to 3.3 hours post dose however the Cmax of the 8-hydroxy-metabolite Ro 47-6936 was found to be 3-fold greater than that of the parent drug, further supporting its role in the *in vivo* activity of Arteflene. 76 *P. falciparum* growth inhibition assays in sera taken from patients showed significant inhibition for volunteers who received 300 mg or higher doses in samples obtained between 30 minutes and 8 hours post dosing which supported the clinical efficacy of Arteflene.76

Phase II clinical trials of Arteflene have been carried out with patient cohorts from Nigeria and Burkina Faso, and Cameroon.77,78 Clinical trials in Nigeria and Burkina Faso investigated the efficacy of Arteflene as a monotherapy in patients aged 12 to 16 years with mild malaria. In 89.5 % of patients parasitaemia was found to be reduced by 50 % or more 48 hours post dose with 52.6 % of patients being completely free from parasites.78 Additionally, alleviation of fever was seen in 89.5 % of patients with a clinical cure observed in 75 % over the same period.78 Despite this, the efficacy observed was less than expected by the investigators, attributed to inadequate storage of the Arteflene suspension. 78

Trials in Cameroon investigated clinical efficacy of 25 mg/kg Arteflene in a cohort of 30 patients aged 12 to 42 years with an initial *P. falciparum* count of >5000 parasites per microlitre.77 Mean parasite count was observed to fall from 21,406 to 157 after 48 hours with 80 % of patients completely free of parasites. Similarly, clinical cure was observed in 70 % of patients. 77 In contrast to the high clinical cure rate, 20 % of patients were found to be smear positive for *P. falciparum*,casting doubt on whether single dose 25 mg/kg Arteflene is an effective treatment for *P. falciparum* malaria. 77 Further to this, 25 mg/kg Arteflene was investigated in comparison to 15 mg/kg Mefloquine in the treatment of *P. falciparum* malaria in children. High grade resistance was observed in 40 % of the arteflene cohort while no high-grade resistance was observed in the mefloquine treated cohort, adding further weight to the argument against 25 mg/kg Arteflene monotherapy.79 Following clinical trials, Arteflene has not been developed further – this may be due to poorer clinical efficacy in comparison to semi-synthetic ARTs, a relatively high recrudescence rate and high grade resistance observed in clinical trials.

# Mechanism of Fe(II) Activation and Postulated Parasiticidal Intermediate

O’Neill and coworkers59 have described the mechanism of action of arteflene **17**, through mimetic studies using ferrous chloride, demonstrating that reaction of **17** with FeCl2 induces homolytic cleavage of the peroxide bridge (with a single electron donation from ferrous iron) in peroxide oxygen **O2**, generating an *O*-centred radical **25**. This intermediate radical then rearranges to provide a stable enone system **26** and a secondary *C*-centered radical **27**, which, under the reaction conditions, undergoes polymerisation. Generation of the diol **30**  happens when a two-electron reduction occurs, by the association of Fe2+ with peroxide oxygen **O1** (inducing a homolytic cleavage of the peroxide)to produce an *O*-centered radical **28** where its rearrangement is not possible. Instead, the oxy radical undergoes further reduction to the anion **29** followed by protonation to give the corresponding diol (**Figure 5, a**).80 This reaction pathway was confirmed by EPR spectroscopy, wherein the secondary carbon-centred radical **27** was spin trapped by 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and 3,5-dibromo-4-nitrosobenzenesulfonic acid sodium salt (DBNBS).80 Cazelles *et al.*81 also reported a study of arteflene reaction with MnII(TPP), in the presence of borohydride, followed by generation of adduct with the alkyl radical and 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) (**Figure 5, b**).



**Figure 5**: (a) Ferrous-Mediated degradation of Arteflene; (b) DMPO, DBNBS80, and TEMPO81 adducts.

# Fenozan-50F



**Figure 6**: Structure representation of the 1,2,4-trioxane antimalarial Fenozan-50F

Fenozan-50F (**31, Figure 6**) was first reported in 1992 in the investigation of *cis*-fused cyclopenteno-1,2,4-trioxanes (fenozans), following work by Jeffords group on the extensive synthetic methodology for the synthesis of 1,2,4-trioxanes.82–84 A series of fenozans were synthesised using the methods reported by Jeffords, as summarised in **Scheme 3**.82,83 Fenozan-50F was synthesised from 1,4-bis(4-fluorophenyl)-cyclopentadiene **35,** previously prepared by condensation of 4-fluorophenacyl bromide (**32**) with ethyl-acetoacetate to afford **33** which upon treatment with base cyclised to give the substituted cyclopentenone **34.** Grignard addition of 4-fluorophenyl magnesium bromide into **34** and subsequent heating yielded **35**. Dye-sensitised photoaddition of singlet oxygen yielded the endoperoxide **36** which was condensed with cyclopentanone in the presence of catalytic Me3SiOTf to yield Fenozan-50F (**31**) as an enantiomeric mixture which was separated by chiral HPLC.85 Fenozan-50F has been investigated extensively in rodent models of malaria.



**Scheme 3:** Synthesis of the 1,2,4-trioxane antimalarial Fenozan-50F **31**.

Fenozan-50F was screened against chloroquine (CQ) resistant W2 and CQ sensitive D6 strains of *P. falciparum* and was found to be equipotent, with IC50 values of 2.01 and 2.07 ng/ml respectively. In comparison to Artesunate, Fenozan-50F was found to be 4 and 2 fold less potent against W2 and D6 respectively.85 In the *P. berghei* mouse model it was found to possess potent ED50 value of 2.5 mg/kg/day over 4 days by subcutaneous administration, as well as possessing equal activity in the oral route with an ED50 of 2.6 mg/mg/day over 4 days.83,85 In the *P. berghei* model, after treatment of two mice with a single dose of 30 mg/kg Fenozan-50F complete reduction in parasitaemia was observed in one mouse by day 5 and to 0.8 % compared to baseline in the other. Despite both treated animals surviving at least 33 days, reoccurrence of parasitaemia was observed in both. 86 Furthermore, resistance acquisition studies found that the resistance index (ED90 resistant strain/ED90 of parent line) for Fenozan-50F was 3.4, suggesting a low propensity to acquire resistance.87

