Trends in Parasitology

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REVIEWS

Review

Ozonide Antimalarial Activity in the Context of Artemisinin-Resistant Malaria

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The ozonides are one of the most advanced drug classes in the antimalarial development pipeline and were designed to improve on limitations associated with current front-line artemisinin-based therapies. Like the artemisinins, the pharmacophoric peroxide bond of ozonides is essential for activity, and it appears that these antimalarials share a similar mode of action, raising the possibility of cross-resistance. Resistance to artemisinins is associated with *Plasmodium falciparum* mutations that allow resistant parasites to escape short-term artemisinin-mediated damage (elimination half-life ~1 h). Importantly, some ozonides (e.g., OZ439) have a sustained *in vivo* drug exposure profile, providing a major pharmacokinetic advantage over the artemisinin derivatives. Here, we describe recent progress made towards understanding ozonide antimalarial activity and discuss ozonide utility within the context of artemisinin resistance.

Highlights

First-line artemisinin-based combination therapies, containing semisynthetic peroxide antimalarials known as artemisinins, are failing in the Greater Mekong Subregion due to the emergence of artemisinin-resistant parasites.

The short *in vivo* half-life of artemisinins may allow resistant parasites to overcome artemisinin-induced damage until the drug is depleted.

Fully synthetic peroxide antimalarials, known as ozonides, have a similar mode of action to the artemisinins, but some have a prolonged *in vivo* half-life.

OZ439 (artefenomel) is the first long half-life peroxide to be tested clinically. It offers a potential single-dose oral cure for malaria when combined with a suitable partner drug.

Antimalarial combinations containing OZ439 should be investigated as therapeutic options for the treatment of artemisinin-resistant infections.

Ozonides: Synthetic Antimalarials Based on the Peroxide Bond of Artemisinins

Despite a decline in malaria mortality over the past decade, malaria continues to be a major global health problem and caused an estimated 219 million cases and 435 000 deaths in 2017, most of which were attributed to the deadliest malaria parasite species, *Plasmodium falciparum* [1]. Antimalarial treatment with **artemisinin-based combination therapies (ACTs)** (see Glossary) is the first-line option for uncomplicated infections caused by *P. falciparum* [2], and these highly efficacious medicines have contributed significantly to the decline in malaria incidence and mortality over the past two decades [1]. However, in recent years progress in reducing the burden of malaria has stalled in most malaria-endemic regions and the emergence of drug resistance severely threatens malaria control and elimination efforts [1]. Drug-resistant malaria parasites now limit the efficacy of all currently available antimalarial drug classes, including the artemisinin-based therapies [3]. Artemisinin resistance in *P. falciparum* was first reported in 2008 in clinical isolates from the Pailin province of western Cambodia [3] and has since been detected, with increasing prevalence [4], in Greater Mekong Subregion countries [5] and recently in eastern India [6], but is yet to emerge in Africa (Box 1).

The artemisinin derivatives used for the treatment of malaria are highly potent semisynthetic derivatives of the natural product artemisinin, the active constituent of a traditional Chinese herbal remedy extracted from the *Artemisia annua* plant. Although a series of highly potent semisynthetic derivatives were developed, artemether, artesunate, and **dihydroartemisinin (DHA)**, (Figure 1) are the predominant derivatives that are currently used for the treatment of falciparum malaria. Distinguishing features of the artemisinins include their fast onset of action and broad-spectrum activity against **asexual malaria blood stages**, which leads to the rapid reduction of parasite burden and resolution of symptoms in infected individuals [7]. All artemisinins have a sesquiterpene lactone backbone containing an **endoperoxide bond** that is essential for antimalarial activity [7]. The mechanism of action of the artemisinins is generally thought to involve activation of the peroxide bond by a reduced iron source, with the resulting drug-derived radicals inducing

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Box 1. *K13*-Mediated Artemisinin Resistance in the Greater Mekong Subregion

Artemisinin resistance in *P. falciparum* was first reported in 2008 in clinical patients from the Pailin province of western Cambodia, a region synonymous with the emergence of multidrug-resistant parasites [3,86]. In this region, artemisinin resistance compromises the therapeutic efficacy of all five World Health Organisation (WHO)-recommended ACTs (failure rate of more than 10%) [87]. Retrospective analysis showed that resistance likely emerged in Cambodia prior to 2001 and the widespread deployment of ACTs [87]. Starting with the epicentre in western Cambodia, resistant parasites spread or emerged independently throughout the Greater Mekong Subregion and are now firmly established in Vietnam, Laos, Thailand, and eastern and central Myanmar [5,88]. Resistance is emerging in northwestern Myanmar along the border with India [89] and in China at the Myanmar–China border [90,91]. There are now reports detailing the emergence of artemisinin resistance in eastern India [6,92]. Work is currently under way as part of the Tracking Resistance to Artemisinin Collaboration (TRAC) to further monitor the westward extension or emergence of the artemisinin-resistance phenotype.

Clinically, artemisinin-resistant parasites exhibit delayed clearance (parasite clearance half-life >5 h or day 3 positive parasitaemia) from the peripheral blood of patients treated with an ACT or artesunate monotherapy [5,61]. Although delayed parasite clearance following an ACT does not always lead to treatment failure, high failure rates are commonly observed in areas where there is concomitant resistance to artemisinin and the partner drug [93]. Periodic examination of blood smears shows that artemisinin-resistant *P. falciparum* parasites persist in the blood of infected individuals even after a standard 3-day treatment with a usually curative ACT. Parasite clearance times were found to correlate with increased *in vitro* survival of early ring-stage parasites (0–3 h post invasion) that are exposed to a 6 h pulse of DHA, mimicking the short *in vivo* elimination half-life of artemisinins [62,94]. More recently, a genetic molecular marker for artemisinin resistance was mapped to the *P. falciparum Kelch13* (*K13*) locus [95] and this gene has been associated with *in vitro* and *in vivo* resistance to artemisinins [88,96].

Multiple SNPs have been found throughout the *K13* gene [88,97–99]. Of the more than 200 known *K13* alleles, most of the nonsynonymous SNPs located within the propeller domain of this protein are associated with delayed parasite clearance [5,91,93,94,96,100]. Only a handful of these candidate mutations are validated by *in vitro* data as having a causal role in artemisinin resistance in Southeast Asian field isolates [93], including the F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, and C580Y mutations [93–96]. Some nonsynonymous *K13* mutations outside of the propeller region have also been linked to delayed parasite clearance in clinical studies from Southeast Asia, but these mutations have not been validated by *in vitro* experiments [93,100]. Low-frequency nonsynonymous *K13* mutations also exist in Africa but are not associated with clinical or *in vitro* artemisinin resistance [93,99,101,102]. Further investigations are needed to confirm whether a newly identified African *K13* polymorphism (M579I) from *P. falciparum* parasites – that exhibit a delayed *in vivo* clearance phenotype and increased *in vitro* ring-stage survival – represents the emergence of artemisinin resistance in Africa [103].

