Mode of Action of Synthetic Antimalarial Peroxides

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Prof. Pascal Mäser (Fakultätsverantwortlicher und Dissertationsleiter) und

Prof. Dominique Soldati-Favre (Korreferentin)

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Prof. Dr. Martin Spiess

Dekan der Philosophisch-

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Summary

Malaria is one of the most widespread infectious diseases which caused an estimated 212 million cases and 429,000 deaths worldwide in 2015. Today, artemisinin-based combination therapy (ACT), a combination of the fast-acting artemisinin with a longer lasting drug, is recommended to treat uncomplicated *Plasmodium falciparum* infections. Artemisinin is of highest antimalarial potency and selectivity. However, as a natural product, artemisinin and its derivatives also have drawbacks. In 2004 Jonathan Vennerstrom reported the development of synthetic peroxides that might overcome these shortcomings. A first-generation synthetic peroxide, OZ277, was registered with piperaquine for combination therapy in India in 2012 and the next-generation ozonide OZ439 is being tested in Phase IIb clinical trials in combination with piperaquine or ferroquine. The exact mode of action of synthetic peroxides and artemisinins, both thought to have similar modes of action, is not known. Recently, prolonged parasite clearance rates in patients after treatment with artesunate or ACTs were published, indicating a starting artemisinin resistance. Therefore, the fear of cross-resistance of artemisinin-resistant clinical isolates against OZ439, the leading candidate in the drug pipeline, overshadowed its superior properties.

In this PhD thesis the mode of action of synthetic peroxides was further elucidated. First, I used monoclonal antibodies raised against the adamantane-portion of ozonides to perform subcellular localization studies and to identify alkylation signatures in *P. falciparum*. Since it was not possible to identify the alkylated parasite proteins with antibodies, I set up a novel approach using click chemistry with newly synthesized alkyne derivatives of antimalarial peroxides. Further, the potential of cross-resistance by an artemisinin-resistant clinical isolate to OZ439 was tested.

I showed that alkylation of proteins by OZ277 and OZ439 takes place in the cytoplasm and other structures such as the nucleus and the food vacuole, in agreement with previous findings. Comparing the alkylation signatures of artemisinin and ozonides, I identified common targets to

both drugs, such as the protein PFNF54_01699. Overall, the ozonides had a larger target space than artemisinin. In addition, we found, that there is no cross-resistance *in vitro* of an artemisinin-resistant clinical *P. falciparum* isolate to OZ439, indicating that OZ439 has the potential to circumvent artemisinin resistance. Nevertheless, larger clinical studies are needed to investigate if OZ439 is effective against artemisinin-resistant malarial parasites.

Abbreviations

AA2	artemisinin-alkyne 2
ACT	artemisinin-based combination therapy
Cam3.II^{C580Y}	Cambodian isolate carrying a C580Y mutation
Cam3.I^{R539T}	Cambodian isolate carrying a R539T mutation
deoxyAA2	deoxy-artemisinin-alkyne 2
EMP	erythrocyte membrane protein
IRS	indoor residual spraying
ITNs	insecticide treated mosquito nets
K13	Kelch 13 propeller domain
kDa	kilodalton
MDR1	multidrug resistant protein 1
OZ	ozonide
RBC	red blood cells
Swiss TPH	Swiss Tropical and Public Health Institute
TAMRA	6-carboxytetramethylrhodamine
WHO	World Health Organization

1. Introduction

1.1. Malaria

Malaria is one of the most important tropical diseases as it caused an estimated 212 million cases and 429,000 deaths worldwide in 2015 (World Health Organization (WHO) Malaria Report, 2016). The majority of malaria deaths (70% of the global total) affect children below the age of five, which means that every two minutes a child dies from malaria (WHO Malaria Report, 2016). Malaria cases are mostly restricted to tropical and subtropical areas, whereby the highest numbers of cases and deaths occur in Africa (90% and 92%, respectively in 2015), followed by South-East Asia and the Eastern Mediterranean Region (WHO Malaria Report, 2016). The infectious disease is caused by protozoan pathogens of *Plasmodium* spp., first discovered in 1880 by Charles Louis Alphonse Laveran, belonging to the phylum *Apicomplexa* (WHO Malaria Report 2016; Hempelmann et al., 2013; Cox, 2010). Of the five different *Plasmodium* species, namely *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*, that cause malaria in humans, *P. falciparum* leads to the most severe form of malaria, called malaria tropica. It was responsible for 99% of malaria deaths worldwide in 2015 (WHO Malaria Report 2016).

Plasmodium falciparum has a complex life cycle including an insect vector as the first host and a human being as an intermediate host. During a blood meal of female mosquitoes belonging to the genus *Anopheles*, sporozoites are injected with the saliva into the human host. They penetrate blood vessels and enter the bloodstream where they quickly reach hepatocytes in the liver. The parasite divides asexually in the hepatocytes, a process called schizogony, resulting in tens of thousands of merozoites which are released into the bloodstream and invade red blood cells (RBCs). Inside the erythrocytes they develop into ring forms, trophozoites and finally schizonts. This process takes 48 hours and is called erythrocytic schizogony. It ends with the rupture of schizonts, each containing 16-32 merozoites, which then will invade new RBCs. A small fraction of merozoites develop into

male and female gametocytes, the stage infective for mosquitoes. When these are taken up during a blood meal, the sexual reproduction takes place in the mosquito, resulting in up to 10,000 sporozoites which migrate to the salivary glands and can infect new human hosts (Mawson, 2013; Cowman et al., 2016; Phillips et al., 2017). Clinical symptoms of malaria manifest after rupture of infected RBCs and include fever, chills, headache, muscle aches, anemia and digestive symptoms (Mawson, 2013; Cowman et al. 2016).

1.2. Prevention and treatment of malaria

Since malaria transmission is reported in 91 countries, prevention strategies are indispensable (WHO Malaria Report 2016). The main prevention strategies are vector control with insecticide treated mosquito nets (ITNs) and indoor residual spraying (IRS), which both effectively reduce malaria transmission in the African Region (WHO Malaria Report 2016, Lengeler, 2000). While it is estimated that ITNs are responsible for 50% of the decline in parasite prevalence among children 2-10 years of age between 2001 and 2015 (WHO Malaria Report 2016), the impact of IRS is difficult to be valued because of limited randomized trial data. Nevertheless, it is assumed that IRS have a similar impact than ITNs (WHO Malaria Report 2016). Currently, twelfe insecticides belonging to four different chemical classes exist, but emerging resistance of *Anopheles* mosquitoes to insecticides is a major problem. Therefore, efforts were undertaken to screen 4 million compounds, resulting in 3 new insecticides entering development primariliy to be used for ITNs (MalEra, 2017). Another prevention strategy is intermittent preventive treatment of malaria in pregnancy, which reduces perinatal mortality, and intermittent preventive treatment in infants, providing protection against malaria (WHO Malaria Report 2016). An up to 80% reduction of the incidence of clinical cases and severe malaria in children below the age of five can be achieved by seasonal chemoprevention of young children (3-59 months) in areas where malaria is seasonal (WHO Malaria Report 2016; MalEra, 2017). A further prevention strategy is the development of a vaccine. RTS,S/AS01 is the sole candidate having completed phase III testing and shown protection, albeit only partial (WHO Malaria Report 2016; Vandoolaeghe et al., 2016). After four doses, clinical incidence was reduced by 39% and severe malaria by 31.5% in young children 5-17months of age (WHO Malaria Report 2016; MalEra, 2017). Thus, chemotherapy remains a main pilar of the global fight against malaria. For the effective treatment of malaria, several drugs exist, with artemisinin-

combination therapy (ACT) being the recommended first-line treatment of uncomplicated malaria tropica (WHO Malaria Report 2016, Phillips et al., 2017).

1.3. Artemisinin

In the 1950s malaria parasites resistant to chloroquine, which was widely used to treat malaria, emerged, resulting in an urgent need for new drugs (Tu, 2011; Cowman et al., 2016).

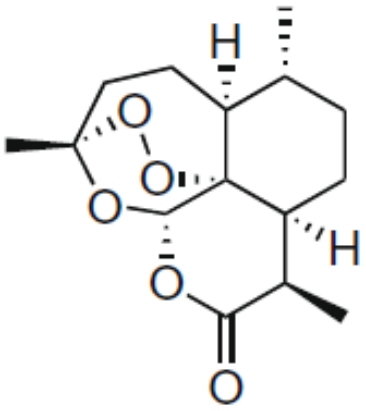

Figure 1 Chemical structure of artemisinin

During the Vietnam war, the US army launched a drug discovery program that resulted in the development of mefloquine (Su et al., 2015; Kitchen et al., 2006). In China, the Project 523 was launched in 1967, in which more than 2,000 Chinese herb preparations used in traditional remedies were investigated (Tu, 2011; Su et al., 2015). Youyou Tu and coworkers found, that extracts of *Artemisia annua*, a plant also called sweet wormwood or qinghao, showed promising inhibition of parasite grow. But these data were not reproducible and an intense review of literature was done. This resulted in the idea that the high temperatures used in the extraction could have destroyed the active ingredient (Tu, 2011). In 1971, a natural extract was found which was active against mice infected with *P. berghei* and monkeys infected with *P. cynomolgi*, when using lower temperatures for extraction. Clinical efficacy of this extract was also tested in patients infected with *P. falciparum* or *P. vivax*, showing fast disappearance of symptoms. In 1972 the active ingredient was isolated and called qinghaosu or artemisinin (Tu, 2011; Klayman, 1985; Su et al., 2015). The discovery of artemisinin was appreciated in 2015 with the Nobel prize in physiology or medicine awarded to Youyou Tu (Su et al., 2015). Since it was found that artemisinin is poorly soluble in water, efforts to modify the structure were undertaken. Beside the discovery of dihydroartemisinin by Youyou Tu, which showed improved water-solubility and treatment efficacy, other artemisinin derivatives such as artesunate, artemether or arteether were synthesized (Tu, 2011, Su et al., 2015, White, 1994, Meshnick, 2002). Today, fixed dose ACTs are the first-line

treatment of uncomplicated *P. falciparum* infections. Thereby Coartem (consisting of artemether and lumefantrine) and Coarsucam (amodiaquine-and artesunate) are the main combination therapies used. For severe malaria tropica, intramuscular injections of artesunate are recommended (Anthony et al., 2012; Wells et al., 2015; White, 2008).

1.4. Mode of Action

Already in 1975, the stereo-structure of artemisinin was investigated and found to be a sesquiterpene lactone, which differed from the structures of all perviously used antimalarial drugs, indicating a different mode of action (Tu, 2011; Klayman, 1985; Meshnick, 2002). When seven other sesquiterpene lactones isolated from *A. annua*, all lacking the peroxy group, were tested and found to be inactive, it was evident that the endoperoxide bridge was essential for antimalarial efficacy (Klayman, 1985). This finding was supported by the observation that only small amounts of radiolabeled deoxyarteether were taken up by parasites compared to radiolabeled arteether (Asawamahasakda et al., 1994), and by the fact that deoxyartemisinin, lacking the peroxide bridge, is over 1000-fold less active against *P. falciparum* than artemisinin (Kaiser et al., 2007). The exact mode of action is still discussed controversially (O`Neill et al., 2010). The iron- dependent alkylation hypothesis is one of the proposed modes of actions of artemisinins. It is suggested that there is a reductive cleavage of the peroxide bridge in artemisinins by ferrous heme or free Fe (II) derived from heme. The reducing agent is suggested to be released by the hemoglobin digestion which takes place in the food vacuole. Toxic free radicals are generated which then damage specific intracellular targets by alkylation (Tilley et al., 2016; O`Neill et al., 2010; Meshnick et al., 1993; Creek et al., 2009). This mode of action model is supported by the fact that artemisinin localizes to the food vacuole and mitochondria, therefore direct contact of artemisinin and heme possibly takes place in the food vacuole (Maeno et al., 1993; Crespo et al., 2008). Further, artemisinin activity is only found in hemoglobin-degrading pathogens, but not in pathogens (or life-cycle stages) that do not degrade hemoglobin (Kaiser et al., 2007). *P. falciparum* young ring stages, in which hemoglobin digestion just began and less heme was produced, were slightly less sensitive than trophozoites or schizonts (Skinner et al., 1996; Ter Kuile et al., 1993; Maerki et al., 2006; Klayman, 1985; Kaiser et al., 2007). Also, young gametocytes, having a greater heme content, were more susceptible to artemisinins

than more mature gametocytes (Dechy-Cabaret et al., 2012; Kaiser et al., 2007). Sporozoites, which do not digest hemoglobin, are not susceptible to artemisinins (Kaiser et al., 2007). Another point, supporting the iron-dependent alkylation hypothesis is the finding, that the formation of carbon-centered radicals is critical to the activity of artemisinin and that the interaction of artemisinin with parasite targets is irreversible (Fügi et al., 2010; Abiodun et al., 2013). Further, antimalarial endoperoxides appeared to react specifically with malarial proteins (Asawamahasakda et al., 1994).

1.5. Artemisinin Resistance

Although artemisinin and its derivatives are highly potent and fast acting, prolonged parasite clearance rates in patients after treatment with artesunate or ACTs were observed along the Thai-Cambodian border in 2006 (Noedl et al., 2008; Dondorp et al., 2009). WHO defines the "delayed parasite clearance" as "partial resistance" (WHO Malaria Report, 2016). Today, this type of artemisinin resistance has emerged in five countries in the Greater Mekong subregion, namely Cambodia, Myanmar, Vietnam, Lao PDR and China (WHO Malaria Report, 2016; Zaw et al., 2017; Paloque et al., 2016). It was found that mutations in the Kelch 13 propeller domain protein (K13) confer to ring-stage parasites the ability to enter a quiescent stage upon exposure to artemisinin. When drug pressure is removed, normal growth is reached again quickly (Ariey et al., 2014; Paloque et al., 2016). Several different mutations in the K13 gene were found, all having emerged independently, since same mutations were found in different geographic locations (Takala-Harrison et al., 2015). Individual point mutations in K13 were shown to be sufficient for artemisinin resistance by Zinc-finger-based genetic engineering of *P. falciparum* isolates (Straimer et al., 2015). Recently, K13 mutant alleles were mapped worldwide, whereby 108 non-synonymous mutations were identified out of 1250 *P. falciparum* isolates, mostly in samples obtained from Asia (Ménard et al., 2016). Although some K13 mutations were found in Africa, none of these had been associated with clinical artemisinin resistance, except one recent report of a migrant worker who showed delayed parasite clearance after ACT treatment (Tilley et al., 2016; Zaw et al., 2017; Ménard et al., 2016). Since infections in patients showing delayed parasite clearance because of artemisinin resistance can still be cleared by the partner drug of ACTs, treatment failures of ACTs are limited to Asia, where resistance to several of the partner drugs of ACTs was reported. Today, partial resistance to mefloquine and amodiaquine, high resistance against piperaquine and little evidence of lumefantrine resistance was observed in Cambodia, resulting in high failure rates after ACT

treatment of four different ACTs in Cambodia (WHO Report, 2016; Blasco et al., 2017). The emergence of resistance occurs often in areas with high parasite loads, low transmission and low host immunity resulting in high numbers of patients having symptoms and seeking treatment (Talisuna et al., 2012). Therefore, reasons for the lack of artemisinin resistance in Africa could be the higher degree of acquired immunity because of permanent exposure to *P. falciparum* in endemic regions resulting in control of drug-resistant infection by the acquired host immunity (Blasco et al., 2017, Tilley et al., 2016). Further the occurrence of polyclonal infections in Africa select against parasites resistant to drugs having reduced growth-rates (Tilley et al., 2016; Blasco et al., 2017; Paloque et al., 2016). In addition, high numbers of chronic asymptomatic infections result in lower drug pressure in Africa (Blasco et al., 2017; Tilley et al., 2016).