Fenozan-50F was investigated for prophylactic action by challenging mice with *P. berghei* after receiving a single dose of 100, 300, 600 mg/kg subcutaneously and 600, 1000 and 3000 mg/kg orally. In the subcutaneous arm, survival was seen to days 12, 16 and 15 respectively, suggesting that a prolonged response was present. All mice dosed orally had died by day 8, suggesting either poor absorption or rapid excretion. Additionally, Fenozan 50F was found to possess modest activity against gametocytes with gametocytocidal dose (GD50 and GD90) values of 8 and 70 mg/kg, respectively.86 Interaction of Fenozan-50F in combination with other antimalarial partner drugs was investigated in the *P. yoelii* infected mice model, against several resistant and sensitive strains. In combination with CQ, Fenozan-50F appeared to potentiate the antimalarial activity in the CQ resistant strain but not the sensitive strain. It was also found to be potentiating in combination with Mefloquine and halofantrine in the CQ resistant strain. Antagonism was observed between Fenozan-50F and pyronaridine against the CQ resistant strain. Combination with mefloquine similarly displayed antagonism in a mefloquine resistant strain of *P. yoelii.*88

Presently, Fenozan-50F has not been tested in humans. However, the *in vivo* data obtained through extensive testing in the mouse model suggests that Fenozan-50F whilst likely be effective, the risk of recrudescence, the poor chemoprotective effect following oral dosing and the potential for rapid elimination would suggest that a single dose of Fenozan-50F would be unlikely to cure malaria.83,86 Instead, it is likely that multiple doses would be required, falling short of the single dose cure outlined in the Medicines for Malaria (MMV) target candidate profile, known as TCP1.89

# Mechanism of Iron Bioactivation and Generation of Radical and Carbocation Intermediates

The reaction of Fenozan-50F **31** with Fe(II) chloride promotes a homolytic cleavage of the endoperoxide bond, wherein a single-electron transfer by the ferrous ion gives the radical anion **37**. Protonation of the radical anion affords an *O*-centred radical **38**, that, upon subsequent capture of a H-atom from the medium (SH), generates the pentanoate **40** product. Alternatively, alkoxy radicals **37** or **38** can undergo a β-scission pathway, providing primary carbon-centred radicals **41** and **42**, which can react with parasite proteins and heme, conferring its antimalarial action (**Scheme 4**). A second, oxidative step can happen in the presence of higher amounts of ferrous iron, where oxidation of the primary carbon-centred radical **42** generates a primary carbocation **43**, most probably stabilized as the bicyclic oxonium species **44**. Reaction of **44** with water produces the alcohol **45**, which can dimerize to species **46**. Carbon-centred radical and carbocation intermediates may eventually induce cellular oxidative stress and promote protein alkylation, conferring its antimalarial action (Scheme 4).85,90



**Scheme 4:** Proposed mechanisms for ferrous-mediated degradation of Fenozan-50F.

# DU1301- A Member of the Fenozan Class of 1,2,4-Trioxane



**Figure 7:** Structures of trioxaquine antimalarials DU1102 and DU1301

A second class of trioxane containing antimalarials are the trioxaquines, which combine a trioxane containing component and a 4-aminoquinoline moiety. The rationale behind these compounds is to afford a hybrid approach combining the alkylating properties of the trioxane component and the improved penetration afforded by the 4-aminoquinoline. Initial synthesis of a series of trioxaquines was reported in 2000, focussing on the SAR of linker length between the 4-aminoquinoline and cis-fused cyclopenteno-1,2,4-trioxanes similar to the motif seen in Fenozan-50F.91 The optimum linker length was determined to be 2 methylene units, affording the lead compound DU1102 (**47, Figure 7**) with an IC50 against *P. falciparum* of 43.1 nM and showing no significant difference between CQ sensitive and resistant strains, indicating no cross resistance with CQ. DU1102 was found to show a low level of cross resistance with Pyrimethamine which, along with poor aqueous solubility of DU1102 citrate, indicates issues for further development.92,93

To expand the SAR understanding of the trioxaquines, a series of further trioxaquine analogues were synthesised exploring a wider range of starting materials, varying the diene utilised for initial endoperoxide formation and the diketone used for condensation.93 Additionally, significant interest was placed on the separation of individual diastereomers, as previously DU1102 had only been screened as a mixture of stereoisomers.92,93 The use of the abundant natural product α-terpinene (**49, Scheme 5**) as a starting material afforded the compound DU1301(**48, Figure 7**). Initial tetraphenylporphyrin (TPP) promoted photooxidation of **49** affording the endoperoxide **50** which was condensed with cyclohexane-1,4-dione to yield an intermediate trioxane as a mixture of diasteromers and enantiomers. The major diastereomer, **51**, was separated by selective precipitation and coupled by reductive amination with the 4-aminoquinoline component **53** to afford **48**.As the reductive amination was not stereoselective a mixture of diastereomers – the *cis,cis* and the *trans,cis* diastereomers are shown in Scheme 5, **B**. The enantiomers were separated by initial enrichment to a 70:30 mixture of *trans,cis*: *cis,cis* stereoisomers by selective precipitation followed by column chromatography.93



**Scheme 5**: A) Synthesis of the trioxaquine DU1301 from α-terpinene B) Structure of cis,cis and trans,cis-stereoisomers of DU1301 – only one enantiomer for each is shown

DU1301 citrate was screened *in vitro* against clinical isolates of *P. falciparum* including CQ sensitive, CQ resistant and extensively CQ resistant strains. Screening was carried out with both the mixture of diastereomers and those separated by column chromatography.93 IC50 values were determined to range between 5 and 19 nM with no significant difference between individual diastereomers and the diastereomeric mixture. *In vivo* studies were carried out in the *P. vinckei* mouse model with **48** administered either by intraperitoneal or oral routes. ED50 values were determined as 5 and 18 mg/kg for intraperitoneal and oral routes respectively.93 The fact that the oral ED50 is only 4 fold lower than that of the intraperitoneal suggests reasonable bioavailability however no formal drug metabolism and pharmacokinetic (DMPK) testing data is available. **48** was also assessed for mutagenesis in an *e. coli* SOS response assay which determined that **48** did not promote the SOS response indicating a low potential for mutagenicity. 93

# Mechanism of Action Studies

Studies with DU1301 **48** confirmed a dual mechanism of action for trioxaquines. Loup *et al.*55 have shown that **48** was five times more effective as inhibitor of β-hematin (hemozoin) formation than chloroquine. Additionally, **48** appears to efficiently alkylate heme,94 due to its trioxane ring, which favours the production of an alkylating carbon-centred radical entity. Homolytic cleavage of O-O bond primarily generates the *O*-centred radical **54** at **O2**, followed by a β-scission to form a primary carbon-centred radical **55**. The alkylating ability of trioxaquine **48** toward Fe(II) heme was assessed in similar conditions as reported for artemisinins (**Scheme 6**).95