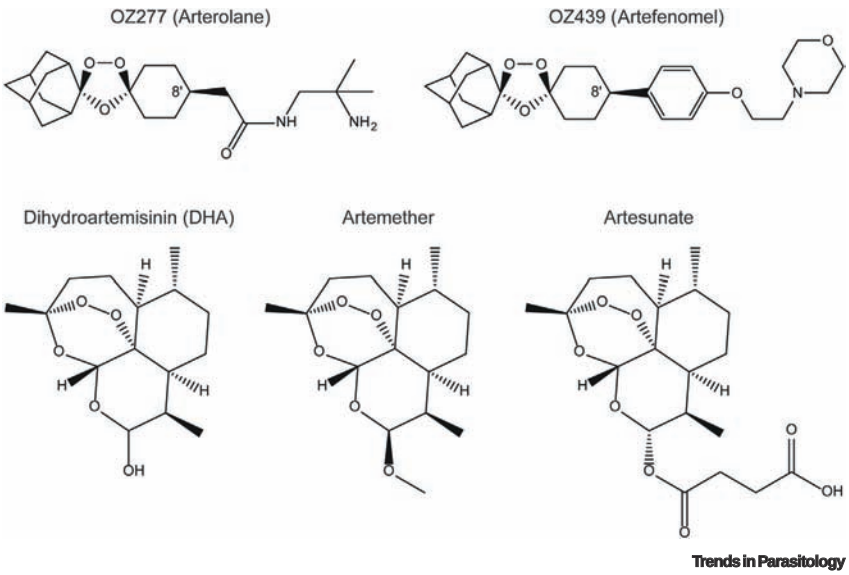

Figure 1. Chemical Structures of the Peroxide Antimalarials. The fully synthetic ozonide antimalarials, OZ277 (arterolane) and OZ439 (artefenomel), and the clinically used semisynthetic artemisinin derivatives, dihydroartemisinin (DHA), artemether, and artesunate, are shown.

Glossary

Artemisinin-based combination therapies (ACTs): these first-line treatments for uncomplicated *P. falciparum* infection combine a short-acting artemisinin derivative with a long-lived partner drug in a 3-day treatment course. There are five ACTs currently recommended by the WHO.
Asexual malaria blood stages: the phase of the *Plasmodium* life cycle in which parasites reside and asexually develop within the human red blood cell. For *P. falciparum*, the parasite matures over 48 h, developing from the early ring stage to form trophozoites and eventually schizonts. Clinical symptoms occur during the blood stage of infection, and most current antimalarials act on this stage to clear parasitaemia and relieve malaria symptoms.
Dihydroartemisinin (DHA): a semisynthetic artemisinin derivative and the active *in vivo* metabolite of clinically used artemisinins.
Elimination half-life: the time required for the concentration of drug in the body to decrease by half.
Endoperoxide bond: an oxygen bridge (-O-O-) that connects two carbon atoms within a molecule. This pharmacophore is an essential structural component for ozonide (and artemisinin) antimalarial activity.
Haemoglobin digestion: an essential catabolic process whereby the malaria parasite imports up to 75% of host red blood cell haemoglobin for degradation into its amino acid constituents. Haemoglobin digestion is performed by a series of proteolytic enzymes and provides a source of amino acids for parasite protein synthesis, as well as serving additional nonanabolic functions. A specialised acidic organelle, known as the digestive vacuole, is the major site of haemoglobin digestion within the parasite.
Haemozoin: the parasite converts toxic free haem that is released as a by-product of haemoglobin digestion into the insoluble, nontoxic and unreactive waste product, haemozoin. Synthesis of haemozoin is the main mechanism for haem detoxification in the parasite.
K13: the *P. falciparum* Kelch protein K13 (gene ID: PF3D7_1343700) is a genetic marker of *P. falciparum* artemisinin resistance in the Greater Mekong Subregion.
Ozonides: class of synthetic peroxide antimalarials in which the active

widespread oxidative damage and promiscuous alkylation of parasite biomolecules [8–10]. It has been reported that temporal accumulation of damaged protein and artemisinin-mediated inhibition of parasite proteasome function overwhelms the parasite cellular stress response mechanisms, eventually causing parasite death [11,12]. Despite their potent activity, the artemisinins have a relatively short *in vivo* **elimination half-life** (typically ~1 h in humans) [13], necessitating a 3-day treatment course in combination with a longer-acting partner drug to prevent parasite recrudescence.

The synthetic **ozonides** (or 1,2,4-trioxolanes) were originally designed to maintain the potent activity of the artemisinins, but to overcome supply limitations associated with the artemisinin starting material that resulted in significant cost fluctuations. The most promising ozonide to emerge from the first-generation series was OZ277 (later known as RBx11160 or arterolane, Figure 1). OZ277 was found to be fast acting, have comparable activity to clinically used semisynthetic artemisinins in *in vitro* assays against various *P. falciparum* strains, and be highly effective following oral dosing to *Plasmodium berghei*-infected mice, which was the primary preclinical *in vivo* efficacy model in use at the time [14,15]. In 2012, OZ277 became the first synthetic peroxide antimalarial to reach the market when it was approved for use as a fixed-dose combination with piperaquine (Synriam™) in India. Clinical studies with OZ277 [16–18] demonstrated that the elimination half-life was only two- to threefold longer than that of DHA [13] and the exposure in malaria patients was reduced compared with that in healthy volunteers [19]. Continued work on the ozonide series identified 'next-generation' compounds that had significantly longer elimination half-lives yet maintained the potency of the first-generation compounds both *in vitro* and *in vivo* [20,21]. OZ439 (later known as artefenomel, Figure 1) was selected for development, and Phase I and II clinical studies confirmed the long elimination half-life in humans, and demonstrated comparable exposure in healthy volunteers and malaria patients [22,23]. Based on its promising efficacy in preclinical models and its extended pharmacokinetic profile, OZ439 was proposed as a potential single-dose oral cure of uncomplicated malaria in combination with an effective partner drug [20]. OZ439 was originally combined with piperaquine [24] but is now in Phase IIb clinical trials in combination with the novel 4-aminoquinoline, ferroquine (ClinicalTrials.gov Identifier: NCT02497612).