1.6. Disadvantages of Artemisinin

Besides the problem of recently reported artemisinin resistance artemisinins are facing other disadvantages. Since artemisinins are natural products extracted from the leaves of *A. annua*, they are dependent on the availability of the plant (Tu, 2011). The first total synthesis of artemisinin was reported by Schmid and Hofheinz in 1983 (Schmid et al., 1983), followed by several other groups describing total or partial syntheses of artemisinin over the past decades (Wang et al., 2014). Also, a method to scale up artemisinin synthesis was described by Lévesque and Seeberger (Lévesque et al., 2012), but total or partial synthesis of artemisinin is a complicated process, involves several steps (Avery et al., 1992), results in low yields, or includes high costs of starting material (White, 2008; Wang et al., 2014). Therefore, no reasonable alternative for a large-scale production is available at the moment. Also, efforts were undertaken to scale up plant production since artemisinin concentration in plants is relatively low. However, Youyou Tu and coworkers had already found that artemisinin is only present in leaves of the species *Artemisia annua* but not in other species of the genus *Artemisia* (Tu, 2011). Therefore, cultivation and processing of *Artemisia annua* plants were even reported in partnerships in India to provide sufficient artemisinin for malaria treatment (Kumar et al., 2005). Another possibility is breeding new varieties as reported in 2010 (Graham et al., 2010). Nevertheless, artemisinin and its derivatives are much more expensive than other antimalarial drugs; they cost, for example, 10 times more than chloroquine (White et al., 2008).

Another disadvantage of artemisinins is their poor bioavailability. Further, their short *in vivo* half-life, which results in recrudescence of parasitemia after 5 days of artemisinin monotherapy in spite of multiple applications, is an additional disadvantage. Since artemisinins are short-lived, reducing the high numbers of parasites for two asexual parasite cycles only, artemisinins are co-administered with longer half-life drugs (White, 2008; Tilley et al., 2016; Meshnick, 2002).

1.7. Synthetic peroxides

The next generation of antimalarials must overcome the drawbacks of artemisinins such as the cumbersome production, the limiting pharmacokinetics, the poor bioavailability and costs. In 2004, Vennerstrom et al. reported the identification of synthetic peroxides, with OZ277 as development candidate, which overcome the disadvantages of artemisinins (Vennerstrom et al., 2004). A series of over 700 peroxides was synthesized and tested by a consortium consisting of Jonathan Vennerstrom`s group at the University of Nebraska (USA), Monash University (Australia), Swiss Tropical and Public Health Institute (Swiss TPH, Switzerland), Basilea Pharmaceutica AG (Switzerland) and F. Hoffmann-La Roche (Switzerland; Vennerstrom et al., 2004; Mäser et al., 2012). The synthesized peroxides showed best activity when a trioxolane heterocycle was stabilized by two additional ring structures, one adamantane ring and one phenyl ring. This turned out to be an ideal balance between a sterically hindered and a sterically unhindered peroxy oxygen atom for attack by iron (II) species (Vennerstrom et al., 2004).

1.8. OZ277

Efficacy and pharmacokinetic properties of synthetic trioxolanes were optimized leading to the clinical candidate called OZ277 or arterolane. OZ277 is a synthetic 1,2,4-trioxolane with a structural simplicity and an economically feasible and scalable synthesis. Further, OZ277 showed, in contrast to artemisinin and its derivatives, superior antimalarial activity and an improved biopharmaceutical profile (Vennerstrom et al., 2004). But OZ277 failed in phase II clinical trials because of reduced plasma exposure in malaria patients compared to healthy volunteers when administered as monotherapy. Nevertheless, OZ277 in combination with piperaquine showed rapid parasite clearance with reduction of malaria symptoms, hence it was registered for antimalarial combination therapy in India in 2012 (Mäser et al., 2012; Patil et al, 2014).

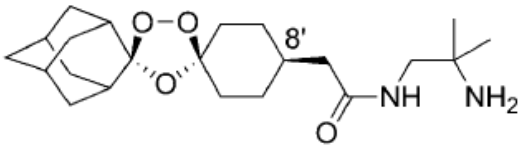

Figure 2 Chemical structure of OZ277

1.9. OZ439

OZ439, also called artefenomel, is a next-generation ozonide containing a cis-8`-phenyl substituent in contrast to the cis-8`-alkyl group in OZ277. It was designed to provide a single-dose oral cure in humans and indeed, it was the first compound of all synthetic peroxides and artemisinin derivatives which completely cured *P. berghei* infected mice with a single oral dose of 20 mg/kg. Further, OZ439 was shown to be >50-fold more stable to Fe(II)-mediated degradation, suggesting that this is the reason for its prolonged plasma profiles in malaria patients (Charman et al., 2011). In addition, excellent prophylactic activity, superior to that of mefloquine, was demonstrated with OZ439 (Charman et al., 2011). Also, a rapid onset of action and activity against all asexual *P. falciparum* blood stages was shown *in vitro* (Charman et al., 2011; Kim et al., 2017). Phase I clinical trials of OZ439 showed good safety and pharmacokinetic profiles in healthy volunteers (Möhrle et al., 2012; McCarthy et al., 2016). Furthermore, good safety profiles were also observed in malaria patients in phase IIa studies (Phyo et al., 2016). OZ439 showed fast clearance of *P. falciparum* and *P. vivax* parasites in malaria patients and its long half-life suggests to use OZ439 in combination with another drug as single-dose oral cure (Phyo et al., 2016). OZ439 is now tested in Phase IIb clinical trials with piperaquine and additionally also in combination with ferroquine by Sanofi (Wells et al., 2015).

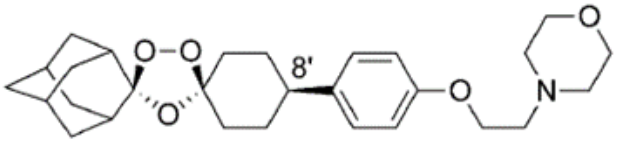

Figure 3 Chemical structure of OZ439

1.10. Mode of Action

Although synthetic peroxides have a different structure to artemisinin and its derivatives, except for the peroxide bridge, it was suggested that both have a similar mode of action (Vennerstrom et al., 2004; Jefford, 2001; Kaiser et al., 2007). As in artemisinin, it was found that the peroxide bridge is essential for antimalarial activity in OZ277 (Kaiser et al., 2007). Further, OZ277 was 1,000 fold more active against hemoglobin-degrading *P. falciparum* parasites than against parasites that do not degrade hemoglobin (Kaiser et al., 2007). This supports the iron-dependent alkylation hypothesis. The synthetic peroxides OZ277 and OZ439 were found to be active against all asexual blood stages of *P. falciparum in vitro* (Märki et al., 2006; Hofer et al., 2008; Charman et al., 2011; Kim et al., 2017). Localization studies showed that in some parasites OZ277 localizes to the parasite cytosol and in some parasites to the food vacuole (Uhlemann et al., 2007). It was shown that for the activity of OZ277 and artemisinin the formation of carbon-centered radicals is essential (Fügi et al., 2010; Tang et al., 2005). Further it was found that the antimalarial properties of both, artemisinin and OZ277, derived from irreversible interactions with parasite targets (Fügi et al., 2010; Abiodun et al., 2013). Different studies showed that OZ277 activation was dependent on free iron, heme or ferrous heme (Creek et al., 2008; Creek et al., 2009). Together, all these facts corroborate the iron-dependent alkylation hypothesis.

1.11. Objectives

The aim of this PhD thesis was to further elucidate the mode of action of synthetic peroxides in *P. falciparum* by combining cellular, biochemical and molecular approaches. The chronology of the chapters in this thesis follows the working process.

First, monoclonal antibodies recognizing the adamantane portion of OZ277 and OZ439, which were kindly provided by Dr. Hugues Matile (Hoffmann-La Roche AG, Basel, Switzerland), were used to face the following objectives:

- Investigate the localization of OZ277 and OZ439 in *P. falciparum* using monoclonal antibodies specific for adamantane-based antimalarials

- Identify the alkylation signature of ozonides in parasite cultures incubated with OZ277 and OZ439 using the monoclonal antibodies

Since increasing numbers of reports with prolonged parasite clearance rates following artemisinin treatment came up in South-East Asia, concerns arose if synthetic peroxides could overcome the emerging artemisinin-resistant phenotypes. Therefore, we set up the next objective:

- Check if analogies in the chemical structure of artemisinins and ozonides result in cross-resistance in artemisinin-resistant clinical isolates

Next, we were looking for an alternative approach to the antibodies in order to identify the alkylation signature of ozonides. Prof. Dr. Jonathan Vennerstom (University of Nebraska, USA) had the idea to use a click chemistry approach and synthesized OZ molecules and artemisinins with an alkyne structure which are capable of performing click chemistry. This resulted in the following objective:

- Establish the click chemistry method with the newly synthesized synthetic peroxides and identify the alkylation signatures of ozonides by mass spectrometry

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2. Monoclonal Antibodies that Recognize the Alkylation Signature of Antimalarial Ozonides OZ277 (Arterolane) and OZ439 (Artefenomel)

Joëlle Jourdan[1,2], Hugues Matile[3], Ellen Reift[1,2], Oliver Biehlmaier[4], Yuxiang Dong[5], Xiaofang Wang[5], Pascal Mäser[1,2], Jonathan L. Vennerstrom[5], and Sergio Wittlin[1,2*]

[1]Swiss Tropical and Public Health Institute, Socinstrasse 57, CH-4002 Basel, Switzerland

[2]University of Basel, CH-4003 Basel, Switzerland

[3]F. Hoffmann- La Roche Ltd., Basel, Switzerland

[4]Imaging Core Facility, Biozentrum, University of Basel, CH-4003 Basel, Switzerland

[5]College of Pharmacy, University of Nebraska Medical Center, 986025 Nebraska Medical Center, Omaha, NE, USA

*Corresponding author

tel: 41 61 284 81 36

email: sergio.wittlin@unibas.ch

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Abstract

The singular structure of artemisinin, with its embedded 1,2,4-trioxane heterocycle, has inspired the discovery of numerous semisynthetic artemisinin and structurally diverse synthetic peroxide antimalarials, including ozonides OZ277 (arterolane) and OZ439 (artefenomel). Despite the critical importance of artemisinin combination therapies (ACTs), the precise mode of action of peroxidic antimalarials is not fully understood. However, it has long been proposed that the peroxide bond in artemisinin and other antimalarial peroxides undergoes reductive activation by ferrous heme released during hemoglobin digestion to produce carbon-centered radicals that alkylate heme and parasite proteins. To probe the mode of action of OZ277 and OZ439, we now describe initial studies with monoclonal antibodies that recognize the alkylation signature (sum of heme and protein alkylation) of these synthetic peroxides. Immunofluorescence experiments conducted with ozonide-treated parasite cultures showed that ozonide alkylation is restricted to the parasite, as no signal was found in the erythrocyte or its membrane. In Western blot experiments with ozonide-treated *P. falciparum* malaria parasites, distinct protein bands were observed. Significantly, no protein bands were detected in parallel Western blot experiments performed with lysates from ozonide-treated *Babesia divergens*, parasites that also proliferate inside erythrocytes, but in contrast to *P. falciparum*, do not catabolize hemoglobin. However, subsequent immunoprecipitation experiments with these antibodies failed to identify the *P. falciparum* proteins alkylated by OZ277 and OZ439. To the best of our knowledge, this shows for the first time that antimalarial ozonides, like the artemisinins, alkylate proteins in *P. falciparum*.

Keywords. alkylation, artemisinin, immunofluorescence, monoclonal antibody, ozonide, *Plasmodium falciparum*.

Introduction

The discovery of artemisinin (ART) from *Artemisia annua*[1] gave rise to the semisynthetic artemisinins dihydroartemisinin (DHA), artemether (AM), and artesunate (AS), which as ART combination therapies (ACT), are the preferred treatment for uncomplicated *Plasmodium falciparum* malaria[2] (Figure 1). The singular structure of ART, with its embedded 1,2,4-trioxane heterocycle, inspired the discovery of additional semisynthetic artemisinins and structurally diverse synthetic peroxide antimalarials.[3-6] One of these, ozonide (1,2,4-trioxolane) OZ277,[7] also known as arterolane maleate, was introduced in 2012 to the Indian market as a combination product with piperaquine phosphate (Synriam®).[8-10] More recently, the 'next generation' ozonide OZ439 (artefenomel)[11,12] has progressed to Phase IIb trials (Figure 1).

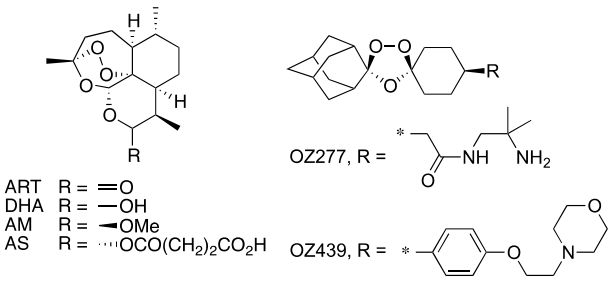


Figure 1. Artemisinin and ozonide structures.