**Scheme 6**: Proposed mechanisms for ferrous-mediated degradation of trioxaquine DU1301 **48**, generating the heme adducts **56** and **57**.95

# PA1103/SAR116242-The First Peroxide Drug Hybrid Clinical Candidate



**Figure 8**: Structure representation of the trioxaquine PA1103/SAR116242

PA1103/SAR116242 (**58, Figure 8**) is a trioxaquine antimalarial initially developed by Palumed and more recently under development by Sanofi.96 The discovery of **58** is the culmination of synthesis and evaluation *in vitro* of a library comprising 120 trioxaquines and trioxaloquines (hybrid molecules containing a trioxolane moiety). The selection was narrowed down by further screening *in vivo* and DMPK studies, leading to the proposal of **58** for further development.97 Synthesis of **58** required the preparation of the intermediate trioxane **63** which has been synthesised by two routes – cobalt catalysed peroxysilylation of **59** or molybdenum catalysed perhydrolysis of the epoxide **61**. **60** or **62**,respectively, were condensed with cyclohexan-1,4-dione to afford **63**. Much like DU1301, the intermediate trioxane was coupled to the 4-aminoquinoline core (**64**) (synthesised by amination of **52**), by reductive amination affording **58** as a mixture of diastereomers, which could be separated by chiral-HPLC for structure determination and screening (**Scheme 6**).97



**Scheme 7:** Synthetic route to PA1103/SAR116242

Trioxaquine **58** was screened against various clinical isolates of *P. falciparum* with IC50 values ranging between 7 to 24 nM. Further *in vitro* screening against a freshly isolated multidrug-resistant isolate from Gabon determined an IC50 of 19.8 nM – outperforming CQ but performing more poorly than artesunate in the same assay.97 *In vivo* studies were carried out on CQ resistant and CQ sensitive strains of *P. vinckei* in the mouse model. CD50 values were determined to be 30 and 32 mg/kg/day over 4 days for CQ resistant and sensitive strains, respectively. In CQ resistant model **58** outperformed CQ and artesunate (>100mg/kg and 100mg/kg respectively), suggesting no cross resistance to CQ, while in the CQ sensitive strain **58** performed poorer than CQ but similar to artesunate (16 and 32 mg/kg respectively).97 A *P. falciparum* humanised mouse model was used to measure parasite reduction by treatment with **58** against W2 CQ resistant and 3D7 CQ sensitive strains of *P. falciparum.*97In the 3D7 model, parasite reduction at day 4 was found to be 100 % at 64 mg/kg/day while in the W2 model parasite reduction was found to be 77 % and 96 %, at 32 and 64 mg/kg/day, respectively.97

Compound **58** has been assessed for DMPK parameters including human microsomal and hepatocyte stability, cytochrome P450 (Cyp450) induction or inhibition and caco-2 permeability.97 **58** was found to possess low clearance in human liver microsomes but moderate-high clearance in human hepatocytes, however it is notable that in both assays **58** outperformed artesunate. No evidence of Cyp450 induction or inhibition was observed, suggesting that drug-drug-interactions are unlikely to be an issue. Caco-2 permeability assays yielded similar values to that of CQ, suggesting that the desired enhanced permeability was attained and supporting the likelihood of oral bioavailability – though no PK studies have been carried out *in vivo.* 97Screening for cardiotoxicity in a hERG inhibition assay revealed that **58** possesses a a low IC50 of 1.5 μM which suggests that QT interval elongation may be an issue. QT elongation is a known adverse effect of CQ with a hERG IC50 of 3 μM – 2 fold higher than **58** which suggests that this might be a common occurance.97–100 Following publication of PA1103/SAR116242 as a drug candidate, no additional information has been recorded on further preclinical testing or first into man profiling .

# Trioxolane Antimalarials

# Arterolane (OZ277)



**Figure 9:** Structure representation of the trioxolane antimalarial Arterolane (OZ277, **65**) and of the 4-aminoquinoline Piperaquine **66**

The trioxolane antimalarials are among the most widely explored class of synthetic endoperoxide antimalarials utilising an 1,2,4-trioxolane moiety as the alkylating endoperoxide warhead.101 The discovery of the pharmacophore initially involved the measurement of potent antimalarial activity of simple spiro-adamantyl and cyclohexyl trioxolanes. The first generation of molecules developed by the Vennerstrom group were closely based on the initial prototype molecules with the addition of solubilising group containing amides on the 4” position on the cyclohexyl ring allowing the development of improved drug-like properties. Arterolane (**65, Figure 9**) was the most potent analogue synthesised of first-generation compounds.101 Griesbaum co-ozonolysis is a key reaction in the synthesis of trioxolane antimalarials, involving the condensation of a carbonyl oxide generated from adamantan-2-one O-methyl oxime (**67**) with a cyclohexanone derivative (**68**) to afford the dispiro 1,2,4-trioxolane 69, with the syn compound as the major diastereomer. Studies by Vennerstrom into the cis-trans selectivity of Griesbaum co-ozonolysis showed that an ethyl ester equivalent of **69** was synthesised in a cis:trans ratio of 4.5:1.102 Hydrolysis of the ester and subsequent formation of the benzotriazole activated ester **70** allowed divergent synthesis of a range of analogues, including Arterolane (Scheme 8).101



**Scheme 8**: Synthesis of Artelorane

Arterolane was found to possess potent *P. falciparum* IC­50 values of 0.91 nM and 1.0 nM against the CQ sensitive strain NF54 and CQ resistant strain K1, outperforming artesunate.101 In *vivo* studies for 30-day survival in the *P. berghei* mouse model showed cures in 66 % of mice, following doses of 3 mg/kg/day over 3 days. In contrast, a similar survival study with a single dose of 3 mg/kg resulted in an average of 9-day survival.89,101 Though arterolane possessed improved PK in comparison to semi-synthetic artemisinins artemether and artesunate the bioavailability is still low with an oral bioavailability of 35 %, which likely contributes to the low survival after single oral doses in the mouse model used.101