In the current climate of artemisinin resistance, alternative treatment strategies are urgently needed to ensure that adequate malaria control is maintained into the future. Clinical deployment of triple combination ACTs is one short-term approach currently being investigated to overcome artemisinin resistance and extend the clinical lifespan of the artemisinins [25] (ClinicalTrials.gov Identifier: NCT02453308). However, new antimalarial drugs that overcome the clinical limitations of currently used artemisinin-based therapies are required to ensure successful malaria treatment in the advent of widespread ACT treatment failure. This review summarizes the current understanding of mechanisms associated with ozonide antimalarial action in *P. falciparum*. We also examine recent clinical and experimental studies that explore ozonide efficacy and cross-resistance potential with the artemisinins and highlight gaps in the existing literature in relation to the potential utility of ozonide-based therapies in the current environment of artemisinin resistance.

Iron-Mediated Activation of the Ozonide Endoperoxide Bond

The mechanism of action of ozonide antimalarials is peroxide-bond dependent. Similar to the artemisinins, ozonides are thought to be activated to cytotoxic species by an intraparasitic iron-based activator that induces reductive cleavage of the pharmacophoric peroxide bond [26]. Inorganic Fe(II) has been shown to cleave the peroxide bond of simple ozonides by preferential attack of the O1 oxygen atom adjacent to the cyclohexane ring, which is less sterically hindered than the O2 atom next to the adamantane substructure [27]. The resulting oxygen-centred radicals rearrange by β-scission into carbon-centred radicals, which are thought to be the primary toxic

compounds are characterised by a 1,2,4-trioxolane ring flanked by an adamantane on one side and a cyclohexane substituent on the other. The cyclohexane may be substituted with various side chains at the 8′ position to improve antimalarial, pharmaceutical, and pharmacokinetic properties.

Parasite clearance half-life: the time required for parasites in the blood to decrease by half during the log-linear phase of parasitaemia reduction.

Parasite reduction ratio (PRR): an estimation of the *in vivo* killing rate of antimalarials, which is measured as the ratio between parasitaemia when drug treatment is initiated and 48 h later, corresponding to one asexual parasite life cycle.

Ring stage: the earliest phase of the parasite intraerythrocytic asexual life cycle, in which the parasite takes on a ring-shaped morphology. In *P. falciparum* this stage lasts approximately 20 h, beginning immediately after red blood cell invasion and ending prior to the microscopic appearance of haemozoin.

Ring-stage survival assay (RSA): a method to determine the susceptibility of *P. falciparum* to a short exposure of an artemisinin derivative, and a useful tool for measuring the degree of artemisinin resistance *in vitro*. The *in vitro* RSA is performed on culture-adapted 0–3 h postinvasion ring-stage parasites, whereas the *ex vivo* RSA is performed on isolates freshly collected from malaria-infected patients. Parasites are pulsed with an artemisinin derivative (typically 700 nM of DHA) for 6 h to reflect the *in vivo* drug exposure in patients treated with an artemisinin, and parasite survival is determined 48–72 h later by comparison with an untreated culture. This method can be extended to antimalarials other than artemisinin derivatives but is most relevant for drugs that, like artemisinins, exhibit a short *in vivo* half-life.

Short-pulsed treatment: treatment in which parasites are exposed to a very short drug treatment before the drug is washed off and subsequent analysis of growth inhibition. This approach contrasts with routine assessment of antimalarial activity that is performed by exposing parasites to drug treatment for 48–72 h, corresponding to at least one asexual parasite life cycle. Pulsed treatment is useful for evaluating parasite stage- and time-dependence of antimalarial activity.

species responsible for ozonide activity. Within the parasite, haem released through the process of **haemoglobin digestion** is the most abundant form of intraparasitic iron [28]. Although most intraparasitic haem is sequestered as inert **haemozoin**, some haem can escape this detoxification process [28] and is available for peroxide bond activation. Chemical inhibition of parasite haemoglobin digestion using the cysteine protease inhibitor, E64d, has been shown to completely abrogate the antimalarial activity of **short-pulsed treatment** with OZ277 and OZ439 [29]. In comparison, chelation of free iron using iron chelators (which effectively deplete free intracellular iron available to the parasite) had a negligible impact on the potency of short-pulsed ozonide exposure [29,30] and only moderately antagonised ozonide activity when both ozonide and chelator pressure were maintained throughout the entire parasite asexual life cycle [31].

Recently, a quantitative assessment of intraparasitic ozonide stability found that haemoglobin-derived free haem (but not labile free iron) induced ozonide degradation and that drug degradation was positively correlated with both parasite asexual life cycle stage and load [30]. As shown previously [29], inhibition of antimalarial activity by inclusion of E64d also resulted in a substantial reduction in chemical degradation, providing evidence that ozonide activation and degradation are linked [30].

Collectively, this work shows that, like the artemisinins [7], a haemoglobin digestion product, most likely free haem, is the biologically relevant iron source responsible for intraparasitic ozonide peroxide bond activation. The observation that ozonide activity correlates with parasite asexual life cycle stages when haemoglobin digestion is most active also supports this hypothesis [29,30,32,33]. Alternatively, specific mitochondrial [34] or iron-independent activation mechanisms [35], and the direct inhibition of parasite targets [36], have also been proposed for the artemisinins, although additional mechanisms of action have not been demonstrated for the ozonides.

Indiscriminate Cellular Targeting of Ozonide-Derived Radicals

Ozonide-derived radicals are proposed to indiscriminately damage parasite components by inducing oxidative damage or covalently reacting with susceptible intracellular targets, thereby disrupting multiple essential parasite functions leading to parasite death. Similar to the artemisinins [7–9], promiscuous targeting of parasite proteins is thought to be a key feature of ozonide-mediated activity, and parasite stage- and concentration-dependent alkylation of a range of *P. falciparum* proteins by ozonide-derived radicals was recently described using monoclonal antibodies for OZ277 and OZ439 [37]. Protein alkylation, as detected by distinct bands on western blots, was found to be ozonide specific, indicating that OZ439 and OZ277 may alkylate distinct protein targets within *P. falciparum*; however, the identity of the protein targets detected, and their relevance to ozonide antimalarial activity, were not determined in this study. More recently, the application of a chemical proteomics methodology identified over 60 protein targets of ozonides after *in situ* treatment of **trophozoite stage** *P. falciparum* cultures with alkyne or azide functionalised ozonide click chemistry probes [38]. These probes were found to covalently modify a diverse range of parasite proteins involved in many vital cellular processes, and the probe-labelled proteins were predominantly localised to the cytosol and digestive vacuole of the *P. falciparum* parasite. The alkylation profile shared more than 80% similarity with a corresponding artemisinin probe, supporting the hypothesis that these drugs act by a similar, promiscuous targeting, mechanism of action [38]. Furthermore, preferential targeting of proteins with a glutathione binding motif suggested that free thiol groups of cysteine residues may act as the target for ozonide protein alkylation. The diverse alkylation profile indicated that ozonide parasiticidal activity likely involves disruption to a number of critical biochemical pathways; however,

Synchronised parasite cultures: cultures in which all the parasites of an *in vitro* culture are within a narrow age range in the asexual blood stage of the life cycle. *P. falciparum* is usually asynchronous during *in vitro* growth; therefore, generation of synchronised cultures is necessary when assessing stage-specific parasite responses.
Trophozoite stage: the period of the asexual blood stage life cycle in which the malaria parasite is the most metabolically active, growing significantly in size and consuming vast amounts of host cell haemoglobin. This phase of asexual growth is defined as the period after the microscopic appearance of haemozoin and prior to the onset of nuclear division.

metabolomic profiling of OZ277- and OZ439-treated *P. falciparum*-infected red blood cells revealed no major alterations in parasite metabolism [39].