The peroxide bond in ART and antimalarial synthetic peroxides is essential for antiplasmodial activity,[6,13] suggesting a chemistry-driven mechanism of action. A considerable amount of data[4,14-24] demonstrates that the activity of antimalarial peroxides does not derive from reversible interactions with parasite targets and that the peroxide bond in ART and other antimalarial peroxides undergoes reductive activation by ferrous heme released during hemoglobin digestion to produce carbon-centered radicals that alkylate heme and parasite proteins (Figure 2). This is accompanied by disruption of the parasite digestive vacuole including lipid peroxidation.[25-27] This mechanism accounts not only for the high antiplasmodial potency and specificity of peroxides, but

also for their weak and peroxide-bond independent activities against pathogens that do not degrade hemoglobin such as other protozoa, bacteria, and fungi.[13,28,29]

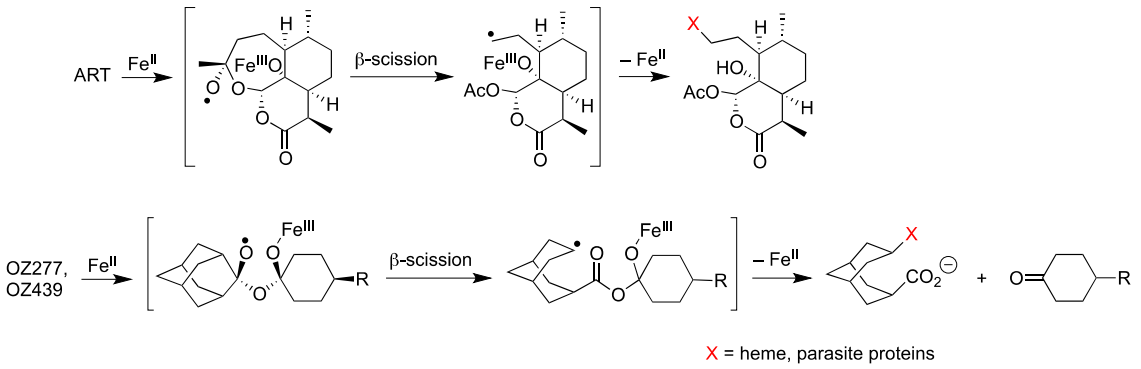
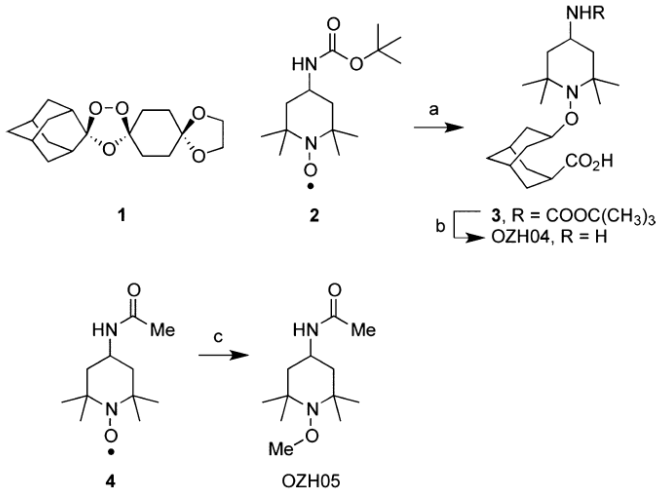


Figure 2. Alkylation reactions of ART and ozonides OZ277 and OZ439.

Electron transfer from heme to the peroxide bond antibonding σ* orbitals of ART and antimalarial ozonides produces short-lived alkoxy radicals (Figure 2). For ART, rearrangment via β-scission forms a primary carbon-centered radical; for OZ277 and OZ439, rearrangement via β-scission forms a secondary carbon-centered radical. As these two ozonides have the same spiroadamantane substructure, they produce the same bicyclic carboxylic acid signature of ozonide alkylation – be that with heme or with proteins. Since we had good success in capturing the ozonide-derived secondary carbon-centered radical with the stable nitroxide radical TEMPO and its analogs,[7,22,30] we decided to capitalize on this finding and synthesized OZH04 as a potential hapten for this ozonide-derived bicyclic carboxylic acid with OZH05 as a control (Scheme 1). We now describe the creation of monoclonal antibodies to OZH04 and their application in immunofluorescence and Western blot experiments.

Results and Discussion

Our first approach was to synthesize OZH04 by reductive amination of 7-(4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy)bicyclo-[3.3.1]nonane-3-carboxylic acid,[30] but the workup of this reaction was difficult, so we elected to access OZH04 by a two-step procedure (Scheme 1). Thus, reaction of prototypical ozonide **1** with ferrous iron in the presence of 4-((*tert*-butoxycarbonyl)amino)-2,2,6,6-tetramethylpiperidinyl-1-oxyl (**2**) led to formation of intermediate **3** in 26% yield. We synthesized OZH04 as its dihydrochloride salt (92% yield) by deprotection of **3** with HCl. OZH05 was synthesized in 64% yield by exposing 4-acetamido-2,2,6,6-tetramethylpiperidinyl-1-oxyl (**4**) to a mixture of cuprous chloride and aq. hydrogen peroxide according to the method of Dichtl et al.[31]



Scheme 1. Synthesis of *N*-alkoxyamines OZH04 and OZH05. (a) $Fe(OAc)_2$, 1:1 CH_2Cl_2:CH_3CN, $Fe(OAc)_2$, 35 °C, 24 h; (b) 6N HCl, THF, rt, 12 h; (c) 50% H_2O_2, CuCl, acetone, rt, 12 h.

The monoclonal antibodies OZH04-2/2 and OZH04-1/8 were raised in Naval Medical Research Institute (NMRI) mice injected subcutaneously with OZH04 hapten coupled to KLH (keyhole limpet hemocyanin). After the third boost, blood was collected and the serum was tested for the presence of antihapten antibodies by ELISA using BSA-conjugated OZH04 antigen to coat the ELISA-plates. Animals with strong immune responses were selected for fusion to PAI myeloma cells.

In order to determine if the monoclonal IgG1 antibodies raised against the OZH04 hapten were binding to *P. falciparum* parasites that had been exposed to OZ277 or OZ439, NF54 cultures were treated with either of the two ozonides, DHA or DMSO, and immunofluorescence experiments were performed. The two monoclonal antibodies OZH04-2/2 and OZH04-1/8 gave positive signals after incubation with parasites exposed to either OZ277 or OZ439 (Table 1, see 2 top rows). No immunofluorescence signals were detected with DHA-treated parasites, 0.1% DMSO or an unrelated IgG1 control antibody. An antibody raised against the cytosolic protein GAPDH served as a positive control.

antibody	specificity	OZ277	OZ439	DHA	DMSO
OZH04-2/22	hapten OZH04	+	+	–	–
OZH04-1/8	hapten OZH04	+	+	–	–
IgG1 antibody	negative control	–	–	–	–
GAPDH	positive control	+	+	+	+

Table 1: Immunofluorescence experiments with *P. falciparum* cultures treated with 10 μg/mL OZ277, 10 μg/mL OZ439, 10 μg/mL DHA or 0.1% DMSO for 2 h. Primary antibodies used were OZH04-2/2, OZH04-1/8, IgG1 negative control or GAPDH positive control. Goat-anti mouse Alexa 488 was used as secondary antibody. + indicates fluorescence and – indicates no fluorescence.

Competition experiments with hapten OZH04 and control hapten OZH05 (Scheme 1) showed that the antibodies OZH04-1/8 and OZH04-2/2 specifically recognize the bicyclic carboxylic acid alkylation substructure, or alkylation signature, of ozonides OZ277and OZ439 (Table 2).

hapten	OZ277 and OZH04-2/2 or -1/8	DMSO and OZH04-2/2 or -1/8
OZH04	–	–
OZH05	+	–
no hapten	+	–

Table 2: Immunofluorescence experiments with *P. falciparum* cultures treated with 10 μg/mL OZ277 or 0.1% DMSO for 2 h. For competition, 33 μM of the hapten OZH04 or OZH05 were combined with the primary antibodies OZH04-2/2

(0.33 μM) or OZH04-1/8 (0.33 μM) and incubated at RT for 1 h. The secondary antibody was goat-anti mouse Alexa 488. + indicates fluorescence; – indicates no fluorescence.

In co-localization studies, synchronized *P. falciparum* trophozoites were treated with 10 μg/mL OZ277 or DMSO and directly labelled antibodies OZH04-2/2-Alexa 488 and GAPDH-Alexa 594 (Figure 3a). These studies showed that OZ277 alkylation (green signal) is clearly restricted to the parasite, as no signal was found in the erythrocyte or its membrane. Within the parasite, OZ277 alkylation co-localized with the cytoplasm (red signal) and to a weaker extent also with other structures such as the nucleus (blue signal) and the hemozoin-filled interior of the food vacuole (dark round structure). A similar co-localization pattern was also observed when rings and schizonts were treated with OZ277 (Supporting Information Figure 1). Immunofluorescence experiments performed with OZ antibody that was not directly labelled with Alexa, but was instead incubated with a secondary antibody containing the labels Alexa 488 or Alexa 594, resulted in much more pronounced signals in the food vacuole (dark round structure) (Figures 3b,c). The explanation for this phenomenon is unclear. One possibility is that the fluorescence signal of the OZ antibody directly labelled with Alexa dye is quenched in the food vacuole by hemozoin, a biocrystallized form of hematin, the latter being a well-known acceptor for energy transfer.[32]

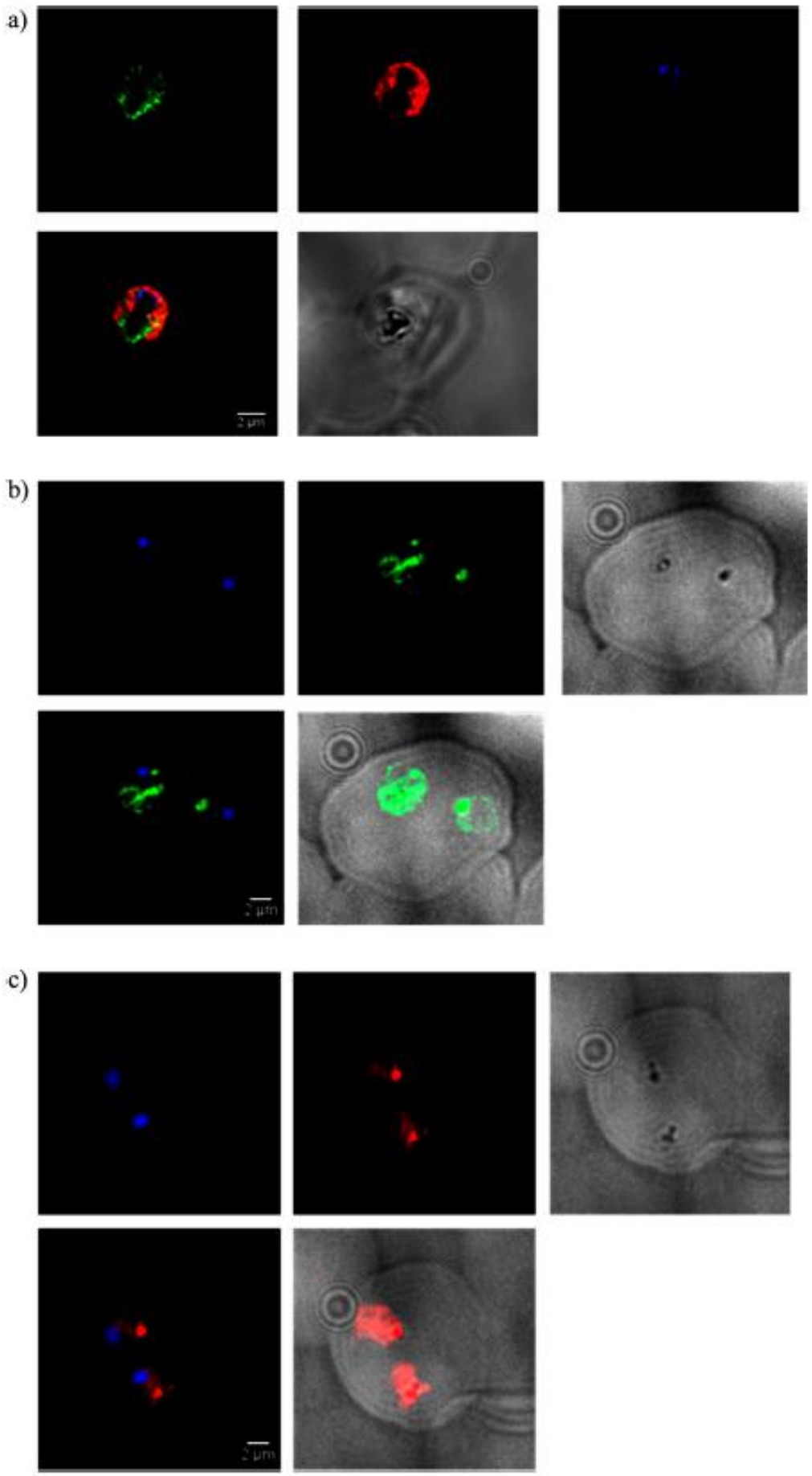

a)
b)
c)

Figure 3: Immunofluorescence studies with P. falciparum trophozoites that were treated with 10 µg/mL OZ277 for 2 h. a) In immunofluorescence co-localisation studies, the blood smears were fixed with 5% formaldehyde and 0.01% glutaraldehyde, permeabilized with 0.5% Triton-X-100 and blocked with 1% BSA in PBS for 1 h. The primary antibodies used were directly labelled OZH04-2/2 (Alexa 488, green signal) and GAPDH (Alexa 594, red signal). For both, the exposure time was 2 s. Nuclei were stained with DAPI (blue signal, exposure time of 0.05 s). The bottom row left shows the merge of OZH04-2/2, GAPDH and DAPI, and on the bottom row right, the reference image (exposure of 1 s). Scale bar 2 µm. b) Same as a), except that unlabelled primary antibodies OZH04-2/2 were used. After 6 washes with PBS, 20 µg/mL of the secondary antibody goat anti-mouse Alexa 488 (green signal) was incubated for 1 h at RT. The exposure time was 0.1 s. Nuclei were stained with DAPI (blue signal, exposure time of 0.05 s). The bottom row left shows the merge of OZH04-2/2 and DAPI, and the bottom row right shows the merge of OZH04-2/2 and the reference image (exposure of 1 s). Scale bar 2 µm.

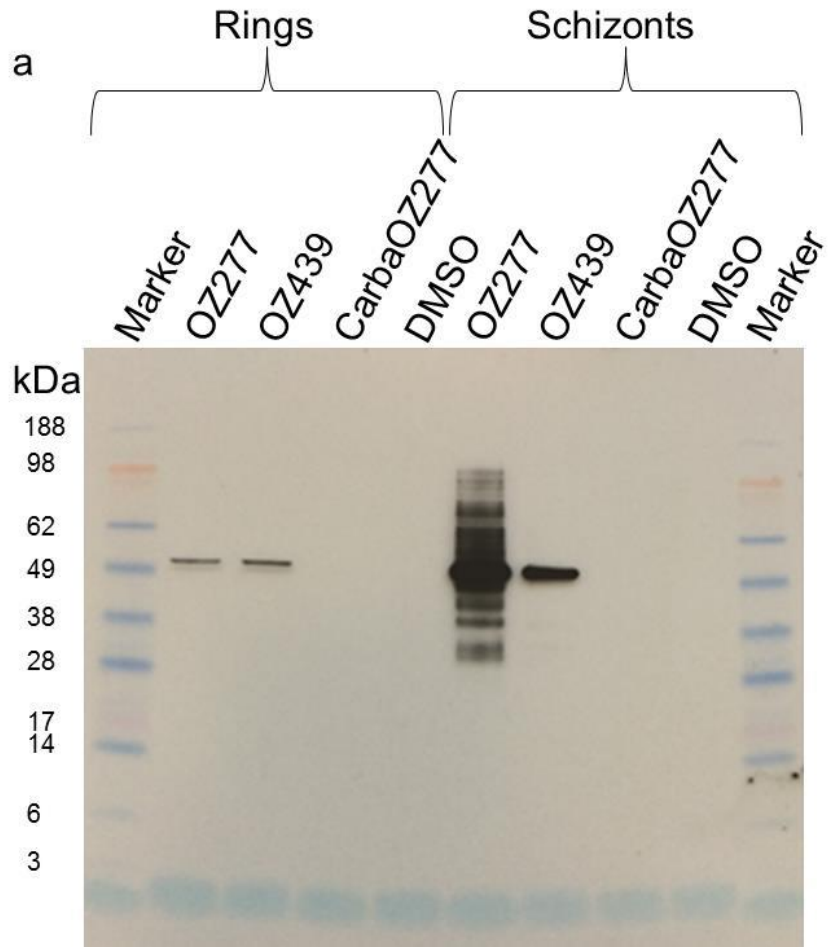
c) Same as b), except that the secondary antibody used was goat anti-mouse Alexa 594 with an exposure of 1s (red signal).