Arterolane was progressed to phase I clinical trials, with results published in 2013.103 An initial phase I safety and PK study involved 64 healthy young males and a separate branch of elderly males and females. It was observed that Cmax was reached 2-3 hours post dose, with a second peak appearing at around 5 hours post dose. The elimination half-life was found to be relatively short, at 2-4 hours, consistent with preclinical findings.101,103 Additionally, the bioavailability of Arterolane appeared to increase with dose, suggesting that metabolising enzymes or transporters involved in elimination might become saturated at a certain concentration. More concerningly, Arterolane displayed a food effect, with enhanced absorption observed after a high fat breakfast, compared to the fasted state- this may present as viable PK when administered to patients.103 Nevertheless, in phase I trials Arterolane was tolerated by all volunteers and was carried on for phase II clinical trials.104

Phase II clinical trials were carried out in malaria infected patients aged 13 to 65. This trial investigated the relationship between the PK parameters and antimalarial activity of Arterolane through measurement of overall exposure by area under the curve (AUC) and time taken to clear 50 % of parasites (PC50).104 Screening at 50, 100 and 200 mg/kg showed PC50 times of 12, 9 and 9.4 hours, respectively, indicating that doses of at least 100 mg are required to deliver maximum benefit of Arterolane therapy while doses over 100 mg would be expected to have limited advantage. At all doses tested, however, recrudescence was observed at a rate of 37, 30 and 27 %, for 50, 100 and 200 mg doses, respectively. Additionally, it was observed that the blood exposure and half-life in malaria patients were approximately 3-fold and 2-fold lower than in healthy volunteers, respectively.104

Initial theories as to why the blood exposure was reduced were cytochrome P450 (CYP450) metabolism to inactive metabolites and Fe(II) mediated inactivation. Metabolism studies identified two inactive CYP450 metabolites with hydroxylation of adamantane.105 However, i*n vivo* studies in a rat model into the effects of pre-treatment with the CYP450 inhibitor 1-aminobenzotriazole (1-ABT) on the pharmacokinetics of OZ277 found that the half-life and blood exposure were increased; however the improvement was not large enough to suggest a majority clearance by CYP450.106 This implies that the main cause of OZ277 poor PK is due to cleavage of the peroxide bond by liberated Fe(II) in the malaria infection.106 However, going some way to mitigate this, Arterolane was developed as a formulation with piperaquine (**66, Figure 9**) as a long acting partner, which was found to increase the AUC of Arterolane 1.5- to 2-fold – largely due to the impact of piperaquine as a CYP3A4 inhibitor which retards the *in vivo* metabolism of Arterolane. 107

Arterolane was released for the treatment of malaria in a fixed dose combination with piperaquine (known as Synriam) by Ranbaxy Laboratories (now merged with Sun Pharma) and was approved for use in India and some African countries.107,108

# Artefenomel (OZ439)



**Figure 10** Structure representation of the second generation trioxolane antimalarial Artefenomel (OZ439) and ozonide **72**

Having attributed the lower *in vivo* activity of Arterolane to degradation of the peroxide bond by Fe(II), Vennerstrom’s group carried out studies on 4” substituted dispiro-1,2,4-trioxolanes investigating the effect of 4” substitution on stability to Fe(II) mediated degradation.109 First order degradation rate constants and *P. falciparum* IC50 values were determined for a variety of substituents comprising a range of functionalities. It was found that, compared to the 2-amino-2-methylpropyl acetamide moiety of arterolane, a 4” phenyl substituent (**72,** **Figure 10**) increased stability to Fe(II) mediated degradation 150-fold. The rationale for this is the increased preference of the phenyl group for equatorial conformation which yields a greater energetic preference for the peroxide bond to take a less Fe(II) accessible axial conformation allowing greater stability. Additionally, the introduction of the phenyl substituent was not found to significantly decrease antimalarial potency.109 Extension of the 4”-phenyl moiety with the addition of a morpholine containing solubilising group allowed enhanced drug-like properties yielding the compound Artefenomel (OZ439, **71,** **Figure 10**).106

As with Arterolane, the key reaction in the synthesis of Artefenomel was the Griesbaum co-ozonolysis of a cyclohexanone with adamantan-2-one O-methyl oxime. The acetyl-protected cyclohexanone derivative **74** (**Scheme 9**) was synthesised from the parent phenol (**73**) by treatment with acetic anhydride and triethylamine.106 Griesbaum co-ozonolysis with **67** afforded the acetyl protected trioxolane **75**. The diastereoselectivity of the synthesis of 4” phenyl 1,2,4-trioxolanes by Griesbaum co-ozonolysis was shown by Vennerstrom to possess a *cis:trans* selectivity of >20:1 outperforming the equivalent step for synthesis of Arterolane.102 The acetyl protecting group was removed by alkaline hydrolysis affording the free phenol **76** which was coupled with *N*-2-chloroethyl morpholine hydrochloride to afford Artefenomel.106



**Scheme 9**: Synthesis of the second generation trioxolane antimalarial Artefenomel

Incubation of **71** in healthy rat blood showed a first-order degradation rate constant 15 fold lower than that of OZ277, indicating that the desired reduction in Fe(II) reactivity had been achieved. *In vitro*, Arterolane was found to possess potent antimalarial activity against K1 CQ resistant and NF54 CQ sensitive strains of malaria with IC50 values of 1.6 and 1.9 ng/ml respectively, indicating that despite reduced Fe(II) sensitivity, **71** retains Fe(II) reactivity sufficient for antimalarial effect. 106 More notably, OZ439 was screened in a 30 day *P. berghei* infected mouse survival model and was found to cure 3/3 mice after treatment with a single dose of **71** at 30 mg/kg. Moreover, exposure of mice pre-treated with a single dose of 30 mg/kg **71** to *P. berghei* displayed a significant protection, with 100 % of mice with no detectable parasites after 30 days – in the same assay Arterolane showed an average of 7 day survival with 0 % protection. 106 These *in vivo* results were supportive of the idea that **71** could be a potential single-dose cure for malaria, with rapid and persistent antimalarial activity. Furthermore, the improved *in vivo* activity correlates with improved PK properties with respect to Arterolane and dihydroartemisinin, with rat blood clearance of 33 mg/ml/kg, a lengthened elimination half life of 23 hours and a vastly improved oral bioavailability of 76 %.106 Artefenomel has recently been carried forward to clinical trials in humans. 33,110–113

Results of first-in-man studies for initial PK, safety and food effect in healthy male and female adults were published in 2012. **71** was investigated in single or multiple rising doses ranging from 50 to 1600 mg and in oral capsule or oral dispersal formulations.110 Maximum plasma concentration of both oral formulations was found to occur at around 3 hours with elimination half-lives of around 30 hours. The oral dispersal however was found to yield a greater plasma exposure of the drug. Unlike Arterolane, no dose proportionality was observed. In the multiple rising dose studies, no accumulation of **71** was observed. 110 When investigating food effect, it was found that significant differences existed between fed and fasted states. It was found that in the fed state maximum plasma concentration was obtained later at 5 hours and a greater plasma exposure was achieved. 110 Nevertheless, no serious adverse events were seen and Artefenomel was carried forward for phase 2 clinical trials.