In addition to parasite proteins, ozonides may also react with intraparasitic small molecules and damage parasite lipids and membrane structures. The reaction with biological haem is proposed to lead to ozonide activation and, given the high abundance of haem within the *Plasmodium* parasite, and its likely close proximity with activated drug, haem represents a potential biologically relevant target. Ozonides react rapidly with free haem in a simplified test system to form ozonide–haem adducts, and the extent of haem alkylation was found to be correlated with *in vitro* activity for a series of ozonide analogues [40,41]. There are currently no reports demonstrating haem alkylation in ozonide-treated *Plasmodium* parasites, but haem adducts from artemisinin-treated parasites have been described [10]. Unlike the artemisinins [42,43], *in vitro* alkylation of thiol-containing small molecules, such as cysteine and glutathione, have not been reported for ozonides. Whether ozonide alkylation of biological haem or other small molecules, such as thiols, is an important process in the antimalarial mechanism of action of the ozonides within *Plasmodium* spp. has not been thoroughly explored. Fluorescently labelled ozonide probes have been shown to accumulate within digestive vacuole-associated neutral lipid bodies (NLBs), which are lipids thought to be involved in haemozoin crystallisation [44]. As haemoglobin-derived haem is the most likely iron source responsible for peroxide bond activation [29], it has been proposed that the ozonides are activated by haem associated with NLBs or that ozonide-derived haem adducts accumulate within the NLBs, promoting oxidative damage of membrane lipids [44].

Figure 2 shows a schematic model that captures the available information regarding haemoglobin-derived haem activation of the ozonide peroxide bond, followed by indiscriminate alkylation or oxidative insult of vital intraparasitic components. A similar promiscuous targeting effect, involving oxidative damage and protein and haem alkylation, has also been proposed for the artemisinins [7] and provides a basis for artemisinin activity inducing disruption to multiple parasite biochemical pathways [45,46]. This mechanism of action is consistent with parasites engaging a generalised stress response in an attempt to overcome artemisinin-mediated damage [12,47].

Parasite Stage and Drug Exposure Time Impacts Ozonide Parasiticidal Activity

A complex relationship between parasite stage, drug concentration, and exposure time appears to govern ozonide antimalarial activity. The exposure of tightly **synchronised parasite cultures** to short-pulsed ozonide treatment was found to distinguish stage- and time-dependent differences in ozonide activity [29]. Although the ozonides display activity against all stages of the *P. falciparum* asexual life cycle [29,32,33], parasites are most sensitive to short-pulsed treatment during the trophozoite stage when there is a higher level of readily available haemoglobin-derived haem for peroxide bond activation [29]. **Ring stage** parasites were relatively insensitive to 1–3 h ozonide pulses but exhibited significantly improved sensitivity when the treatment duration was extended to more clinically relevant exposure periods (>6 h) [29,32,33]. Extending the duration of ozonide treatment reduced the difference in sensitivity between relatively insensitive mid-ring stage parasites and highly susceptible trophozoite stage parasites [29,32,33]. A similar exposure time dependence of parasiticidal activity has also been observed for artemisinin derivatives [48,49], further highlighting the importance of an adequate duration of exposure for peroxide-mediated parasite killing.

The temporal *in vitro* response to ozonide treatment was also found to vary depending on ozonide structure, such that the time required to render parasites 50% nonviable was longer for OZ439 compared with OZ277 [29]. Consistent with differential iron reactivity between first- and second-generation ozonides [50], it is possible that the *cis*-8′-phenyl substituent of OZ439 restricts access to the peroxide bond to a greater extent than the less bulky *cis*-8′-alkyl group of