Previously, ultrastructural autoradiographic studies of [^{3}H]-dihydroartemisinin-treated parasites have shown that the drug and its alkylation reaction products are present in the parasitophorous vacuole membranes, digestive vacuole membranes and mitochondria.[15] Other work indicated that a fluorescent TAMRA OZ277 conjugate was associated with the food vacuole and the parasite endoplasmic reticulum,[33] although in this study, fluorescence signal was derived from the parent ozonide, not from its alkylation products. To ascertain if ozonide alkylation localizes to specific parasite membranes, further studies will be required.

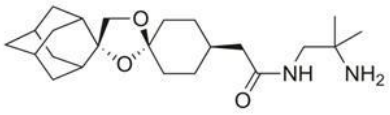
Western Blot experiments demonstrated that monoclonal antibody OZH04-2/2 recognizes distinct *P. falciparum* protein bands (Figure 4a) in parasites treated with OZ277 or OZ439. This indicates that the two ozonides alkylate parasite proteins. Ring stages showed one prominent band at ~50 kDa. In schizont stages, a variety of bands ranging from ~28 to ~98 kDa could be found after OZ277 treatment. After longer film exposure times, the same pattern of bands was also observed in schizonts treated with OZ439. We conclude that the extent of ozonide alkylation seems to be higher in schizonts vs. rings, consistent with the greater hemoglobin digestion that has occurred in the former.

The same bands were also found when the concentrations of the ozonides were lowered to 100 ng/mL, which at a parasitemia of 8-10%, corresponds to the IC99 (concentration where 99% of parasite growth is inhibited compared to untreated control parasites) (Supporting Information Figure 2). However, Western blot experiments performed at these lower ozonide concentrations were not practical as they required longer film exposure times, where false-positive signals can become an issue. Based on this, 10,000 ng/mL was found to be the most practical concentration.

No bands could be observed in parasite cultures treated with carbaOZ277, the nonperoxidic analogue of OZ277 (Figure 4)[13] or when uninfected erythrocytes were used (Supporting Information Figure 3). This data indicates that the antibody binding was specific for alkylated proteins.



b



CarbaOZ277

Figure 4: Western blot with *P. falciparum* cultures synchronized for rings and schizonts. Cultures were treated with 10 µg/mL OZ277, OZ439, CarbaOZ277 or a DMSO control (0.1%) for 2 h. All samples were normalized for their protein content. Antibody OZH04-2/2 and a film exposure time of 1 min were used.

Additionally, no protein bands were detected in Western blot experiments performed with lysates from ozonide-treated *Babesia divergens* (Supporting Information Figure 4), parasites that also proliferate inside erythrocytes, but in contrast to *P. falciparum*, do not catabolize hemoglobin.[34] This underscores the hemoglobin-digestion dependent activity of antimalarial peroxides.

Heme has been shown to be an alkylation target of the antimalarial ozonides.[17] Using monoclonal antibodies that specifically detect ozonide alkylation, we demonstrate here that ozonides OZ277 and OZ439 also alkylate parasite proteins as revealed by distinct bands on Western blots (Figure 4), with the most prominent band at ~50 kDa. To the best of our knowledge, this shows for the first time that the ozonides, like the artemisinins, alkylate accessible proteins in *P. falciparum*.

Artemisinin has previously been shown to alkylate as much as half of the food vacuole-associated heme[15,35] but also proteins. Among the identified target proteins was the *P. falciparum* translationally controlled tumor protein (TCTP) homologue.[16] Parasite redox-active flavoenzymes,[36] mitochondrial reductive activation,[37] and the PfATP6, a sarcoplasmic reticulum calcium ATPase, have also been suggested as targets of artemisinins, although we have shown that OZ277 does not inhibit the latter.[33] To better understand the roles that heme and protein alkylation play in the antimalarial activity of the ozonides OZ277 and OZ439, the distinct bands on the Western blots will need to be identified. However, we could not identify alkylated proteins in our immunoprecipitation experiments with the monoclonal antibodies OZH04-2/2 or OZH04-1/8. We

suggest that the antibodies were not able to bind to the alkylated proteins under the non-denaturing conditions required for those experiments. Due to lack of binding of the antibodies to the native proteins, an alternative, antibody-independent approach using click chemistry is currently underway.

Methods

Monoclonal antibody preparation

Hapten OZH04 was coupled to KLH (keyhole limpet hemocyanin, Thermo Scientific 77600, Imject mcKLH) and BSA (bovine serum albumin, Thermo Scientific 77110 Imject BSA) respectively, by crosslinking with glutaraldehyde based on the method of Onica et al.[38]

0.1 mL of OZH04 dissolved at 57.5 mg/mL in DMSO was mixed with 20 mg of the respective carrier protein in 0.2 M Na_2HPO_4 pH 8.0, and 1 mL of 0.2% glutaraldehyde in 0.2 M Na_2HPO_4 was then slowly added with agitation. After 1 h incubation at RT, the reaction was stopped by adding 0.25 mL 1 M glycine pH 8.0 and dialyzed against PBS overnight.

Naval Medical Research Institute (NMRI) mice were immunized with 100 µg subcutaneous injections of KLH-conjugated OZH04 emulsified in aluminum hydroxide gel (Alhydrogel-2%, Brenntag Biosector) containing CPGOGN as previously described.[39] After the third boost, blood was collected and the serum was tested for the presence of antihapten antibodies by ELISA using BSA-conjugated OZH04 antigen to coat the ELISA-plates. Animals with strong immune responses were selected for fusion.[40]

Parasite cultivation

P. falciparum strain NF54 (Origin: Airport, Netherlands; Provider MR4, MRA-1000) asexual blood stages were cultivated in a variation of the medium previously described,[41-43] consisting of RPMI 1640 supplemented with 0.5% ALBUMAX® II, 25 mM Hepes, 25 mM $NaHCO_3$ (pH 7.3), 0.36 mM hypoxanthine, and 100 µg/mL neomycin. Human erythrocytes served as host cells. Cultures were maintained at 37 °C in an atmosphere of 3% O_2, 4% CO_2, and 93% N_2 in humidified modular chambers. Fifty, ninety and ninety nine percent inhibitory concentrations (IC_{50}, IC_{90} and IC_{99}) of OZ277 in ng/mL against unsynchronized NF54 parasites were 0.91, 1.7 and 2.7 when determined at the standard parasitemia of 0.3% parasitemia.[7] At about 10-30x higher parasitemia, the inhibitory

concentrations were found to be 10-30x higher, which is consistent with the so called "incoculum effect". The term refers to an increase in the amount of drug necessary to inhibit microbial growth with greater numbers of microorganisms per milliliter. This effect has been previously observed with antimalarial compounds such as chloroquine and artesunate, which show enrichment in *P. falciparum*-infected red blood cells.[44,45] Also, OZ277 has been shown to partition into *P. falciparum*-infected red blood cells.[46]

Immunofluorescence

P. falciparum NF54 cultures (5% (v/v) hematocrit, 8–10% parasitemia) were either synchronized with 5% D-sorbitol for trophozoites[47] prior to treatment or used as mixed cultures. The cultures were treated with 10 µg/mL OZ277, 10 µg/mL OZ439 and 0.1% DMSO for 2 h. Cultures were washed once with PBS and pelleted RBCs were smeared on glass slides. The blood smears were fixed with prechilled methanol (-20°C; 100%) and blocked with 1% BSA in PBS for 1 h.

Primary antibodies OZH04-2/2, OZH04-1/8, IgG1 (Hoffmann-La Roche AG, Basel, Switzerland) and GAPDH (glyceraldehyde-3-phosphate-dehydrogenase, a gift from Paola Favuzza, Swiss TPH, Basel, Switzerland) were incubated for 1 h at RT with concentrations of 50 µg/mL. After 6 washes with PBS, the secondary antibodies goat anti-mouse Alexa 488 (Invitrogen) or goat anti-rabbit Alexa 594 (Invitrogen) were incubated with concentrations of 20 µg/mL for 1 h at RT. Control experiments, performed with secondary antibody only, resulted in no detectable immunofluorescence signals (not shown).

For competition experiments, 33 µM of OZH04 or OZH05 were combined with the primary antibody OZH04-1/8 (0.33 µM) or OZH04-2/2 (0.33 µM) and incubated at RT for 1 h. As secondary antibody, goat anti-mouse Alexa 488 (20 µg/ml) was incubated for 1 h at RT.

For co-localization studies, blood smears were fixed with 5% formaldehyde and 0.01% glutaraldehyde and permeabilized with 0.5% Triton-X-100. Both antibodies, OZH04-2/2 and

GAPDH, were directly labelled with Alexa Fluor 488 succinidyl ester (Life Technologies) and Alexa Fluor594 succinidyl ester (Life Technologies), respectively. 50 µg/ml of OZH04-2/2 directly labelled with Alexa 488 was incubated for 1 h at RT. After washing 6 times with PBS, 50 µg/mL of GAPDH directly labelled with Alexa 594 was incubated for 1 h at RT. Vectashield Hard Set with DAPI (Vector Laboratories) was added to all slides after washing 6 times with PBS. The slides were analysed with a Widefield Delta Vision core microscope based on an Olympus IX71 stand, using a 60x/1.42NA oil objective. Basic image analysis (e.g. contrast and brightness adjustments) were done with ImageJ Fiji and images were analysed with deconvolution (SoftWorx 4.1.2; enhanced ratio aggressive; Number of cycles:10).

Western blots

P. falciparum lysates were prepared from NF54 cultures (5% (v/v) hematocrit, 8–10% parasitemia) synchronized with 5% D-sorbitol for rings and schizonts.[47] Cultures were treated with 10 µg/mL OZ277, 10 µg/mL OZ439, 10 µg/mL CarbaOZ277 or 0.1% DMSO for 2 h. After centrifugation, pellets of samples were resuspended in 1.5 mL 0.1% saponin in PBS and incubated for 10 min on ice. Samples were washed twice with PBS and pellets were incubated for 10-15 min with 50 µL completed Ripa-Lysisbuffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP40, 2 mM EDTA, EDTA-free protease inhibitor mixture tablets (Roche Applied Science)) on ice. After centrifugation, extracts were used for Western blot. For normalizing protein contents in ring- and schizont samples, a BCA protein assay was performed using a BCA protein assay kit from Merck Millipore (Product No. 21285-3) with BSA as standard prior to the Western blot.

NP40 extracts were diluted 1:2 with 2x LDS sample buffer (50% 4x LDS sample buffer, Invitrogen; 20% mercaptoethanol; 30% ddH_2O), heated for 10 min at 70 °C, loaded onto polyacrylamide gels (10 µL of samples; 4-12% Bis-Tris-polyacrylamide Gels, Invitrogen) and run for 35 min (120 mA; 200 V) using 1x MES SDS Running Buffer (20x MES DS Running Buffer, Invitrogen) as running

buffer. Gels were transferred onto nitrocellulose membranes (0.2 µm pore size; 100% nitrocellulose, Invitrogen) and blocked for 1 h with 3% milk powder blocking solution (3% milk powder in TBS-Tween buffer pH 8.0; TBS-Tween buffer: 20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH adjusted to 8.0 with HCl). 1 µg/mL of OZH04-2/2 antibody, diluted in 0.5% milk powder blocking solution, was added to the membrane and incubated for 1 h at rt. Membranes were washed 3 times for 5 min with TBS- Tween buffer and incubated with polyclonal rabbit anti- mouse immunoglobulin horseradish peroxidase 1:5000 (1.3 g/L; DAKO; diluted in 0.5% milk powder in TBS-Tween buffer) for 1 h at RT. Membranes were washed 4 times for 5 min with TBS-Tween buffer. Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) was added to the membrane, incubated for 5 min and the Western blot was developed using a Carestream Kodak Biomax light film (Sigma).

Supporting Information. Synthesis and characterization of OZH04 and OZH05.

Immunofluorescence experiments with *P. falciparum* rings, trophozoites and schizonts treated with OZ277 (SI Figure 1). Western Blot experiments with mixed *P. falciparum* cultures treated with a range of OZ277 concentrations (SI Figure 2). Western blot and Coomassie gel for uninfected erythrocytes (SI Figure 3) or *Babesia divergens* (SI Figure 4) treated with OZ277, OZ439, CarbaOZ277 or a DMSO control. This material is available free of charge via the Internet at http://pubs.acs.org.

Abbreviations

ACT, artemisinin combination therapy; AM, artemether; ART, artemisinin; AS, artesunate; BCA, bicinchoninic acid assay; BSA, bovine serum albumin; CarbaOZ277, nonperoxidic analogue of OZ277; DAPI, 4',6-diamidino-2-phenylindole DNA fluorescent stain; DHA, dihydroartemisinin; DMSO, dimethyl sulfoxide; ELISA, enzyme linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehyderogenase; IC, inhibitory concentration; KLH, keyhole limpet hemocyanin; NF54, *P. falciparum* strain (Netherlands Airport strain); NMRI, naval medical research institute mice; OZ277, arterolane; OZ439, artefenomel; OZH04, hapten for ozonide-derived bicyclic carboxylic acid; OZH04-1/8, monoclonal antibody raised against OZH04; OZH04-2/2, monoclonal antibody raised against OZH04; OZH05, control hapten; PBS, phosphate buffered saline; RBC, red blood cells; RT, room temperature; TEMPO, 2,2,6,6-tetramethyl-piperidine 1-oxyl. α-CRT, chloroquine resistance transporter.

Conflicts of Interest. The authors declare no competing interests.

Author Information. Sergio Wittlin, Swiss Tropical and Public Health Institute, Socinstrasse 57, CH-4002 Basel, Switzerland.

Tel: 41 61 284 8136 E-mail: sergio.wittlin@unibas.ch

Author Contribution. X.W. and Y.D. synthesized the haptens, H.M. created the monoclonal antibodies, J.J., H.M., E.R., O.B., P.M. and S.W. analyzed the data. J.J., H.M., P.M., J.L.V., and S.W. wrote the paper.

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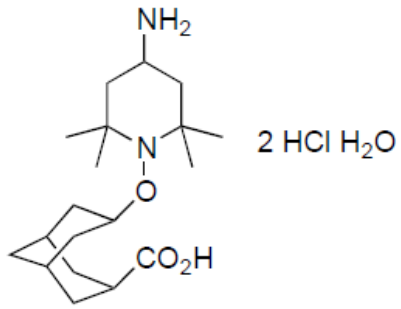
SUPPORTING INFORMATION

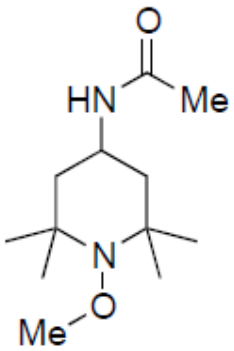
Synthesis and characterization of OZH04 and OZH05.

Melting points are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a 500 MHz spectrometer. Combustion and HPLC analysis confirmed that the target compounds possessed purities ≥ 95%.