Artefenomel **71** was initially investigated as a monotherapy in an open-label phase 2 trial in patients with *P. falciparum* and *P. vivax* malaria.Patients were treated with **71** in single doses of200, 400, 800 or 1200 mg followed by definitive treatment after 36 hours or sooner if no reduction in parasitaemia was observed – the definitive treatments were mefloquine or primaquine for *falciparum* and *vivax* malaria respectively. The key finding of this study was the median parasite clearance time which was measured at 4.1 – 5.6 hours and 2.3 – 3.2 hours for *falciparum* and *vivax* malaria respectively – these values corroborate the fast-acting mechanism of action of **71**.111 Additionally, consistent with prior studies, an elimination half-life of 46 – 62 hours suggesting a lengthy duration of action. 111 While no severe adverse events were recorded during this trial, two patients presented with elongated QTcF intervals compared to baseline, however the authors suggest that this is due QTcF shortening due to malaria infection – this is supported by the absence of QTcF elongation in dog studies.111



**Figure 11**: Structure representation of partner drugs DSM265, Piperaquine and Ferroquine, investigated in combinations wtih Artefenomel

Combination therapies including Artefenomel alongside a longer acting partner drug are of particular interest and have been the subject of a number of clinical trials. The earliest such study published in 2015 investigated fixed dose combinations of **71** with the long acting antimalarial DSM265 (**77, Figure 11**).112 DSM265 is a *plasmodium* dihydrooratate dehydrogenase inhibitor which displays both blood and liver stage activity.114 Combinations of 200 mg **71** with 50 or 100 mg DSM265 were investigated. Both doses displayed a rapid parasite clearance with half lives consistent with prior studies. Recrudescence was observed in both cohorts, occurring in 5/8 patients who received 100 mg DSM265 and in 5/5 patients who received 50 mg DSM265, in addition to **71**. 112

A later double-blind phase 2b trial of 800 mg **71** with either 640, 960 or 1440 mg piperaquine (**66, Figure 11**) was carried out on patients from Africa and Vietnam, with 85 % of the cohort being children.113 The primary end point of the trial was adequate clinical and parasitological response (ACPR) which was found to be 68.4 – 78.6 % with no significant differences between dosage of piperaquine. Notably, the parasite clearance in the patient cohort from Vietnam was found to be slower than the African cohort – this was attributed to a higher prevalence of K13 mutation conferring resistance to artemisinin. 113 This observation may imply a level of cross resistance of **71** with artemisinin derivatives.

A third study investigating the combination of **71** with ferroquine (**78, Figure 11**) is currently undergoing and results have not yet been published.33

As highlighted in early clinical trials of **71**, a significant food effect exists for OZ439 where enhanced bioavailability is observed in the fed state. As such, several investigations have been carried out on the relationship between physicochemical properties of **71** and solubility when formulated as the mesylate salt.115 In studies, both the mesylate salt and the free-base form of **71** have been observed to form insoluble aggregates and other solid-state structures. Interestingly, the mesylate salt displays a counterintuitive decreasing solubility at lower concentrations where, through dilution, the pH is effectively increased. At concentrations above the critical micellar concentration, the mesylate artefenomel-mesylate exists as soluble micelles whereas at lower concentrations it exists as a mixture of micelles as well as free-base aggregates. 115 As well as this, upon treatment with HCl, artefenomel mesylate can undergo anion transfer to produce the insoluble hydrochloride salt. Further neutralisation of the hydrochloride salt yields initially an unstable free base which rapidly converts to a more stable crystalline structure. These conditions are comparable to those of gastric fluid and the upper small intestine and as such similar conversions of **71** would be expected as gastric and upper small intestinal pH varies between fed and fasted state, providing insight into the food effect on **71** bioavailability. 115

Additional studies have been carried out on the effects of co-administration of **71** with milk with the hypothesis that co-administration would increase bioavailability of **71** similar to food effects seen in clinical trials.110,116 *In vitro* studies simulating the administration of **71** and simultaneous digestion of milk showed that the first unstable free-base form was partially solubilised by milk lipids liberated during digestion thus accelerating the conversion to the more stable free-base form 2.116 Additionally, compared to lipid free administration, a lower amount of overall crystalline free-base form was observed, potentially due to formation of colloidal structures with digested lipids. Notably, these effects were not observed in the absence of lipid digestion.116 While co-administration with milk is promising for increased bioavailability during monotherapy, investigations into co-administration with Ferroquine and milk showed a reduced bioavailabily of **71** when Ferroquine is included in the assay. This was attributed to competitive binding of Ferroquine to digested milk products which decreases the barrier to dissolution of ferroquine while preventing dissolution of **71**.117

As an amphiphilic molecule, **71** is a suitable candidate for nano-formulation and has been investigated as lyophilised and spray dried nanoparticles.118,119 The parent drug and prepared nanoparticles have been assessed for solubility and release characteristics in fasted and fed state simulated intestinal fluid (FaSSIF and FeSSIF respectively). The parent drug was found to possess poor solubility in FaSSIF conditions while both prepared nanoparticles showing similar solubilisation trends in both FeSSIF and FaSSIF conditions.118,119 Between the two formulations, the spray dried formulation displayed greater solubilisation and has the advantage of being more feasible for scalable production.119

# Mechanism of action of dispiro-1,2,4-trioxolanes

In the ozonide class of antimalarials, inorganic Fe(II) has been demonstrated to cleave the peroxide bond by preferential attack of the **O2** oxygen atom, close to the spirocyclohexane substructure, which is less sterically hindered than the **O1** oxygen atom, adjacent to the spiro-adamantane group.101,120 Byproducts expected from the **O1** oxygen attack in the ozonides are residual.120 Mechanistic studies using Fe(OAc)2 were performed on the active spiro-adamantanyl-1,2,4-trioxolane **79** (with the core substructure of OZ277101 and OZ439106), in the presence of the spin-trapping agent TEMPO.101 From the results, the authors postulated the formation of an *O*-centred radical **80**, which, through β-scission, generates the secondarycarbon-centred radical **81**. Carbon radical **81** was then trapped by TEMPO, affording the aminoxy adduct **82**. Lack of evidence in the detection of the products that would result from the β-scission of the spirocyclohexyl group shows that the ferrous ion preferentially associates with the less hindered peroxide oxygen, in this class of endoperoxides (**Figure 12, a** and **b**).95,101,109,120–124