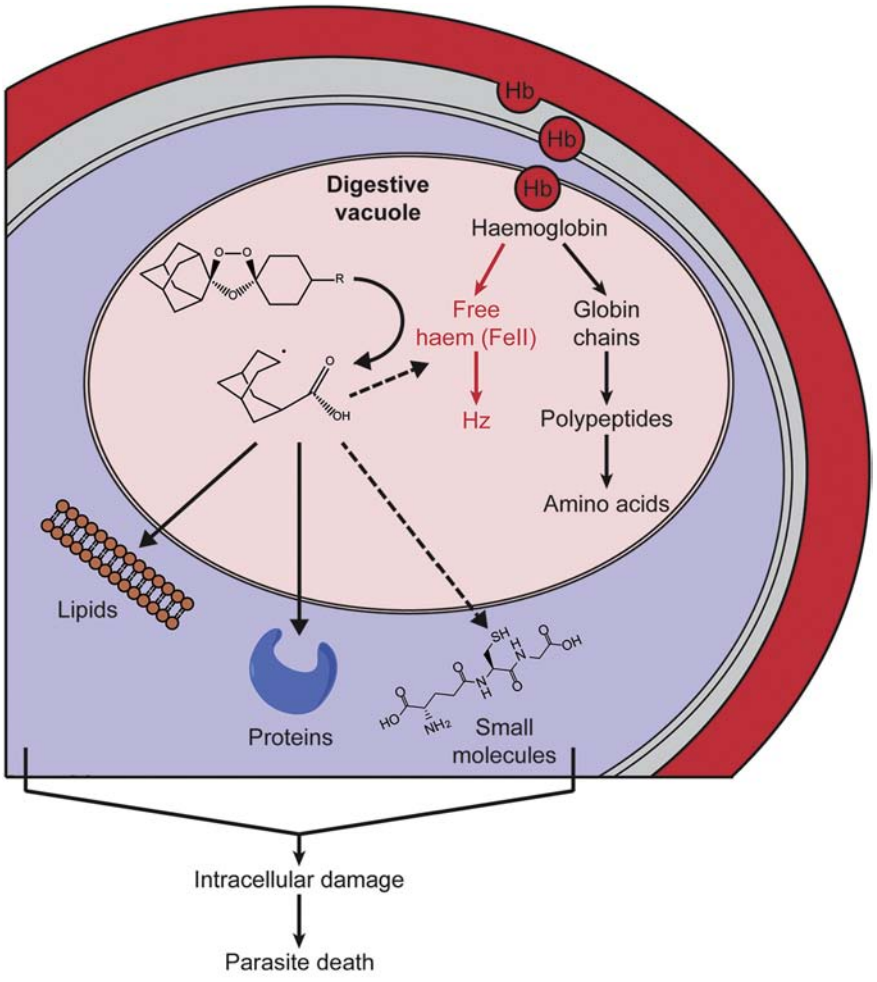

Figure 2. Proposed Mechanism of Action for Ozonide Antimalarials. Parasite haemoglobin digestion releases globin protein chains, which are degraded into their constituent amino acids, and toxic-free haem, which is mostly sequestered into inert haemozoin. Some free haem may escape detoxification and is available to activate the ozonides by cleavage of the peroxide bond, producing reactive radical species that rearrange to form carbon-centred radicals. These free radicals mediate antimalarial activity by indiscriminately reacting with a range of intracellular targets, including proteins and lipids (represented here by the unbroken arrows) and potentially small molecules such as glutathione and haem (represented by the broken arrows). This widespread intracellular damage ultimately leads to parasite death. Abbreviations: Hb, haemoglobin; Hz, haemozoin.

OZ277, resulting in slower haem-mediated activation and lower levels of parasite death with short exposure times. Given the differences in lipophilicity between OZ439 and OZ277, there are also likely to be differences in the bound (to protein or lipid) and free fraction of drug in culture medium (noting that only the free fraction can diffuse across membranes and enter the parasite to have an effect), which may also contribute to the lower *in vitro* activity observed for OZ439 with short pulse exposure. In comparison, the artemisinin derivative, DHA, exerts *in vitro* parasiticidal activity more rapidly than both OZ277 and OZ439 [29,51]. In one *in vitro* study using artemisinin-sensitive parasites (including three recently culture-adapted parasite lines from Cambodia and a reference line from Vietnam), the drug concentration that inhibited 50% survival of very early (0–3 h post invasion) ring-stage parasites exposed to a 4 h pulse of DHA, OZ439, or OZ277 was found to be

3.7 nM, 17.2 nM, or 33.1 nM, respectively [51]. Differences in the toxicity of the activated radicals, as well as differences in binding to Albumax or human serum present in the culture medium, may also impact the time-dependent variations in *in vitro* activity between peroxide antimalarials [29].

The reduced ring stage activities of OZ277 and OZ439 in *in vitro* assays parallels the extended **parasite clearance half-lives** seen in ozonide-treated *P. falciparum*-infected patients (approximately 9 h for OZ277 [17] and 4–6 h for OZ439 [23]) when compared with artesunate (approximately 2–3 h [5,52]). Similarly, in a controlled human malaria infection model, volunteers cleared parasitaemia with a half-life of approximately 3.6 h after a single 500 mg dose of OZ439 [53]. This corresponded to a **parasite reduction ratio (PRR)** at 48 h of 10^4. In the same model, artemether/lumefantrine and piperaquine were found to have PRRs within the range of 10^3–10^4 [54,55], whereas atovaquone/proguanil and mefloquine were slower acting, exhibiting PRRs of approximately 10 and 10^2, respectively [55,56]. Thus, while OZ439 exhibits a slower kill rate compared with the artemisinins, it clears parasitaemia at a similar rate or more rapidly than other existing antimalarials.

Pharmacokinetic Improvements Differentiate OZ439 from OZ277 and the Artemisinin Derivatives

The available evidence indicates that ozonides and artemisinins exert their antimalarial activity by similar mechanisms, both involving the production of toxic radical species that induce widespread cellular damage within treated parasites. However, a defining feature of the second-generation ozonide, OZ439, compared with its first-generation predecessors (including OZ277) and the artemisinin derivatives, is its superior *in vivo* elimination half-life (Table 1). Clinically used artemisinin derivatives are converted to DHA *in vivo*, and DHA in turn undergoes hepatic metabolism to form glucuronidated metabolites [57]. *In vitro*, DHA is also unstable in plasma [58], suggesting the potential for extrahepatic degradation as a contributor to its *in vivo* clearance. Both OZ277 and OZ439 also undergo hepatic metabolism to form predominantly hydroxylated metabolites on the adamantane ring, which are devoid of antimalarial activity [22,59]. *In vitro*, OZ277 degrades relatively rapidly in blood, and the rate of degradation is accelerated in the presence of *P. falciparum*-infected red blood cells [20]. When tested clinically, OZ277 had lower exposure in patients infected with *P. falciparum* compared with healthy volunteers [17,19], which may be related to the differential rates of degradation observed in blood *in vitro*. In comparison, OZ439 shows minimal degradation *in vitro* when incubated with either noninfected or infected blood [20], and shows comparable exposure in healthy volunteers and malaria-infected patients [22,23]. The extended half-life and increase in exposure of OZ439 compared with OZ277 (and DHA) likely results from the combined effect of improved blood stability, higher volume of distribution due to increased lipophilicity, and increased plasma protein binding.

Table 1. *In vivo* Elimination Half-Lives of Synthetic Ozonides and the Clinically Used Semisynthetic Artemisinin Derivative, Dihydroartemisinin

Peroxide antimalarials		*In vivo* elimination half-life (h)
Artemisinin derivative	DHA[a]	<1.5
Ozonides	OZ277[b]	3
	OZ439[c]	46–62

[a]Half-life measured in malaria patients from Thailand and Cambodia who were administered artesunate monotherapy (2 mg/kg). Data derived from Dondorp *et al*. [3].
[b]Half-life measured in malaria patients from Thailand and India who were administered combination therapy of OZ277 (150 mg) and piperaquine (750 mg). Data derived from Valecha *et al*. [16].
[c]Half-life measured in malaria patients from Thailand who were administered 200–1200 mg OZ439. Data derived from Phyo *et al*. [23].

As parasite viability varies as a function of peroxide exposure duration, parasites treated with short-lived peroxide antimalarials, like DHA or OZ277, will be exposed to only short-term radical-mediated damage until the drug is effectively depleted to sublethal levels. In contrast, the longer-lived synthetic peroxide, OZ439, will result in sustained *in vivo* exposure to lethal drug concentrations, which could translate to more effective treatment of clinical malaria infections with less frequent dosing [22,23,29,53,60] (Figure 3). As shown by McCarthy *et al*. using the controlled human malaria infection model, parasitaemia decreased as long as OZ439 concentrations remained above the minimum inhibitory concentration (MIC), which was estimated to be approximately 4 ng/ml and was maintained for >100 h following a single 500 mg dose [53].