7-(4-Amino-2,2,6,6-tetramethyl-1-piperidinyloxy)bicyclo-[3.3.1]nonane-3-carboxylic acid (**OZH04**). **Step 1.** To a solution of adamantane-2-spiro-3'-1',2',4',9',12'-pentaoxadispiro[4.2.4.2]tetradecane (**1**)[1] (0.70 g, 2.17 mmol), 4-((*tert*-butoxycarbonyl)amino)-2,2,6,6-tetramethylpiperidinyl-1-oxyl (**2**) (0.70 g, 2.58 mmol) in CH_2Cl_2 (30 mL) and CH_3CN (30 mL) was added $Fe(OAc)_2$ (0.70 g, 4.03 mmol). The resulting mixture was stirred under Ar and 35 °C for 24 h before being quenched with water (50 mL). After separation of the organic layer, the aqueous layer was extracted with CH_2Cl_2 (2 x 30 mL). The combined extracts were washed with brine (2 x 30 mL), dried over $MgSO_4$ and filtered and concentrated. The residue was purified by flash chromatography (silica gel, 10-50% ether in hexane) to afford 7-(4-((*tert*-butoxycarbonyl)amino)-2,2,6,6-tetramethyl-1-piperidinyloxy)bicyclo-[3.3.1]nonane-3-carboxylic acid (**3**) (mixture of rotamers) as a colorless solid (0.25 g, 26%). ^{1}H NMR ($CDCl_3$) δ 1.17 (s, 6H), 1.20 (s, 6H), 1.44 (s, 9H), 1.00-1.52 (m, 7H), 1.56 (d, J = 14 Hz, 1H), 1.66-1.82 (m, 2H), 2.00-2.28 (m, 6H), 2.40-2.54 (m, 1H), 3.55-3.68 (m, 0.4H), 3.72-3.86 (m, 0.6H), 3.95-4.08 (m, 1H), 4.30-4.40 (m, 0.6H), 6.48 (m, 0.4H); ^{13}C NMR ($CDCl_3$) δ 20.77, 20.99, 25.93, 28.39, 29.21, 29.46, 33.70, 34.15, 35.16, 35.58, 39.70, 39.96) 42.16, 43.16, 45.87, 59.67, 60.25, 75.64, 79.24, 80.36) 155.23, 157.51, 180.79, 181.06. **Step 2.** A

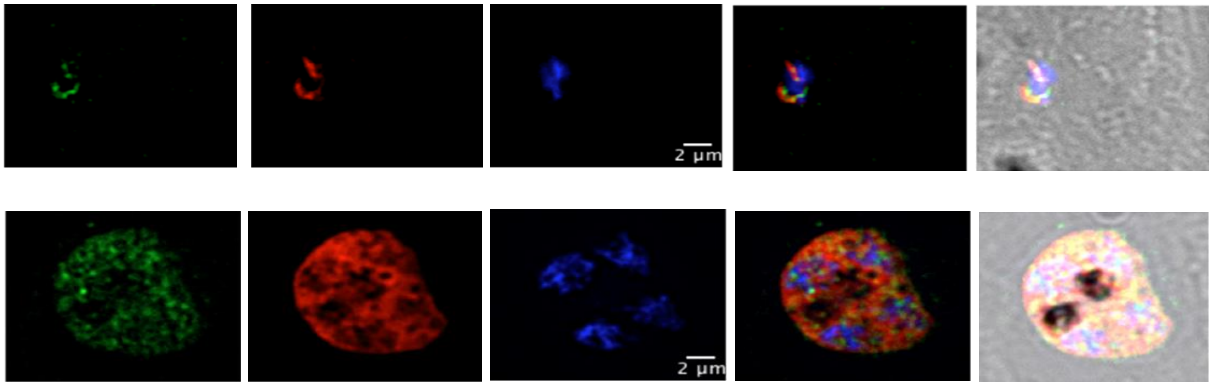
mixture of **3** (0.15 g, 0.34 mmol) and 6 N HCl (0.50 mL) in THF (20 mL) was stirred at rt overnight. After removal of solvent, the residue was washed with CH_2Cl_2 to afford **OZH04** dihydrochloride monohydrate as a white solid (0.12 g, 92%). ^{1}H NMR (D_2O) δ 1.19 (d, J = 13 Hz, 1H), 1.41 (t, J = 12.5 Hz, 2H), 1.49 (s, 6H), 1.57 (s, 6H), 1.50-1.70 (m, 3H), 2.06 (t, J = 13 Hz, 2H), 2.14-2.38 (m, 4H), 2.38-2.44 (m, 4H), 2.55-2.66 (m, 1H), 3.82-3.95 (m, 1H), 4.55-4.68 (m 1H); ^{13}C NMR (D_2O) δ 20.22, 26.26, 27.58, 28.78, 28.85, 35.01, 38.27, 40.37, 41.51, 69.63, 83.83, 181.63. Anal. Calcd for $C_{19}H_{36}Cl_2N_2O_3$ H_2O: C, 53.14; H, 8.92; N, 6.52. Found: C, 52.97; H, 8.63; N, 6.41.



***N*-(1-Methoxy-2,2,6,6-tetramethylpiperidin-4-yl)acetamide (OZH05).** To a solution of 4-acetamido-2,2,6,6-tetramethylpiperidinyl-1-oxyl (1.0 g, 4.33 mmol) in acetone (20 mL) was added CuCl (20 mg, 0.12 mmol) followed by 50% H_2O_2 (1.0 mL). The reaction mixture was stirred at rt overnight and then concentrated. The residue was diluted with CH_2Cl_2 (20 mL), washed with brine (2 x 10 mL), dried over $MgSO_4$ and concentrated to afford **OZH05** as a white solid (0.68 g, 64%). ^{1}H NMR ($CDCl_3$) δ 1.18 (s, 6H), 1.19 (s, 6H), 1.30 (t, J = 12 Hz, 2H), 1.78 (d, J = 11 Hz, 2H), 1.94 (s, 3H), 3.60 (s, 3H), 4.06-4.18 (m, 1H), 5.08 (brs, 1H); ^{13}C NMR ($CDCl_3$) δ 20.45, 23.50, 32.86, 41.01, 45.63, 59.82, 65.36, 169.42. Anal. Calcd for $C_{12}H_{24}N_2O_2$: C, 63.12; H, 10.59; N, 12.27. Found: C, 62.93; H, 10.37; N, 11.93.

SI Figure 1:

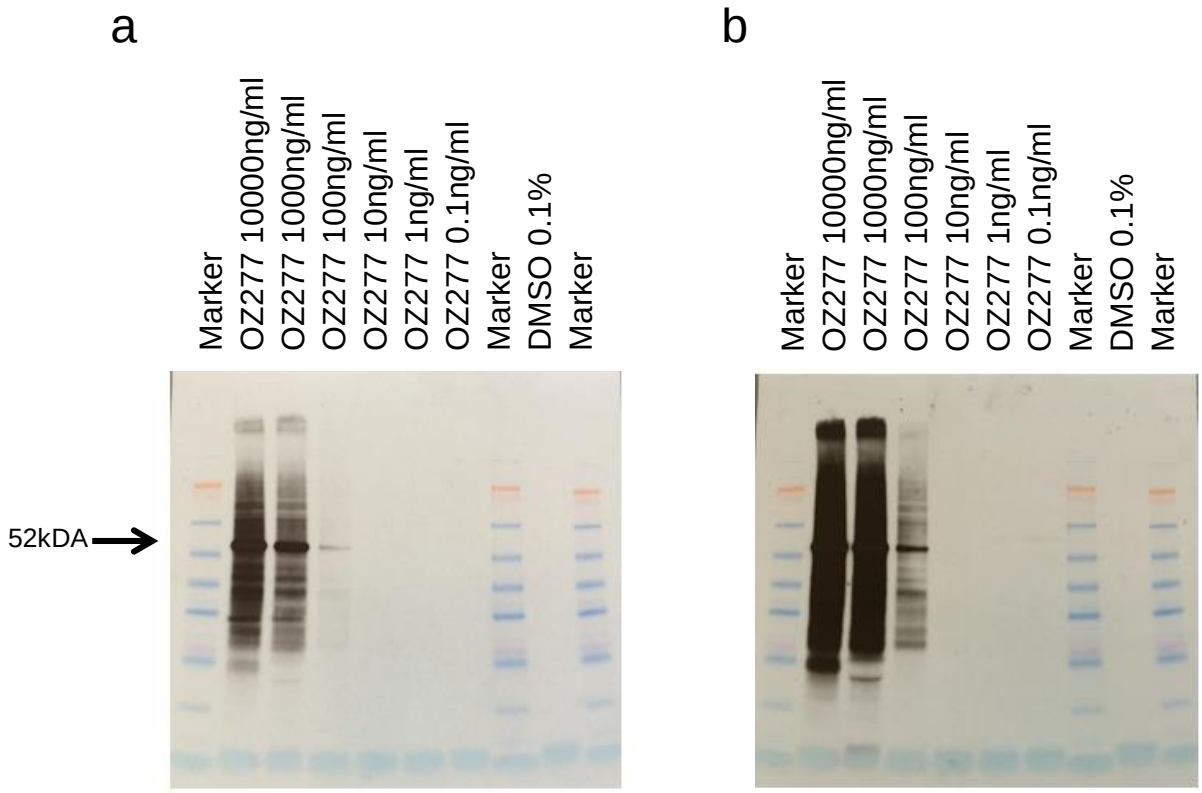
Immunofluorescence co-localisation studies with P. falciparum rings (top row) and schizonts (bottom row) that were treated with 10 µg/mL OZ277 for 2 h. The blood smears were fixed with prechilled methanol (-20 °C; 100%) and blocked with 1% BSA in PBS for 1 h. Scale bar 2 µm. The primary antibodies used were directly labelled OZH04-2/2 (Alexa 488, green signal) and GAPDH (Alexa 594, red signal). The left column shows parasites with OZH04-2/2 with an exposure of 2 s. The next column shows GAPDH with an exposure of 2 s, followed by DAPI staining (blue signal) with an exposure of 0.025 s and a scale bar of 2 µm, and then a merge of OZH04-2/2, GAPDH and DAPI. The last image is a merge of OZH04-2/2, GAPDH, DAPI and the reference image (exposure 0.1 s). Scale bar 2 µm.



SI Figure 2:

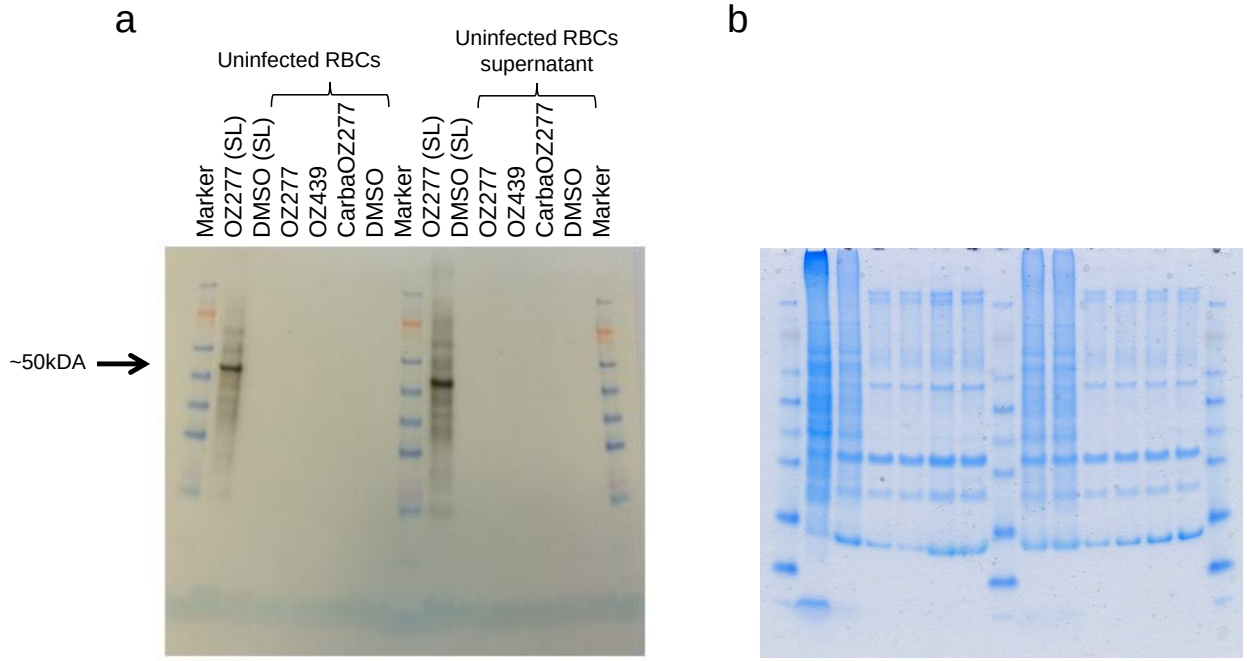
a) Western blot with mixed *P. falciparum* cultures. Cultures were treated with 10,000, 1,000, 100, 10, 1 and 0.1 ng/mL OZ277 or a DMSO control for 2 h. Under these experimental condition, a concentration of 100 ng/mL corresponds to ~1x IC99. Antibody OZH04-2/2 and a film exposure time of 1 s were used.

b) Longer film exposure (1 minute) of same blot.



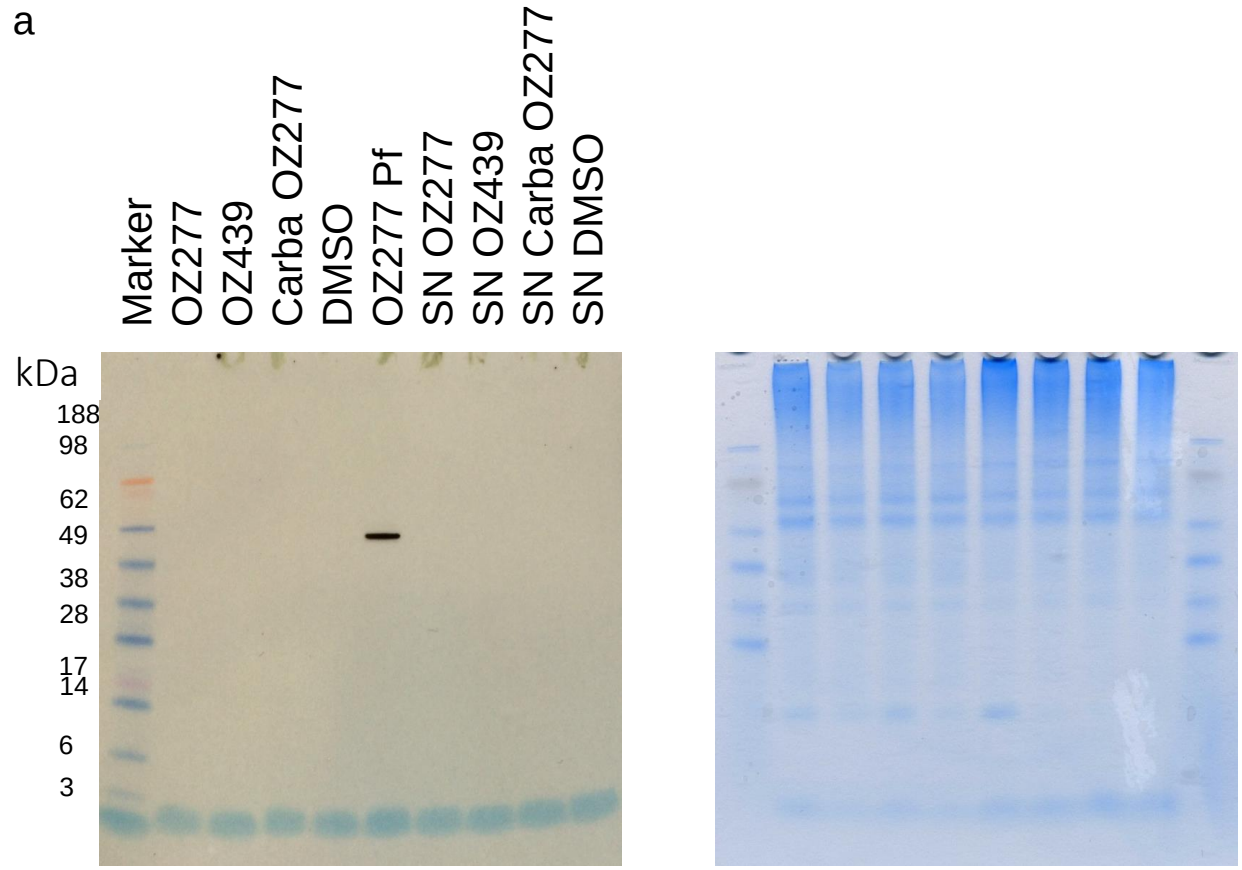
SI Figure 3:

a) Western blot with uninfected erythrocytes (RBCs), which were treated with 10 µg/mL OZ277, OZ439, CarbaOZ277 or a DMSO control for 2 h. Positive control samples from infected erythrocytes (SL, saponin lysate) are loaded next to the left and middle protein marker. Antibody OZH04-2/2 and a film exposure time of 1 s were used. b) Coomassie stained gel. All samples were normalized for their protein content.