In 1,2,4-trioxolanes, the stereochemistry at the 4’’position also plays a part in the rate of bioactivation of the peroxide bond. Dong *et al.*125 showed that the 4’’-*cis* diastereomer of **83** was 40-fold more effective than its 4’’-*trans* diastereomer **84**,*in vivo*. The *trans* diastereomer was found to have a higher plasma clearance and a lower oral bioavailability than its *cis* counterpart, suggesting a higher reactivity.This difference in reactivity can be clarified in terms of the cyclohexane ring's conformational dynamics in these compounds, in which the 4''-*trans* substituent favours the equatorial conformer. Therefore the peroxide bond is more exposed and consequently more susceptible to premature activation (**Figure 12, c**).126

Heme has been reported as an alkylation target of the antimalarial 1,2,4-trioxolanes, as observed in artemisinins. The reaction of OZ277 with heme gave the covalent heme adduct **85**121,122,127 (**Figure 5, d**).



**Figure 12**: (a) Proposed mechanism for iron-mediated degradation of 1,2,4-trioxolane with core substructure of OZ277 and OZ439; (b) TEMPO adduct; (c) OZ277 *cis-trans* diastereomers; (d) Proof of concept for heme alkylation by OZ277.

***Nonspecific alkylation of proteins in ozonides:*** A recent study with monoclonal antibodies and ozonides reported by Jourdan *et al.,*128 has shown that the antimalarial ozonides OZ277 and OZ439 also alkylate proteins in *P. falciparum*. However, unlike for artemisinins, following immunoprecipitation experiments with these antibodies, the authors could not detect the specific alkylated parasite proteins.

Chemical proteomics with activity-based probes of ozonides (**86-94**, Figure 13**)** were first performed by Ismail et al.69 The research group was able to determine that the clickable 1,2,4-trioxolane **(86-89**, Figure 13**)** and ART-probes revealed consistent overlapping profiles, with a total of 53 of the same parasite proteins, with a significant overlap (ca. 90%), both qualitatively and semi-quantitatively.

Alkylation of protein targets did not occur when experimenting with deoxo analogues, showing that the peroxide bond for activity is also crucial to promote protein alkylation. These results gave sound proof for a common mechanism of action between artemisinin and ozonides, also with prospects of a potential cross-resistance issue for the endoperoxides.

Jourdan *et al.*70 also reported a study involving similar dispiro ozonide based probes (**90**-**93**, **Figure 13**)that lead to the identification of 25 parasite proteins, from alkylation of the endoperoxides, in a peroxide-dependent behaviour. However, this study revealed a low degree of overlap in the alkylation signatures between the ozonides used, which were different from those of the previously reported studies. Explanations from the authors to rationalise these results included the nature of the chemical linker or the adamantane attachment site, which could have influenced the target protein binding by the chemical probes, and also differences in approach and controls used during the experiment.

Wei *et al.*129 synthesized dispiro ozonide probes **88** and **94** (**Figure 13**) to explore the protein alkylation capacity of primary and secondary *C*-centred radicals produced by the synthetic ozonides. The results have shown that most of the protein labeling *in situ* found in the study was due to the secondary-*C*-centred radical form of the ozonides, observed in both gel-free and gel-based proteomics methods. These findings corroborate the previous evidence of the preferred association with the less hindered peroxide oxygen, in this class of endoperoxides.



**Figure 13**: Structure representation of ozonide-based probes and their controls (**86-89** from Ismail *et al.*69, **90-93** Jourdan *et al.*70 and **94** Wei *et al.*129)

# 1,2,4,5-Tetraoxane Antimalarials

# RKA182



**Figure 14**: Structure representation of prototype 1,2,4,5-tetraoxane antimalarials and of the first generation tetraoxane antimalarial RKA182. aValues recorded for the di-tosylate salt.

Another synthetically attainable endoperoxide warhead is 1,2,4,5-tetraoxane, which was first reported by Vennerstrom *et al* in 1992, where a series of symmetrical bis-cyclohexyl tetraoxanes were found to possess antimalarial activity comparable to ART derivatives.130 This included WR148999 (**95**, **Figure 14**) which was found to possess an IC50 of 28nM against K1 *P. falciparum* as well as 27.7 day average survival after a single 100 mg/kg subcutaneous dose – however survival after a single oral 100 mg/kg dose was found to be just 7.7 days, indicating poor oral bioavailability.130,131 Further analogues of WR148999 have been reported, however none displayed greatly improved *in vivo* activity following oral dosing.132133 Since, Vennerstrom’s group has focussed mainly on the 1,2,4-trioxolane class of antimalarials, with development of the 1,2,4,5-tetraoxanes being continued by O’Neill.123

The antimalarial potency and stability of the tetraoxane as an endoperoxide warhead is exemplified in the prototype molecule dispirocyclohexyl-1,2,4,5-tetraoxane (**96, Figure 14**). While the analogous dispiro-1,2,4-trioxolane is unstable and shows no antimalarial activity, the tetraoxane equivalent is stable and displayed high antimalarial activity at an IC50 of 25nM. As with the development of OZ277, work by O’Neill saw one cyclohexane ring replaced with an adamantyl group to improve the stability of the tetraoxane core, while the remaining cyclohexyl ring was extended to include a solubilising group.123

In medicinal chemistry optimisation of the tetraoxane scaffold, over 150 molecules were screened for antimalarial activity and DMPK to yield target molecules. RKA182 (**97, Figure 14**) was selected for preclinical development owing to its balance of *in vitro* and *in vivo* activity, as possessing the most favourable PK properties of the synthesised first generation tetraoxane antimalarials. 123

RKA182 and other amide analogues were obtained in a 5-step synthesis (**Scheme 10**), beginning with the initial generation of the dihydroperoxide intermediate **99** by treatment of the cyclohexanone derivative **98** with 30 % hydrogen peroxide and formic acid. This was followed by Re2O7 catalysed condensation with 2-adamantanone **100** to afford the intermediate 1,2,4,5-tetraoxane **101**. Subsequent ester deprotection by treatment with KOH afforded the free acid **102** as a divergent point for the synthesis of a range of analogues. Target amides were synthesised in a one-pot amide coupling by initial formation of a mixed anhydride through treatment with ethyl chloroformate and, subsequently, with the amine of interest; for RKA182, 1-(1-methylpiperidin-4-yl)piperazine was used. 123