Proposed *K13*-Dependent Artemisinin-Resistance Mechanisms

Clinical artemisinin resistance manifests as delayed parasite clearance following treatment with artesunate monotherapy or an ACT [5,61] and is associated with point mutations in the *P. falciparum* **K13** (*Kelch13*) gene (Box 1), and reduced susceptibility in the *in vitro* **ring-stage survival assay (RSA)** [62]. The precise molecular mechanism of *K13*-mediated artemisinin resistance is not fully understood, but it is generally thought that *K13*-mutant parasites have enhanced cytoprotective capabilities (including enhanced antioxidant defence and stress response pathways) that may allow ring-stage parasites to overcome artemisinin-induced damage [12,63,64]. Recent evidence suggests that this might include an augmented

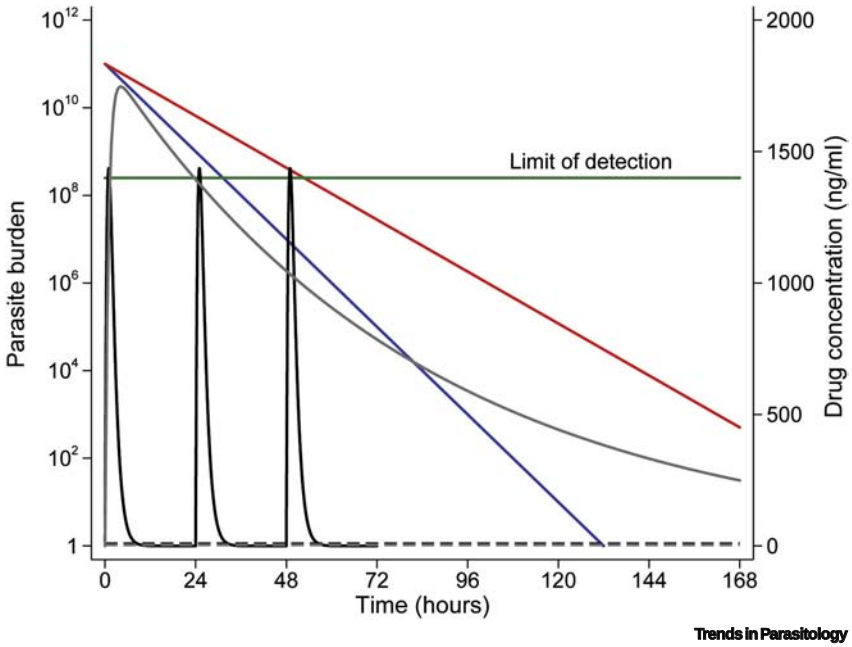

Figure 3. Simulation of the *in vivo* Pharmacokinetic/Pharmacodynamic Profile for Dihydroartemisinin (DHA) and OZ439. *In vivo* drug concentrations [black line for DHA (4 mg/kg per day for 3 days) and grey line for OZ439 (single dose of 800 mg)] and projected decline in parasite burden following treatment of artemisinin sensitive (blue line) and resistant (red line) parasites with DHA. The improved pharmacokinetic properties of OZ439 lead to an extended *in vivo* drug exposure duration and prolonged plasma concentrations that exceed the minimum inhibitory concentration (broken black line for DHA and broken grey line for OZ439). The estimated minimum inhibitory concentrations for DHA and OZ439 were 8.64 ng/ml [110] and 4.1 ng/ml [53], respectively. Pharmacokinetic profile simulations for DHA and OZ439 are based on data derived from Jamsen *et al*. [111] and Phyo *et al*. [23], respectively. Parasite reduction ratio for resistant parasites is based on data derived from Ashley *et al*. [5].

proteostatic stress response that engages the parasite ubiquitin–proteasome system [12,64,65]. Targeting the parasite proteasome with proteasome inhibitors was found to be synergistic with DHA and to sensitize *K13*-resistant parasites to artemisinins *in vitro* [12,66]. Furthermore, longitudinal genomic surveillance of *P. falciparum* isolates from northwest Thailand identified several background mutations in loci associated with the parasite ubiquitin–proteasome system that may be necessary for the spread of *K13* resistance, further implicating this pathway in the artemisinin resistance mechanism [67]. Transcriptomic analysis of *P. falciparum* isolates from malaria-infected patients from Southeast Asia revealed that artemisinin resistance is also associated with an upregulation of mRNA expression for genes involved in protein folding and repair, especially unfolded protein response pathways, as well as regulators of the cellular response to oxidative stress [64]. Increased glutathione levels have been implicated in rodent models of artemisinin resistance [68], adaptive responses against oxidative stress were found to be important in a *K13*-wild-type model of artemisinin resistance [69], and altered glutathione metabolism was recently linked to the *K13*-mediated artemisinin resistance mechanism [63,70]. Although the exact molecular function of K13 remains unknown, these findings are in line with its presumed role in regulating the *Plasmodium* parasite response to stress [7,11,71].

Another potential *K13*-dependent artemisinin-resistance mechanism implicates altered regulation of *P. falciparum* phosphatidylinositol-3-kinase (*Pf*PI3K) and the parasite vesicular transport system as being central to conferring artemisinin resistance [36,65]. It was proposed that *Pf*PI3K is an important target of artemisinins in ring–stage parasites [36], where the lipid product of *Pf*PI3K, phosphatidylinositol-3-phosphate (PI3P), plays an important role in protein trafficking from the parasite endoplasmic reticulum. DHA (the active form of all clinically used artemisinins) was found to directly and potently inhibit *Pf*PI3K and cause depletion of PI3P [36]. Modelling studies predicted that DHA may have selective access to the *Pf*PI3K active site due to proposed shape complementarity with the kinase-binding pocket and that an intact peroxide bond (i.e., nonactivated DHA) was necessary for reversible binding and for enzyme inhibition. However, the highly active artemisinin analogue artemisone (which contains the peroxide bond but is not metabolised to DHA) was not expected to abrogate *Pf*PI3K activity due to comparatively poor structural conformity with the kinase active site [36]. More recently, multiple artemisinin derivatives, including artemisone, as well as the ozonides OZ277 and OZ439, were found to covalently and nonspecifically react with recombinant *Pf*PI3K only after iron-mediated peroxide bond activation and the formation of drug-derived radicals [72]. This study also reported that *in vitro* potency against purified *Pf*PI3K poorly correlated with *in vivo* activity for the peroxide antimalarials tested; however, this could be related to difficulty in accurately quantifying enzyme inhibition for drugs that act covalently [72]. While these findings suggest that inhibition of *Pf*PI3K may be involved in the mode of action of peroxide antimalarials in the ring stage of infection, it is likely that inhibition or damage of other critical molecular targets also plays a role in parasite killing [8,9,38]. In the context of resistance, it was found that impaired K13-mediated ubiquitinylation and subsequent proteasomal degradation of *Pf*PI3K in parasites with a *K13* C580Y mutation led to elevated levels of the kinase and its lipid product, PI3P [36,65]. Synthetic elevation of PI3P levels in the absence of *K13* mutation was also found to induce artemisinin resistance [36]. More recently, K13 was shown to colocalise with PI3P vesicles of the parasite endoplasmic reticulum (and to a lesser extent at the food vacuole and apicoplast), and these vesicles were found to be enriched in proteostatic regulatory proteins of the unfolded protein response pathways [65]. Therefore, it was suggested that *Pf*PI3K may be an important resistance determinant for artemisinins in ring-stage parasites and that elevated PI3P levels in *K13*-mutants induce artemisinin resistance through endoplasmic reticulum-PI3P vesicular expansion of the parasite proteostasis machinery [65]. Interestingly, other parasite determinants of vesicular regulation may also be associated with