SI Figure 4:

a) Western blot of Babesia divergens cultures that were treated with 10 µg/mL OZ277, OZ439, CarbaOZ277 or a DMSO control for 2 h. Streptolysin O was used for lysis as previously described.[2] A positive control sample from P. falciparum infected erythrocytes (saponin lysates) is shown next to the DMSO control sample. Antibody OZH04-2/2 and a film exposure time of 1 min were used. b) Coomassie stained gel. All samples were normalized for their protein content.



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3. In vitro activity of anti-malarial ozonides against an artemisinin-resistant isolate

Fabian Baumgärtner[1,2] , Joëlle Jourdan[1,2] , Christian Scheurer[1,2] , Benjamin Blasco[3] , Brice Campo[3] , Pascal Mäser[1,2], Sergio Wittlin[1,2,*]

[1]Swiss Tropical and Public Health Institute, Socinstrasse 57, CH-4002 Basel, Switzerland

[2]University of Basel, Petersplatz 1, CH-4001 Basel, Switzerland

[3]Medicines for Malaria Venture, ICC, 20 Route de Pré-Bois, PO Box 1826, 1215 Geneva, Switzerland

[*]Corresponding author

Email: sergio.wittlin@unibas.ch

Tel: +41 61 284 81 36

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Abstract

Background

Recently published data suggest that artemisinin derivatives and synthetic peroxides, such as the ozonides OZ277 and OZ439, have a similar mode of action. Here the cross-resistance of OZ277 and OZ439 and four additional next-generation ozonides was probed against the artemisinin-resistant clinical isolate *Plasmodium falciparum* Cam3.I, which carries the K13-propeller mutation R539T (Cam3.I^{R539T}).

Methods

The previously described *in vitro* ring-stage survival assay ($RSA_{0\text{-}3h}$) was employed and a simplified variation of the original protocol was developed.

Results

At the pharmacologically relevant concentration of 700 nM, all six ozonides were highly effective against the dihydroartemisinin-resistant *P. falciparum* Cam3.I^{R539T} parasites, showing a per cent survival ranging from <0.01 to 1.83%. A simplified version of the original $RSA_{0\text{-}3h}$ method was developed and gave similar results, thus providing a practical drug discovery tool for further optimization of next-generation anti-malarial peroxides.

Conclusion

The absence of *in vitro* cross-resistance against the artemisinin-resistant clinical isolate Cam3.I^{R539T} suggests that ozonides could be effective against artemisinin-resistant *P. falciparum.* How this will translate to the human situation in clinical settings remains to be investigated.

Keywords Ring-stage survival assay, Artemisinin, Ozonide, *Plasmodium falciparum,* Cam3.I^{R539T}, Drug resistance

Background

Malaria is one of the most important tropical diseases resulting in 214 million new cases and an estimated 438,000 malaria deaths worldwide in 2015 [1]. The discovery of artemisinin in the 1970s was an important step forward in anti-malarial drug therapy and was recognized with the Nobel Prize in Physiology or Medicine in 2015 [2,3]. Artemisinin and its semi-synthetic derivatives, such as dihydroartemisinin (DHA) (Fig. 1), artesunate and artemether, contain a unique sesquiterpene lactone peroxide (1,2,4-trioxane) structure and artemisinin-based combination therapy (ACT) represents the current first-line treatment of uncomplicated *Plasmodium falciparum* malaria [4,5,6]. Since the starting material artemisinin is a natural product, its production is limited to the availability of the plant [7,4], although several total syntheses of artemisinin have been described [8]. In 2004, Vennerstrom *et al.* reported the discovery of a completely synthetic peroxide anti-malarial containing a 1,2,4-trioxolane (ozonide) pharmacophore named OZ277 (arterolane) (Fig. 1) with anti-malarial activity comparable to the artemisinin derivatives [9,10]. In combination with piperaquine, arterolane was registered for anti-malarial combination therapy in India in 2011 [11,12,13,14]. The next-generation ozonide, OZ439 (artefenomel) (Fig. 1), exhibits an increased pharmacokinetic half-life and good safety profile and is now being tested in phase IIb clinical trials [12,14,15,16,17]. The iron-dependent alkylation hypothesis is one of the proposed modes of action of artemisinin and synthetic peroxides [18,19,20,21] where the peroxide is thought to be activated by the reductive cleavage in the presence of ferrous haem (or free Fe(II) derived from haem) released as a by-product of hemoglobin digestion in the food vacuole [20,22,23,24, 25,26,27]. Thereby carbon-centred radicals are generated, which then alkylate haem and parasite proteins [28,29,30,31,32,33]. The interaction of the artemisinin derivatives or ozonides with parasite targets is irreversible [31,34]. Although the semi-synthetic artemisinins are highly effective, prolonged parasite clearance times were first reported along the Thai–Cambodian border in 2006, suggesting

an emerging artemisinin resistance phenotype [35]. Today, delayed parasite clearance following treatment with artemisinin derivatives has been observed across Southeast Asia [36,37,38,39,40,41]. It was found that mutations in the Kelch 13 propeller domain are associated with ring-stage parasites entering a quiescent state with delayed parasite clearance after exposure to artemisinins [41,42,43,44,45]. When 50% inhibitory concentrations (IC_{50}) were measured using conventional methods such as the [^{3}H] hypoxanthine incorporation assay [46], no difference was observed between artemisinin-resistant and -susceptible strains after treatment with artemisinin or its derivatives [47,48,49,50]. In an effort to correlate the delayed parasite clearance observed *in vivo* with *in vitro* parasite survival, Witkowski *et al.* [48,49] developed a ring-stage survival assay ($RSA_{0\text{-}3h}$) that exploited the differences in susceptibility observed between wild-type and K13 mutants at the early ring stage of the asexual blood cycle following a short pulse of artemisinin treatment. In the $RSA_{0\text{-}3h}$, synchronized young ring stage parasites (0-3 hrs old) are exposed to drugs for six hours, and then cultured in drug free culture medium for 66 hours before relative growth is determined by microscopic analysis [48,49]. Since the structural analogies between artemisinins and ozonides (Fig. 1) suggest that they share similar modes of action, and thus some level of cross resistance [9,10,51,52], the per cent survival of an artemisinin-resistant clinical isolate (Cam3.I^{R539T}) treated with DHA, OZ277, OZ439, and four additional next-generation ozonides (Fig. 1) using the $RSA_{0\text{-}3h}$ as described by Witkowski *et al.* [48,49] was evaluated. Additionally, a sub-set of these compounds was tested in the $RSA_{0\text{-}3h}$ described by Xie *et al.* [53] that also uses tightly synchronized ring-stage cultures, but allows the assay to be performed routinely within a convenient time-frame.

Methods

Parasite cultivation

The artemisinin-resistant *P. falciparum* isolate Cam3.I^{R539T} from Battambang, Cambodia was obtained from BEI Resources [54] with the accession number MRA-1240. The drug-sensitive *P. falciparum* strain NF54 (airport strain from The Netherlands) was provided by F. Hoffmann-La Roche Ltd. Parasites were cultivated in standard cultivation medium, consisting of hypoxanthine (50 mg/l), RPMI (10.44 g/l) supplemented with HEPES (5.94 g/l), albumax (5 g/l), sodium bicarbonate (2.1 g/l) and neomycin (100 mg/l) [55].

Ring-stage survival assays ($RSA_{0\text{-}3h}$)

Ring-stage survival assays ($RSA_{0\text{-}3h}$) were carried out essentially as previously described by Witkowski *et al.* [48], but with a few modifications in the drug-washing procedure to ensure that no residual peroxide was present during the 66-hour post-treatment period [56]. Briefly, zero to three hours post-invasion ring stages were adjusted to 1% parasitaemia and 2.5% haematocrit by adding uninfected erythrocytes, transferred in a total volume of 1 mL into 48-well plates and exposed for six hours to a range of concentrations (700, 350, 175, 88, and 49 nM) of DHA or one of the six ozonides tested in this study. The synthesis of the four next-generation ozonides, OZ493, OZ609, OZ655 and OZ657, will be reported in due course by the laboratory of Prof. Jonathan Vennerstrom (manuscript with ID jm-2016-01586n in the Journal of Medicinal Chemistry is currently in press). After six hours, cultures were transferred to 15 mL conical tubes, centrifuged at 1,400 rpm (400 g) for 2 min and carefully washed two times with 12 mL of culture medium. The complete removal of compound after washing was verified by incubating the supernatant recovered after the last washing step with fresh cultures of NF54 parasites, ensuring that no growth inhibition was detected. After washing, blood pellets were resuspended in complete drug-free culture medium, transferred into new wells and cultured for 66 hours under standard conditions.

Thin blood smears were prepared, methanol-fixed and stained with 10% Giemsa. Per cent survival was assessed using light microscopy, counting the number of parasitized cells in ≥10,000 red blood cells (RBCs) and comparing survival to that of the drug-free dimethylsulfoxid incubation. Microscopy analysis was performed independently by two microscopists, one having more than 15 years of work experience.

Alternative parasite synchronization method

Parasites were synchronized according to Xie *et al.* [53] with 5% d-sorbitol. After 30 and 43 hours, parasites were synchronized a second and third time, respectively, resulting in zero to one-hour old ring-stage parasites. The $RSA_{0\text{-}3h}$ was initiated two hours later.

Standard [^{3}H] hypoxanthine incorporation assay

The *in vitro* anti-malarial activity was measured using the [^{3}H]-hypoxanthine incorporation assay [55]. Results were expressed as the concentration resulting in 50% inhibition (IC_{50}).

Results

The per cent survival of parasites exposed to a concentration range of DHA and six different ozonides (Fig. 1) was determined using the artemisinin-resistant *P. falciparum* Cambodian isolate Cam3.I^{R539T}. As expected, DHA exposure gave a high survival rate ranging from 74 to 33% at concentrations of 49 and 700 nM, respectively (Fig. 2), which is comparable to the observed survival value of 40% at 700 nM published previously [44]. In contrast, when tested at 700 nM, the two ozonides OZ277 and OZ439 showed an approximate 18- to 45-fold increase in potency compared with DHA (Fig. 2). Full and equal potency was observed when DHA, OZ277 and OZ439 were tested in parallel in the RSA$_{0\text{-}3h}$ using the artemisinin-sensitive strain NF54 (Supplementary Table S1). At the lowest concentration (49 nM), OZ277 had poor activity, showing a similar per cent survival to that of DHA, whereas OZ439 was still about five-fold more potent. A possible explanation for OZ439 being more potent than OZ277 could be related to its improved stability in blood as previously described [15]. In those studies, OZ277 or OZ439 were incubated at 37°C in *P. falciparum*-infected human blood. After 2 hours more than 90% of OZ277 was degraded, whereas OZ439 was found to be about 10-20x more stable. A similar and more recent study found similar differences in stability for OZ277 and OZ439 [56]. The same compounds were also tested in a more convenient variation of the standard RSA$_{0\text{-}3h}$ that uses synchronized ring-stage cultures that can be easily produced during normal working hours [53]. As shown in Table 1, this alternative synchronization method gave results that were comparable to those obtained using the standard RSA$_{0\text{-}3h}$.

To investigate further the level of cross-resistance between DHA and the ozonides, four additional next-generation ozonides (OZ493, OZ609, OZ655, OZ657) (Fig. 1) were tested against the Cam3.I^{R539T} parasites. While all six ozonides had a similar IC50 value using a conventional 72-hour [^{3}H] hypoxanthine incorporation assay (Supplementary Table S2), the RSA$_{0\text{-}3h}$ showed that OZ493,

OZ609 and OZ655 were highly potent and completely inhibited the growth of the artemisinin-resistant isolate at the two highest concentrations tested (Fig. 2). At the lowest concentration, potency was comparable to that for OZ439. The overall potency of OZ657 was comparable to that of OZ277.

The $RSA_{0\text{-}3h}$ was recently developed to provide an *in vitro* correlate of the longer *in vivo* parasite clearance times observed after artemisinin treatment in Southeast Asia, which is widely interpreted as a sign of potential artemisinin resistance [57,58]. Provided that the $RSA_{0\text{-}3h}$ does indeed predict the potency of compounds against artemisinin-resistant parasites in malaria patients, the here described data suggest that all of the tested ozonides are highly potent against isolates such as *P. falciparum* Cam3.I^{R539T}. These data are in line with the recent clinical observation that the parasite clearance rate following OZ439 treatment is not significantly affected by resistance-associated mutations in the Kelch 13 propeller region [17] and the recent data published by Siriwardana *et al.* [59], which showed no reduced susceptibility of OZ439 in a different delayed clearance phenotype parasite (Cam3.II) *in vitro*.

Conclusion

In the traditional $RSA_{0\text{-}3h}$, as well as a more convenient variation of the original method, all of the tested ozonides, were highly potent against the artemisinin-resistant isolate *P. falciparum* Cam3.I^{R539T} in contrast to results for DHA. These data indicate that artemisinin-resistant *P. falciparum* infections could be successfully treated with ozonide anti-malarial drugs.

Abbreviations

OZ277: arterolane; OZ439: artefenomel; DHA: Dihydroartemisinin; ACT: Artemisinin combination therapy; RBC: Red blood cell.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

FB, JJ, CS, BB, BC, PM and SW designed the research. FB and CS performed the research. All authors analysed data. FB, JJ and SW wrote the manuscript. All authors had full access to all data in the study and read and approved the final manuscript.