**Scheme 10**: Synthesis of the first generation tetraoxane antimalarial RKA182

The tosylate salt of **97** was found to be a potent antimalarial *in vitro*,with IC50 values of 0.8 and 1.1, against 3D7 CQ sensitive and K1 CQ resistant strains of *P. falciparum*. Moreover, **97** has been screened *in vitro* against clinical isolates for which ACT had failed, displaying in all 11 isolates improved activity compared to ARTs and IC50 values lower than 5 nM.123 Initial *in vivo* results were equally promising, with ED50 and ED90 values of 1.33 and 4.18 mg/kg in the *P. berghei* infected mouse model, after 4 daily doses; these values outperform artemether and artesunate. Survival assays in *P. berghei* infected mice yielded an average of 22 days following 3 x 10 mg/kg doses which, while outperforming artesunate, fell short of the desired single dose cure. 123

The blood stability of **97** was also characterised *in vitro* by incubation with both healthy and infected red blood cells. Percentage recovery of the parent compound after 4 hours was found to be 79 %, with no significant difference between healthy and infected blood cells. Furthermore, comparison of *in vivo* exposure profiles between healthy and infected mice showed no significant difference.123 This contrasts with Arterolane, where in phase 2 clinical trials a significant difference was observed between healthy volunteers and malaria infected patients.104 Further rat PK parameters were obtained, and though **97** possessed good stability to Fe(II) mediated degradation the elimination half-life was found to be just 2.38 hours for the tosylate salt. Bioavailability was similarly poor, at 38 % and 42 % in the rat and mouse models, respectively. 123

# E209



**Figure 15:** Structure representation of the second generation tetraoxane antimalarial E209

Owing to the incompatibility of the PK profile of RKA182 with a single dose cure, further improvements to stability were investigated on the tetraoxane scaffold. Following similar logic to the development to OZ439, the introduction of a 4” phenyl substituent on the cyclohexyl ring was investigated by O’Neill *et al*. The synthesis of several 4” phenyl analogues afforded the molecule E209 (**103,** **Figure 15**) which was selected for preclinical development owing to its activity *in vivo*.134

Two syntheses of E209 have been published to date, the earliest of which was the synthetic route used for the preparation of the initial series of second generation tetraoxanes.134,135 An optimised synthesis (Scheme 11) was published after optimising for yield of the tetraoxane forming step as well as cost of synthesis.135 Initial acetyl protection of 4-(4-hydroxyphenyl)cyclohexan-1-one (**104**) afforded **105** as the substrate for tetraoxane formation. The intermediate dihydroperoxide **106** was formed by treatment of **105** with 50 % hydrogen peroxide and formic acid, and was used in the subsequent Bi(OTf)3 catalysed condensation with **100** without further purification. The intermediate acetyl protected tetraoxane **107** was isolated in around 60 % yield – this represents an improvement on the 46 % yield reported for Re2O7 catalysed methods.134,135 The acetyl protecting group was removed by treatment with LiOH to afford the free phenol **108** which was subsequently coupled with 1-(2-chloroethyl)-4-fluoropiperidine hydrochloride to afford **103**.135



**Scheme 11**: Optimised synthesis of E209, **103**

E209 was shown to have an IC50 of 5.1nM against 3D7 *P. falciparum****,***showing equal activity to OZ439 in the same assay. E209 was also screened in ring stage survival assays against K13 mutant strains of *P. falciparum* posessing C580Y and R539T mutations shown to reduce sensitivity to ART. The C580Y mutation did not appear to confer cross resistance to E209 while the R549T mutant strains did show a modest but significant increase in parasite survival. 134

E209 also displayed potent antimalarial activity in the *P. berghei* infected mouse model, with an ED50 determined to be 4 mg/kg in a 4-day suppressive test after 3 doses. More importantly, E209 displayed a 66 % cure rate in the *P. berghei* infected mice 30 day survival assay, following a single oral dose of 30 mg/kg suggesting that, like Artefenomel, E209might be suitable for a single dose cure – although Artefenomel attained a 100 % cure rate in the same assay.134 E209was also evaluated against 3D7 *P. falciparum* in the humanised mouse model revealing that complete parasite clearance could be achieved with a single dose of 30 mg/kg. Additionally, this assay was used to determine that the optimal rate of parasite killing was achieved with blood exposure between 5 – 10 μg h/ml.134

E209was also assessed for PK and preliminary safety data, both *in vivo* and *in vitro*. *In vitro* microsomal clearance data showed low to moderate clearance values (36, 13, 10 and 25 μl/min/mg protein in rat, mouse, dog and human models repsectively.) *In vivo* PK showed that the bioavailability of E209was improved compared to RKA182, with measured values of 60, 82 and 40 % in rat, mouse and dog models, respectively.123,134

Like Artefenomel, the mesylate salt of E209 was found to possess varied solubility in different media. At pH 7.4 and under FaSSIF conditions E209 mesylate was found to possess poor solubility of < 0.1 and 121 µg/mL after 6 hours incubation respectively, whilst under FeSSIF conditions the solubility after 6 hours was 3870 µg/mL.134 This might suggest that there would likely be a food effect on administration of E209.