reduced artemisinin susceptibility [67,73,74], and longitudinal genomic surveillance of Thai parasite isolates indicated that background mutations in *P. falciparum* phosphatidylinositol-4-kinase may be necessary for the efficient spread of artemisinin resistance [67]. Additional parasite and host factors have also been associated with *K13*-mediated artemisinin resistance (Box 2), further highlighting the complex biochemical mechanisms underpinning artemisinin resistance.

The Potential for Cross-Resistance between Artemisinins and Ozonides

Several studies have been conducted to determine the potential for cross-resistance between the ozonides and the artemisinins. *In vitro* studies by Yang *et al*. showed that the duration of OZ277 and OZ439 exposure necessary for effective killing of artemisinin-resistant *K13*-mutant parasites was greater than in isogenic wild-type parasites, indicating a level of cross-resistance between the artemisinins and the ozonides [29]. Extending the duration of *in vitro* ring-stage exposure to reflect more clinically relevant ozonide exposure periods was found to render *K13* resistant ring-stage parasites susceptible to ozonide treatment, and simulations of *in vivo* response modelled in Cambodian lines expressing wild-type or R539T *K13* suggested that the prolonged elimination half-life of OZ439 could result in significantly improved efficacy against artemisinin-resistant parasites when compared with short half-life peroxides, like DHA and OZ277 [29] (Figure 3).

Other studies have shown that *K13* mutations only confer *in vitro* cross-resistance to OZ277, and most mutant parasite lines remain susceptible to short-pulsed OZ439 treatment [51,75,76]. In a recent study by Straimer *et al*., the degree of *in vitro* susceptibility to ozonides was shown to be dependent on the type of *K13* mutation. When tested against a panel of artemisinin-resistant and -sensitive parasite lines with *K13* mutations (C580Y, R539T, I543T) that were developed on four separate genetic backgrounds, including culture-adapted isolates from Cambodia and a Vietnamese reference line, the level of cross-resistance with ozonides was found to be greatest in parasites with the I543T mutation [51]. Parasites with the C580Y and R539T mutations displayed no apparent cross-resistance to a 4 h pulse with OZ439, while I543T mutants

Box 2. Roles of Other Parasite and Host Factors in *K13*-Mediated Artemisinin Resistance

Resistance to artemisinins is primarily conferred by mutations in *K13* [7], and recent studies indicate that the underlying resistance mechanism may involve perturbation to stress response pathways [11,12] and *Pf*PI3K-mediated vesicular transport systems [36,65] within the parasite. Further to this, additional proteins also show an association with *K13*-mediated artemisinin resistance and may provide a genetic background on which *K13* mutations are more likely to emerge [98,103]. These proteins include apicoplast ribosomal protein S10 precursor, ferredoxin, multidrug-resistance protein 2, and chloroquine resistance transporter. Reduced abundance of haemoglobin-derived peptides was also linked to artemisinin resistance [63], indicating that resistant parasites may have impaired haemoglobin uptake or digestion, potentially producing less haemoglobin-derived haem that is necessary for peroxide bond activation. Reported delays in parasite growth, quiescence, or dormancy may also contribute to artemisinin resistance through decreased peroxide-bond activation and/or minimization of subsequent cellular damage [103,104].

Clinical responsiveness to antimalarials is influenced by additional factors such as patient pharmacokinetic profiles, life cycle stage, distribution of the parasites, and levels of host immunity. Naturally acquired immunity, which develops after repeated exposure to *P. falciparum* and varies across populations, can slow parasite multiplication and accelerate parasite clearance as well as reduce the spread of *K13*-mutant parasites [105]. In Southeast Asia the highest prevalence of parasites with *K13* mutations and delayed parasite clearance is found in areas where immunity is lowest [106]. Studies of immunity in therapeutic efficacy studies of artemisinin derivatives have shown that parasite clearance is faster in patients with higher *P. falciparum* antibody levels [106–109] and that immunity may have the greatest impact on clearance of *K13*-mutant parasites. This may reflect the greater role of immunity in the removal of undamaged *K13* parasites that are less susceptible to pitting (the process whereby the spleen rapidly removes damaged intraerythrocytic ring-stage parasites) [106]. Therefore, immunity should be taken into consideration when determining an individual patient's resistance status, and when validating *K13* mutations associated with resistance to artemisinins or other peroxide antimalarials [106].

displayed significantly increased ring stage survival (3.3% vs 0.7%). Importantly, even though *P. falciparum* parasites with the I543T mutation currently represent a minor population in the Greater Mekong Subregion, it raises the prospect that, if OZ439 were to be deployed in the field, this *K13* allele could rapidly become widespread.

While it is possible that OZ439 is less affected by the mechanisms that cause *K13*-mediated artemisinin resistance, differential *in vitro* susceptibility may also reflect subtle, but important, variations in experimental protocols such as differences in the parasite synchronisation and life cycle stage, parasitaemia, haematocrit, and efficiency of the washing procedures, as OZ439 is particularly lipophilic and difficult to remove from the culture system following short-pulsed drug treatment [30].

Further complicating the understanding of *K13*-mediated artemisinin resistance is a recent study in an *Aotus* monkey malaria model that showed there was no difference in frequency of recrudescent infections or **parasite clearance half-life** following artesunate treatment between *K13* C580Y mutant and wild-type *P. falciparum* parasites, despite resistant parasites displaying enhanced survival in the RSA [77]. Although there are likely to be differences in host–parasite interactions between splenectomised *Aotus* monkeys and spleen-intact humans, the findings from this study indicate that *in vitro* RSA results may not always be predictive of resistance *in vivo*. This complex nature of artemisinin treatment response suggests that factors in addition to RSA sensitivity, such as drug pharmacokinetics, patient immune status, and choice of partner drug, will influence treatment outcomes, and this highlights a critical need for *in vivo* studies to evaluate the efficacy of ozonides in the context of artemisinin-resistant malaria. Recent clinical studies evaluating the therapeutic efficacy of OZ439 as monotherapy, or in combination with the partner drug piperaquine, have displayed mixed results.