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Fig. 1 Chemical structures of dihydroartemisinin (DHA) and the six ozonides investigated

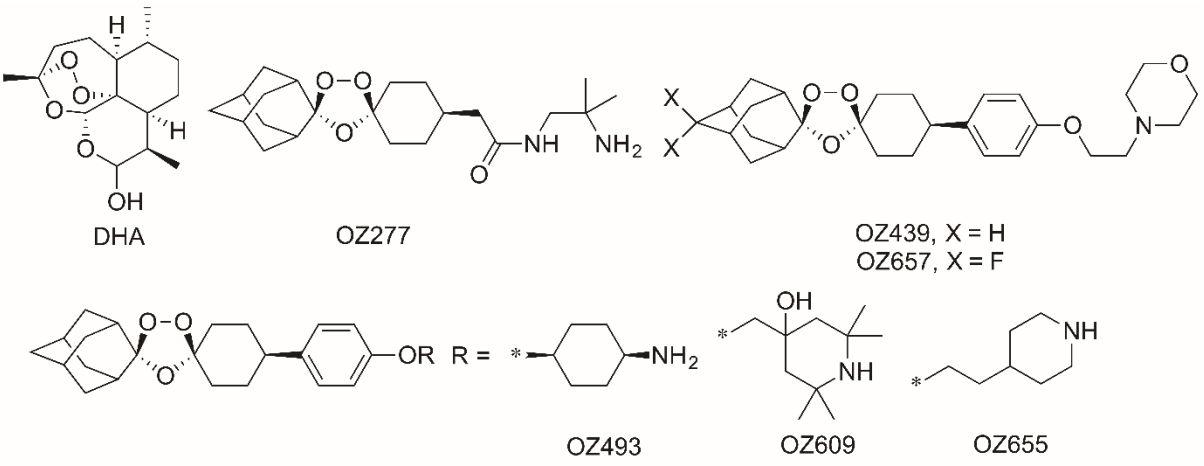
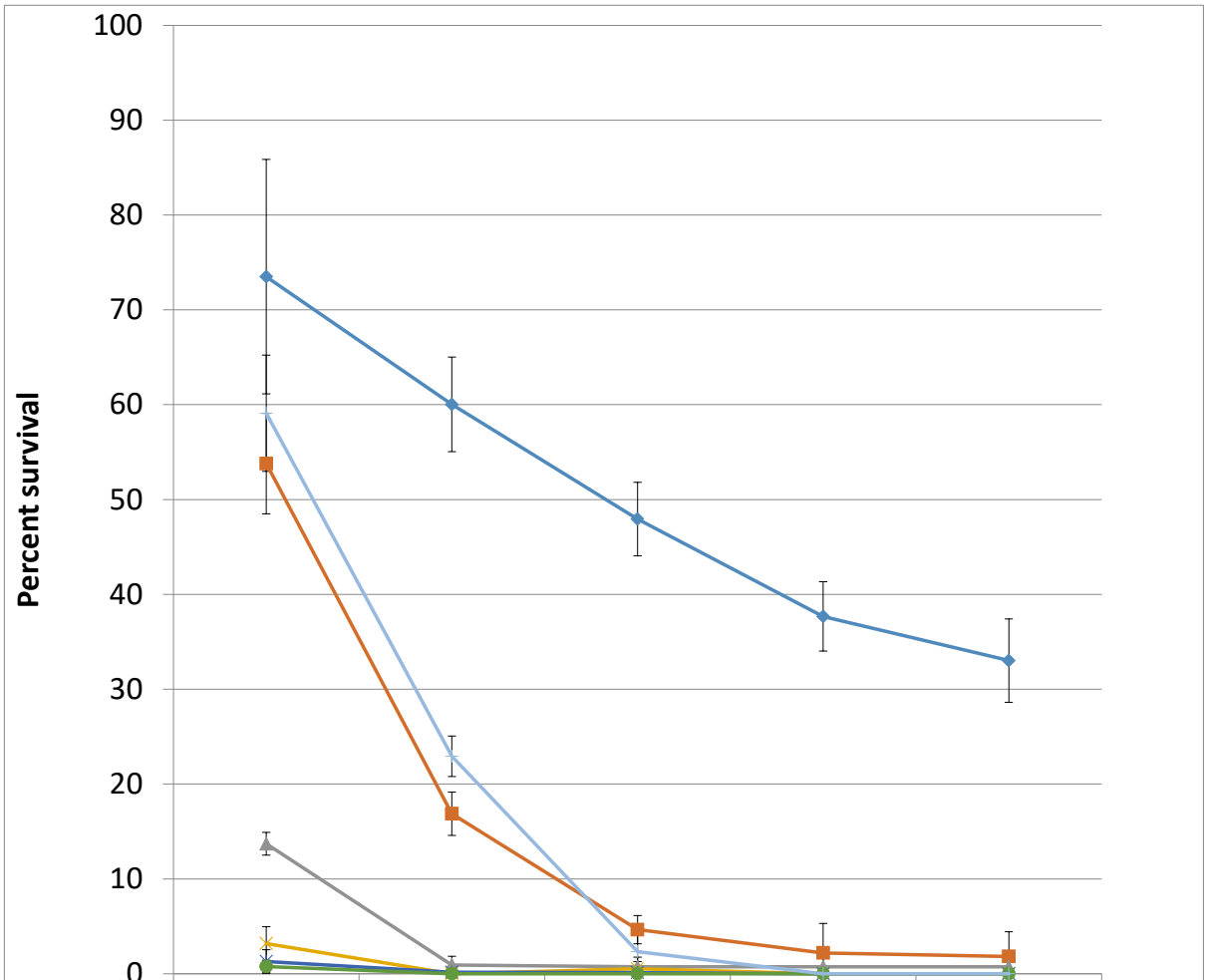


Fig. 2 Mean per cent survival ± standard error (SE) of *Plasmodium falciparum* isolate Cam3.I^{R539T} parasites after a 6-hour exposure to a range of concentrations of dihydroartemisinin (DHA) or six different ozonides

Three biological replicates were performed per compound.



	49 nM	88 nM	175 nM	350 nM	700 nM
DHA	73.50	60.03	47.94	37.68	33.02
OZ277	53.80	16.87	4.65	2.20	1.83
OZ439	13.70	0.93	0.73	0.73	0.73
OZ493	3.19	0.00	0.53	0.00	0.00
OZ609	1.29	0.17	0.12	0.00	0.00
OZ655	0.76	0.00	0.00	0.00	0.00
OZ657	59.10	22.93	2.33	0.00	0.00

Table 1 Mean per cent survival (individual values in brackets) of Cam3.I^{R539T} isolate after 6 hours exposure to a range of concentrations of DHA, OZ439 or OZ277 using the synchronization protocol from Xie *et al.* [53].

Two biological replicates were performed per compound.

Compounds	**RSA values (per cent survival) at different concentrations**		
	175 nM	**350 nM**	**700 nM**
DHA	46 (49,43)	42 (45,39)	37 (39,35)
OZ277	4.0 (4.4,3.6)	2.3 (1.9,2.7)	1.4 (1.7,1.1)
OZ439	<0.01 (<0.01,<0.01)	<0.01 (<0.01,<0.01)	<0.01 (<0.01,<0.01)

Supplementary Table S1 Mean per cent survival (individual values in brackets) of NF54 after 6 hours exposure to 500 nM of DHA, OZ439 or OZ277 using the synchronization protocol from Straimer *et al.* [44.]

Two biological replicates were performed per compound.

Compounds	Per cent survival
DHA	<0.01 (<0.01,<0.01)
OZ277	<0.01 (<0.01,<0.01)
OZ439	<0.01 (<0.01,<0.01)

Supplementary Table S2 Mean *in vitro* IC_{50} values (single values in brackets) for *Plasmodium falciparum* isolate Cam3.I^{R539T} and NF54 in the 72-hour [^{3}H] hypoxanthine assay

Two biological replicates were performed per compound.

Compounds	IC_{50} (nM) Cam3.I^{R539T}	IC_{50} (nM) NF54
DHA	1.5 (1.7,1.3)	2.6 (2.4,2.8)
OZ277	1.8 (1.8,1.8)	1.7 (1.7,1.8)
OZ439	4.6 (4.6,4.6)	4.2 (4.6,3.9)
OZ493	3.6 (3.6,3.7)	3.8 (4.5,3.1)
OZ609	1.8 (1.8,1.8)	1.3 (1.4,1.2)
OZ655	2.8 (2.7,2.8)	2.5 (2.3,2.7)
OZ657	2.9 (2.9,2.9)	2.3 (2.3,2.3)

4. Identification of the Alkylation Signatures of Antimalarial Ozonides and Artemisinin

Joëlle Jourdan[1,2], Hugues Matile[3], Alexander Schmidt[4], Yuxiang Dong[5], Xiaofang Wang[5], Jonathan L. Vennerstrom[5], Remo S. Schmidt[1,2], Sergio Wittlin[1,2] and Pascal Mäser[1,2*]

[1]Swiss Tropical and Public Health Institute, Socinstrasse 57, CH-4002 Basel, Switzerland

[2]University of Basel, CH-4003 Basel, Switzerland

[3]F. Hoffmann- La Roche Ltd., CH-4070 Basel, Switzerland

[4]Proteomics Core Facility, Biozentrum, University of Basel, CH-4056 Basel, Switzerland

[5]College of Pharmacy, University of Nebraska Medical Center, 986025 Nebraska Medical Center, Omaha, Nebraska 68198, United States

[*]Corresponding author

E-mail: pascal.maeser@swisstph.ch

Tel: 41 61 284 83 38

Personal Contribution to the Paper: I tested drug sensitivity, produced and analysed Western blot data, optimized conditions for sample preparation and prepared samples for mass spectrometry. Further, I analysed hit lists and helped writing the paper.

Abstract

Antimalarial peroxides such as the phytochemical artemisinin or the synthetic artefenomel are prodrugs that need to be activated by reductive cleavage of the peroxide bond. This happens inside the malaria parasite, presumably in the food vacuole with ferrous heme as the electron donor. The generated radicals will then alkylate heme itself and proteins. Here we determine the alkylation signatures of artemisinin and synthetic ozonides by chemical proteomics in *Plasmodium falciparum* using alkyne probes that allow to identify the target proteins by click chemistry and affinity purification. Two forms of controls are included: the non-clickable parent peroxides, as well as non-peroxidic derivatives of the alkyne probes. These permitted to stringently determine the target space of the peroxides, identifying a total of 18 proteins for artemisinin and 41 for the synthetic ozonides, with an intersect of 14. We further compare the alkylation signatures of the antimalarial peroxides to each other and to previously published datasets.

Introduction

Malaria remains one of the most prevalent infectious diseases worldwide, with an estimated death toll of 429,000 in 2015 (WHO Report 2016). The most lethal form of malaria, malaria tropica, is caused by *Plasmodium falciparum*. The current first-line treatments for malaria tropica are the artemisinin-based combination therapies, ACTs (White, 2008), such as artemether-lumefantrine (Coartem) or dihydroartemisinin-piperaquine (Artekin; Anthony et al., 2012). Artemisinin, discovered in 1971 by Youyou Tu (Tu, 2011), is a sesquiterpene lactone with an endoperoxide bridge (Figure 1). It is of highest activity and specificity against malaria parasites. However, as a natural product artemisinin has two limitations: its production is laborious and constrained by the availability of the plant *Artemisia annua*, and its pharmacological properties are difficult to improve (Avery et al. 1992, Klayman et al. 1985, Kumar et al 2005). Thus, synthetic antimalarial peroxides are a promising alternative for future antimalarials (Vennerstrom et al., 2004; O`Neill et al., 2017). Vennerstrom and co-workers concentrated on the synthesis of adamantane-based 1,2,4-trioxolanes (Figure 1), herein called ozonides, and presented a first drug candidate, OZ277 or arterolane maleate in 2004 (Vennerstrom et al., 2004; Tang et al., 2004). In spite of its suboptimal stability in *P. falciparum*-infected blood, arterolane maleate was subsequently registered for antimalarial therapy in India in combination with piperaquine phosphate (Synriam®; Mäser et al., 2012; Anthony et al., 2012; Patil et al., 2014; Wells et al., 2015). A next generation ozonide with improved pharmacokinetic properties, OZ439 or artefenomel, is presently being tested in phase IIb clinical trials in combination with ferroquine (Charman et al., 2011; Moehrle et al., 2013; Wells et al., 2015, Phyo et al., 2016 and Blasco et al., 2017).

Artemisinins and ozonides are thought to possess similar modes of action since for both classes, the endoperoxide bridge was found to be necessary for antimalarial activity (Klayman 1985; Kaiser et al. 2007). One of the proposed modes of actions is therefore the iron-dependent artemisinin

alkylation hypothesis (Kaiser et al., 2007; Creek et al., 2008; Meunier et al., 2010), which suggests that ferrous heme, or Fe^{II} derived from heme, is released as a product of hemoglobin digestion in the parasite's food vacuole and leads to a reductive cleavage of the peroxide bond (Klonis et al., 2011; Robert et al., 2013; Klonis e al., 2013; Hartwig et al., 2009). This generates highly reactive radicals that alkylate intracellular targets such as proteins and heme itself (Asawamahasaksa et al., 1994; Meshnick, 2002; Tilley et al., 2011). The alkylation hypothesis is supported by the findings that the digestive vacuole is an important initial site for antimalarial peroxide activity (Crespo et al.; 2008; Uhlemann et al., 2007), and that artemisinin and OZ277 are inactive against pathogens that do not degrade hemoglobin (Kaiser et al., 2007). The formation of carbon-centered radicals was found to be critical for the activity of antimalarial peroxides (Tang et al., 2005; Hartwig et al., 2011, Fügi et al., 2010) and the activities of artemisinin as well as ozonides do not derive from reversible interactions with parasite targets (Fügi et al., 2010; Abiodun et al., 2013).

The elucidation of the mode of action and identification of the intracellular targets of the antimalarial peroxides has become all the more pressing after the emergence of artemisinin-resistant *P. falciparum* isolates in South-East Asia (Noedl et al., 2008; Dondorp et al., 2009), and recently also in Africa (Lu et al., 2017), whose mechanism of resistance is not fully understood either (Blasco et al., 2017). Alkyne-tagged artemisinins and 1,2,4-trioxolanes have been used as chemical probes for activity-based profiling of the peroxide target signatures in *P. falciparum* (Wang et al., 2015; Ismail et al., 2016a; Ismail et al., 2016b), and the alkylation signature of a clickable 1,2,4-trioxolane was found to be similar to that of artemisinin (Ismail et al., 2016b). Here we further sharpen this approach, assessing the alkylation signatures of artemisinin and ozonides in parallel, and including control molecules that allow to focus on those drug-target interactions that depend on the presence of a peroxide bond.