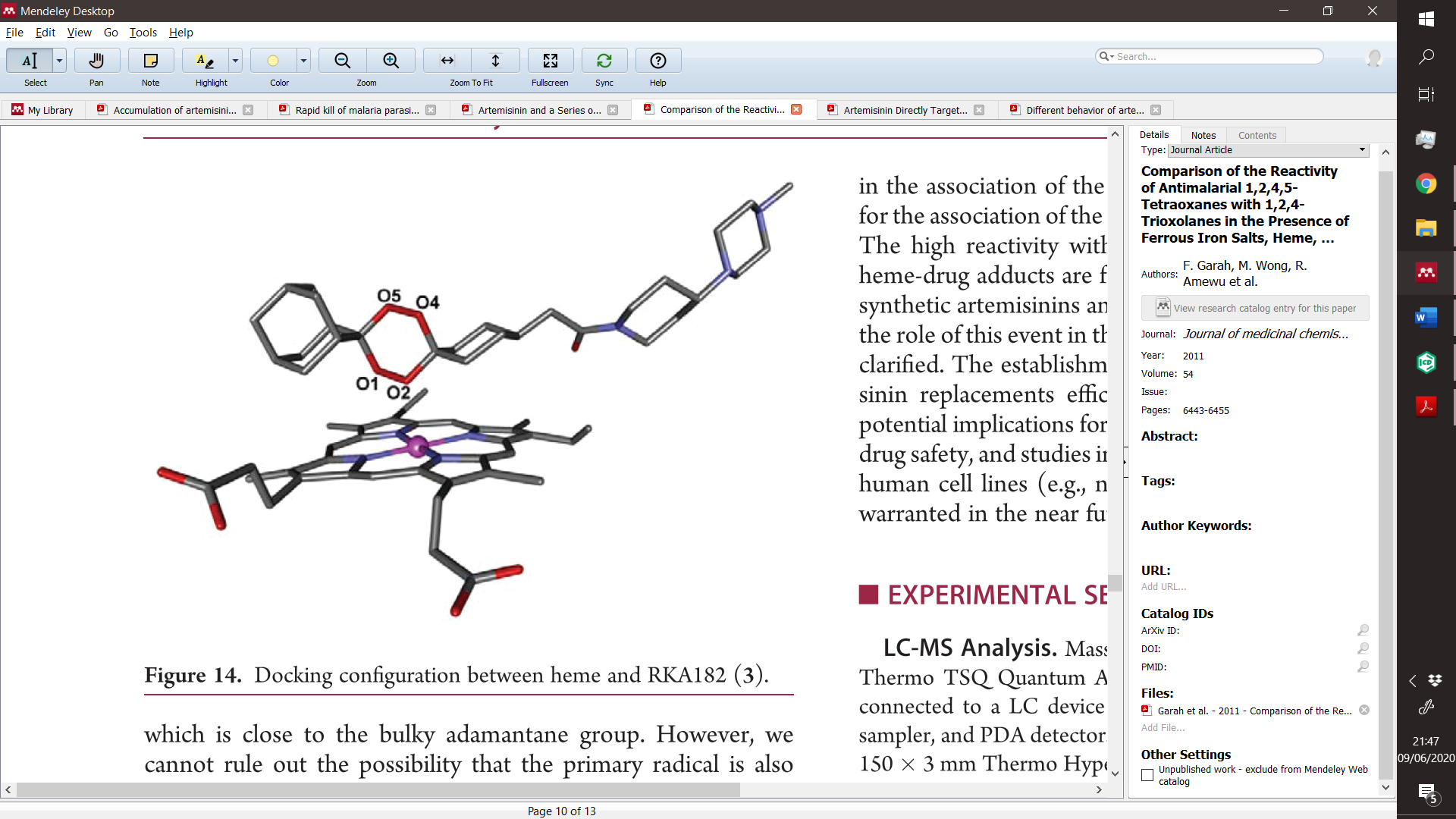
# Mechanism of action of dispiro 1,2,4,5-tetraoxanes

O’Neill and coworkers123 have characterised the potential mediators of the antimalarial 1,2,4,5-tetraoxane activity of RKA182 **97**. From mechanistic studies, using dispiro 1,2,4,5-tetraoxane **109** and ferrous bromide, the authors postulated the formation of regioisomeric alkoxy radicals **110** and **111**, which, through β-scission, isomerised to the primaryand secondary carbon-centred radicals **112** and **113**, respectively. These species were intercepted by TEMPO, generating the corresponding aminoxy adducts **114** and **115** (**Scheme 12, a**). Also, the reaction of **109** with heme gave the covalent adduct **116** (**Scheme 12, b**), the same heme adduct detected from the reaction of ozonide OZ277 (**Figure 12, d)**. Preference of iron for the O2 oxygen is also observed in 1,2,4,5-tetraoxanes, though the reported study shows subtle differences from 1,2,4-trioxolanes. While 1,2,4,5-tetraoxanes lead to the formation of either primary and secondary carbon-centred radical species from the reaction of Fe(II), with OZ277 and further 1,2,4-trioxolanes solely the generation of secondary carbon-centred radical species was observed.120

Modeling studies performed by Garah *et al*.136 with heme and RKA182 have shown that heme preferentially co-ordinates with the 1,2,4,5-tetraoxane via the less hindered oxygen O2 atom, instead of oxygen atom O1 (**Figure 16**). In docking conformations performed by the same group, they have found that the shortest distance was between heme Fe(II) and the O2 of RKA182, with values for the lowest energy conformations in the range of 2.4 Å-2.8 Å. This observation indicated a preference for the heme Fe(II) association with the less-hindered oxygen atom O2 of the O-O bond of RKA182, thus explaining the regioselectivity observed in alkylation and lipid peroxidation experiments.



**Scheme 12**: (a) Proposed mechanisms for (a) the iron-mediated degradation of RKA182 and its TEMPO adducts **114** and **115**; (b) The heme alkylation by **109**.



**Figure 16:** Representation of the docking configuration between RKA182 and heme (source in Garah *et al*.136).

# Conclusions

For decades, the treatment of malaria has been underpinned by ACTs containing a semi-synthetic ART derivative such as Artemether, Artether or Artesunate. Recent research into semi-synthetic ARTs has shed light on their promiscuous mechanism of action through target identification using molecular probe techniques. However, the rise of resistance to ART in South East Asia and the synthetic limitations of the ART scaffold have paved the way for the development of fully synthetic endoperoxide antimalarials.

Several classes of synthetic endoperoxide antimalarials have been described in literature utilising a variety of endoperoxide warheads, including 1,2-dioxanes, 1,2,4-trioxanes, 1,2,4-trioxolanes and 1,2,4,5-tetraoxanes. Since the report on Fenozan-50F in 1993 a number of these compounds have been carried forward to phase I and phase II clinical trials, while also advancing the understanding of the mechanism of action of the varied sources of peroxide through mechanistic studies.

Of the compounds described in this work, only the 1,2,4-trioxolane classes and 1,2,4,5-tetraoxane based antimalarials appear to still be in active development – the most recent publication pertaining to the development of Arteflene, Fenozan-50F, DU1301 or PA1103/SAR116242 was published in 2008. The results of a phase II clinical trial of Artefenomel with the partner drug Ferroquine are yet to be published and work into nanoformulation is underway. The most recent publication on the 1,2,4,5-tetraoxane antimalarials, published in 2018, related to the synthesis and characterisation of benzylmorpholine 1,2,4,5-tetraoxane antimalarials including the second generation tetraoxane antimalarial N205 which displayed similar *in vivo* activity to E209 in the 30 day survival assay.137 Looking forward, the incorporation of synthetic endoperoxide antimalarials into ACT regimens might be the solution to the looming threat of ART resistance.

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