In a Phase IIa exploratory study, single-exposure OZ439 (200–1200 mg) was found to be effective in clearing parasitaemia in patients infected with either *P. falciparum* or *Plasmodium vivax*. Median parasite clearance half-life estimates ranged from 4.1–5.6 h for *P. falciparum* and 2.3–3.2 h for *P. vivax* [23]. Of the 31 patients with *P. falciparum* malaria that had parasites genotyped for *K13* status, 19 were found to be infected with parasites having an artemisinin resistance-associated *K13* mutation (including P441L, N458Y, F446I, P527H, G538V, C580Y, A675A/V, or P667T). These patients cleared parasitaemia slightly (but not significantly) more slowly than patients infected with *K13* wild-type parasites [median parasite clearance half-life of 5.5 h (n = 17) vs 4.4 h (n = 11)]. In other studies, artesunate monotherapy (4 mg/kg) produced parasite clearance half-lives of 2–3 h in artemisinin-sensitive infections and 6 h or longer in resistant infections [5,52,78]. Based on reported parasite clearance half-lives, therapeutic responses to OZ439 appear to be slower than to artesunate against artemisinin-sensitive parasites and marginally faster than artesunate against artemisinin-resistant parasites [23].

In a subsequent multisite study in six African countries and in Vietnam, therapeutic efficacy of a single-dose treatment with OZ439 (800 mg) and piperaquine (640–1440 mg) ranged from 68% to 79% at day 28 post dose, which was below the ≥95% efficacy target [24]. There was a high frequency of artemisinin resistance-associated *K13* mutations in the Vietnamese cohort (70.1%), whereas a low level of *K13* mutation (<2%, none associated with artemisinin resistance) was detected in the African participants [24]. Parasite clearance half-lives were found to be extended in patients infected with artemisinin-resistant parasites (median values were 5.5–8.3 h depending on the *K13*-mutation vs 2.6 h for the wild type) [24]. It is noteworthy that resistance to the partner drug, piperaquine, has emerged in Vietnam [79,80] and could have contributed to the lower than expected efficacy of single-dose OZ439/piperaquine combination therapy; however,

the piperaquine susceptibility status of the *P. falciparum* parasites in this study is unknown. Variable drug exposure was a significant limitation in this study and may explain why substandard efficacy was also observed in the Africa cohort. The high rate of vomiting (29%) may have contributed to lower drug exposure, particularly in young children, due in part to the increased risk of vomiting associated with piperaquine treatment among this age group [81] and the poor tolerability of the formulation that was used for oral administration of OZ439. OZ439 is associated with significant formulation challenges that stem from its poor aqueous solubility profile [82], and alternative formulation approaches are currently being explored to improve the bioavailability and tolerability of orally administered OZ439 [83].

Further clinical studies using optimised OZ439 formulations combined with effective partner drugs, and that include close monitoring of drug pharmacokinetics, will be needed to determine the efficacy of long half-life ozonides in regions that are endemic with artemisinin-resistant parasites. Additional factors, such as potential side effects (e.g., neutropenia [24]) and any selection favouring mutant-*K13* parasites (such as those with the I543T allele) will also need to be carefully considered when assessing the potential clinical utility of OZ439-based therapies. Phase IIb clinical trials of OZ439 in combination with ferroquine, which is active against chloroquine-, mefloquine- and piperaquine-resistant *P. falciparum* [84,85], but has never been clinically deployed as single therapy, are currently ongoing (ClinicalTrials.gov Identifier: NCT02497612).

Concluding Remarks

Combination treatments based on fully synthetic, long half-life ozonides are currently being investigated as novel malaria therapeutics. Ozonides are thought to act by inducing widespread damage to the malaria parasite following specific intracellular activation by a reduced iron source, and this mechanism appears to overlap with that of currently used artemisinins, which are associated with treatment failures due to the emergence of artemisinin-resistant parasites in the Greater Mekong Subregion. All artemisinin derivatives have a short *in vivo* elimination half-life, and this pitfall may allow resistant parasites to escape artemisinin treatment, especially when there is reduced susceptibility to the ACT partner drug. In contrast, the leading ozonide clinical candidate, OZ439, has a prolonged *in vivo* exposure profile, which could offer a significant pharmacokinetic advantage compared with short-acting peroxides in the context of artemisinin resistance. While recent *in vitro* and clinical data suggest that ozonides have reduced activity against ring-stage *K13*-mutant parasites and induce longer parasite clearance times (compared with wild type), other studies have shown that OZ439 is only cross-resistant in parasites that carry the *K13* I543T mutation. The question remains as to whether the extended duration of exposure of OZ439 is sufficient to overcome clinical artemisinin resistance, a question that can only be addressed through further clinical studies (see Outstanding Questions). Additional work to resolve the biologically important molecular targets and parasite biochemical pathways vital for ozonide action and resistance will also be necessary and may aid in the selection of appropriate partner drugs for combination therapy.

Outstanding Questions

To date, most research on the ozonide antimalarial mechanism of action has focused on the protein targets of simple ozonide probes, which have different iron-reactivity profiles and physicochemical properties compared with the second-generation ozonide clinical candidate, OZ439. Could the second-generation ozonides target different intraparasitic proteins compared with their first-generation predecessors (and the artemisinins), and might this have implications for emergence of resistance or artemisinin cross-resistance?

What are the contributions of nonprotein targets, such as haem, glutathione, and lipids, to the mechanism of action of ozonide antimalarials, and what proportion of activated drug targets these chemical species versus proteins?

Are particular intraparasitic components more crucial than others for ozonide activity, and can this knowledge be harnessed to optimise ozonide-based drug combination regimens, protect against the emergence of resistance, or develop new nonperoxide antimalarials?

What are the biochemical consequences of target alkylation that facilitates ozonide-induced parasite death?

What is the underlying molecular mechanism of *K13*-mediated artemisinin resistance?

Even though ozonides and artemisinins appear to exert their activity by similar mechanisms, will the extended *in vivo* half-life of OZ439 (and potentially other long half-life peroxides) translate to improved clinical activity against *K13*-mediated artemisinin resistant parasites?

Acknowledgments

The authors thank Professor Jonathan Vennerstrom for helpful discussions. This work was supported by NHMRC Project Grant #1128003, NHMRC Career Development Fellowship #1148700 (D.J.C.) #1166753 (F.J.I.F.), and NHMRC Senior Researcher Fellowship #1104975 (J.A.S.). This work was supported in part by the Australian Centre of Research Excellence on Malaria Elimination (ID# 1134989).

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