Results

Design and synthesis of alkynes as chemical probes

If the peroxides are indeed activated by reductive cleavage and subsequently alkylate proteins, then the covalent interaction between drug and target will facilitate the identification of the latter. We designed alkyne derivatives of artemisinin and of an archetypal ozonide, OZ03, as chemical probes that can be linked by azide-alkyne Huisgen cycloaddition (click-chemistry; Kolb et al., 2001) to biotin or other markers (Figure 1). In addition to the parent compounds, which allow to discriminate false positive hits, we included a second type of control molecules that possessed the alkyne group but lacked the peroxide bridge: the deoxy form of the artemisinin alkyne, deoxyAA2, and the carba form of OZ727, carbaOZ727 (Figure 1). These allow to discriminate hits that may actually be bound by the probes, but not by means of peroxide reactivity.

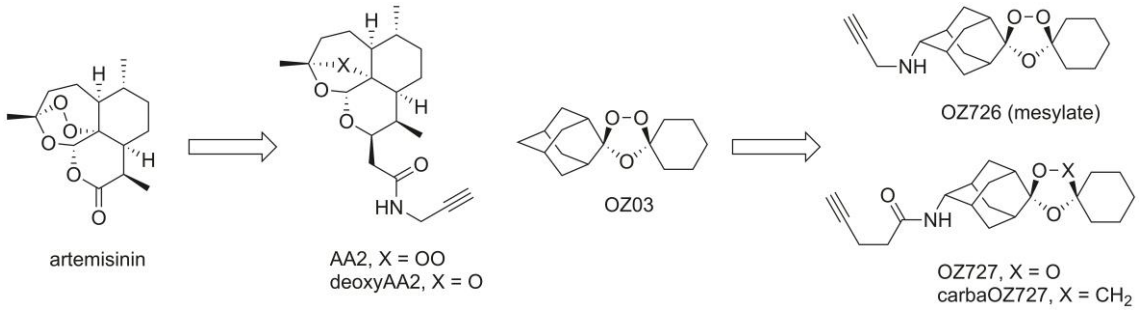


Figure 1. Chemical structures of the parent molecules artemisinin and OZ03 plus the newly synthesized alkyne derivatives as chemical probes.

Bioactivity of the chemical probes

The 50% inhibitory concentrations (IC_{50}) of the newly synthesized alkynes against intraerythrocytic *P. falciparum* in culture were determined using the [^{3}H]-hypoxanthine incorporation assay (Desjardins et al., 1979). The alkynes AA2, OZ726 and OZ727 were highly active against *P. falciparum* NF54 parasites, whereas the nonperoxidic analogues carbaOZ277, carbaOZ727 and deoxyAA2 were at least 1,000-fold less active (Table 1). Thus, as expected, the antiplasmodial

activity was dependent on the presence of the peroxide bridge, while the chosen alkyne modifications did not compromise the antiplasmodial activity of the molecules. In particular, it was interesting to note that the ozonides appeared to permit modification of the adamantane moiety.

	Peroxide?	Alkyne?	IC_{50} [ng/ml]
AA2	Yes	Yes	2.44
Artemisinin	Yes	No	2.28
DeoxyAA2	No	Yes	>10,000
OZ439	Yes	No	2.03
OZ277	Yes	No	0.91
CarbaOZ277	No	No	4,900
OZ03	Yes	No	2.36
OZ726	Yes	Yes	0.74
OZ727	Yes	Yes	0.98
CarbaOZ727	No	Yes	>10,000

Table 1. Mean IC_{50} values of the parent molecules and the newly synthesized alkynes against *P. falciparum* NF54. All assays were performed in three biological replicates.

Protein alkylation by the chemical probes

A series of pilot experiments was carried out to optimize the incubation parameters (Figure S1) and the reaction conditions for click chemistry. In brief (see Material and Methods for details), *P. falciparum* cultures were incubated for 4 h with a chemical probe, followed by harvesting and lysis of the parasites. The protein extracts were then treated with biotin-azide under copper to catalyze the cycloaddition with the alkyne. After acetone precipitation, the samples were run on polyacrylamide gels for Western blotting, using streptavidin-coupled horseradish peroxidase to detect the alkylated proteins.

Parasite cultures treated with 1 µg/ml AA2, OZ726 or OZ727 produced clear signals with reproducible banding patterns, whereas neither the non-clickable parent compounds nor the

nonperoxidic alkynes gave a signal on the Western blot (Figure 2A). The signal of the alkyne OZ726 was outcompeted by increasing ratios of the parent compound OZ03 (Figure 2B), indicating that the parental ozonide and the alkyne derivative have the same binding sites and that binding is saturable. These results confirmed that the peroxides covalently bind to proteins, as was known from previous studies (Meshnick et al., 1993), and that they possess specific alkylation signatures rather than binding unspecifically to all the *P. falciparum* proteins (Asawamahasakda et al., 1994; Fügi et al., 2010; Abiodun et al., 2013). In order to identify these alkylation signatures by mass spectrometry, we tested whether the click chemistry also works for precipitation of the bound proteins with neutravidin-coupled agarose beads. This was indeed the case: protein bands were detected by Western blot in *P. falciparum* cultures treated with 100 ng/ml AA2, whereas no bands were observed in parasite cultures treated with 0.1% DMSO. Negative controls such as the neutravidin beads alone or uninfected erythrocytes without drug gave no signal (Figure 2C).

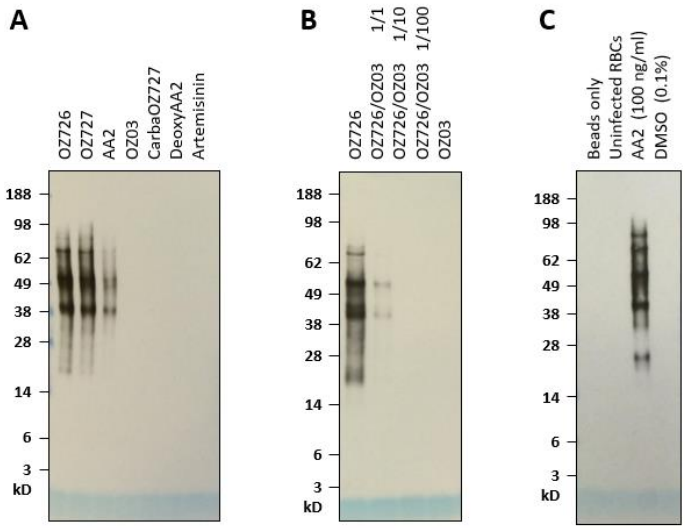


Figure 2. Western blot experiments with *P. falciparum* cultures treated with (A) 1 µg/ml OZ726, OZ727, AA2, OZ03, carbaOZ727, deoxyAA2 or artemisinin for 4 h, protein extracts prepared with 1.2% SDS in PBS, exposure time 1 min, and (B) competing concentrations of OZ726 and the non-clickable parent OZ03 for 2 h, extracts prepared with Ripa-Lysis buffer, exposure time 10 s. (C) shows a precipitation experiment with cultures treated

with 100 ng/ml AA2 in 0.1% DMSO for 4 h and different negative controls. Protein extracts were prepared with 1.2% SDS in PBS and precipitated with neutravidin agarose resin; exposure time 1 min.

Mass spectrometry-based proteomics to identify alkylated proteins

The proteomics experiments were performed at an about 30x higher parasitemia (10%) compared to the hypoxanthine incorporation assays (0.3%). From previous work it is known that a 30x higher parasitemia results in a 30x higher inhibitory concentration (Jourdan et al., 2015). As a consequence, in the here described studies we treated *P. falciparum* NF54 cultures of 7% to 12% parasitemia with 100ng/ml (corresponding to about an IC_{99}) as well as 1000ng/ml artemisinin, AA2, deoxyAA2, OZ03, OZ726, OZ727, carbaOZ727 or DMSO only, each in biological triplicates. The lysed samples were subjected to click-chemistry with biotin-azide as above, incubated with neutravidin agarose beads, and washed extensively (see Figures S2 and S3 for optimization of the washing conditions). The bound proteins were eluted, digested with trypsin, and processed for mass spectrometry as described in Material and Methods. The proteins were identified by their fingerprints of trypsin fragments as compared to the predicted peptides of the proteome of *P. falciparum* NF54 (Uniprot ID UP000030673).

Overall only 59 different proteins were significantly (>1.8 fold, $p<0.05$) enriched in any one of the probe-treated cultures vs. the DMSO-treated control, indicating again that the peroxides have specific alkylation signatures. The results are summarized in Tables S1 and S2. All the proteins that were also obtained with the unmodified parent molecules, 1 (PFNF54_01079, dihydrofolate reductase-thymidylate synthase) for artemisinin and 10 for OZ03 (Table S1), were removed from the list of hits. Next, we removed the hits that were also obtained with the nonperoxidic alkyne probes, 6 for deoxyAA2 but 0 for carbaOZ727 (Table S1). A potentially interesting hit among the proteins fished with deoxyAA2 was 1-cys peroxiredoxin (PFNF54_02028), since another *P. falciparum* peroxiredoxin, PfAOP, had been implicated in artemisinin susceptibility (Djuika et al.,

2017). The removal of all the hits that turned up in any of the control samples yielded a final list of 45 *P. falciparum* proteins that were bound by the chemical probes by means of peroxide activity (Table 2).

Alkylation signatures of artemisinin and ozonides

We used the 100 ng/ml samples to compare the alkylation signatures of artemisinin and the two ozonides. A total of 25 different *P. falciparum* proteins was alkylated by either AA2, OZ726 or OZ727 (Table 2). Unsurprisingly, the largest degree of overlap was between the identified target spaces of OZ726 and OZ727 (Figure 3A). There was also a clear overlap between the identified targets of AA2 with those of OZ726, and to a lesser extent with those of OZ727 (Figure 3A). Only one protein was identified with all three peroxides: PFNF54_01699, which corresponds to PlasmoDB entry PF3D7_0706500. However, this protein is not linked to any publication and does not contain any known domains (according to PlasmoDB). A blastp search with PF3D7_0706500 revealed significant similarity to proteins annotated as erythrocyte membrane proteins in *P. coatneyi* and *P. yoelii*, as well as a PfEMP3-like protein in *P. berghei*. The latter contains an RNA recognition motif (RRM) that was also found by the NCBI algorithm during the blastp search.

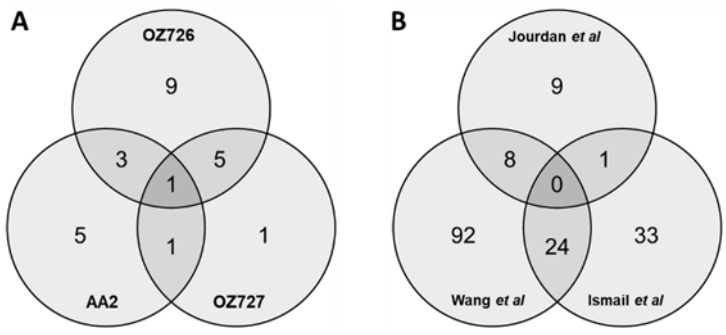


Figure 3. Venn diagrams of (A) all the hits obtained after treatment with 100 ng/ml artemisinin alkyne, OZ726 or OZ727; and (B) and of all the hits identified with an alkyne derivative of artemisinin in the present study compared to three published datasets (Wang et al., 2015; Ismail et al., 2016a and b).

The samples incubated with 1000 ng/ml of the chemical probes, though arguably a less physiological concentration than 100 ng/ml, also yielded a number of interesting hits, and they had a stronger overlap between the artemisinine-alkyne and ozonide-alkyne samples. Nine of the 11 proteins identified with AA2 were also present in the target space of OZ726. However, OZ726 alkylated an additional 16 proteins that were not identified in any of the AA2 1000 ng/ml experiments (Table 2). Thioredoxin peroxidase 1 and a thioredoxin-related protein were identified as alkylation targets of both artemisinin and synthetic peroxides (Table 2). Proteins of interest that were identified with ozonide alkynes but not with artemisinin alkyne were subunits of the V-type ATPase, multidrug resistant protein 1, the proteasome subunit beta type-6, aminopeptidase P and plasmepsin III (Table 2).

Comparison to published datasets

The obtained results (Table 2) were compared with other studies that had also used alkyne derivatives of antimalarial peroxides, followed by click chemistry and mass spectrometry-based proteomics, to identify potential targets (Wang et al., 2015; Ismail et al., 2016a and 2016b). Table 3 lists all the common hits with these studies, Figure 3B focuses on the intersects between the hits obtained with artemisinin-alkynes. The overlap of our results with those of Wang et al. was larger than with those of Ismail et al. Using AA2 (Figure 1), we have identified a total of 18 hits (Figure 3B, Table 2), of which 8 were also identified in the 124 hits by Wang et al (p, assuming a protein space of 3000), while there was only 1 common protein between our hits and the 58 hits obtained by Ismail et al. (Figure 3B). The negative controls were DMSO in Wang et al, and DMSO and nonperoxidic-alkynes in Ismail 2016a.

Discussion

The present study is in agreement with earlier work (Asawamahaska et al., 1994; Jourdan et al., 2015; Ismail et al 2016a and b; Wang et al., 2015) to support the notion that antimalarial peroxides covalently bind target molecules in *P. falciparum* after reductive bioactivation of the peroxide bridge. Heme itself is among these targets (Robert et al., 2005; Creek et al., 2008), and we cannot exclude further non-proteinaceous structures to be alkylated as well. Here we focus on the protein targets of artemisinin and synthetic ozonides, since these are straightforward to identify by mass spectrometry based on their fingerprints after digestion with trypsin. The same approach, using alkyne derivatives as chemical probes, had been taken by two other labs (Wang et al., 2015; Ismail et al., 2016a and b). While there was a clear overlap between the target spaces identified herein and those by Wang et al. (2015), there is less consistency with the data from Ismail et al. (2016 a and b; Table 3). This might be because the two former sets of experiments were performed with unsynchronized cultures of the *P. falciparum* erythrocytic cycle, whereas the latter concentrated on trophozoites. Furthermore, the present study incorporated additional controls in the form of the non-alkyne parent molecules artemisinin and OZ03, plus the aperoxidic, inactive but clickable molecules deoxyAA2 and carbaOZ727 (Figure 1). These turned out to be helpful to scrutinize the obtained hits. Dihydrofolate reductase-thymidylate synthase (DHFR-TS, PF3D7_0417200), for example, had been identified as a candidate target of artemisinin (Ismail et al., 2016a) and was also isolated here with all the clickable peroxides, AA2, OZ726 and OZ727 (Table S1). However, as DHFR-TS was also isolated with the non-clickable controls artemisinin and OZ03, and with the non-peroxidic control deoxyAA2 (Table S1), it was removed from the list of alkylation targets (Table 1). The remaining intersect of the protein sets identified by the different proteomics studies (Table 2) present a robust set of candidate alkylation targets of antimalarial peroxides.

It was also interesting to compare our results to the study by Asawamahaska et al., who had used radiolabeled dihydroartemisinin as a probe to identify four major *P. falciparum* protein bands of 25 kDa, 50 kDa, 65 kDa and >200 kDa, plus two minor bands of 32 kDa and 42 kDa as the alkylation signature (Asawamahasakda et al., 1994). This resembled the pattern observed by Western blot with artemisinin alkyne (Figure 2C), with protein bands at ~25 kDa, ~50 kDa, ~68 kDa, ~32 kDa and 42 kDa. Several *P. falciparum* proteins were identified that match these molecular weights and are alkylated by peroxide probes (Table 2), e.g. a thioredoxin-related protein of 24 kDa, a glideosome associated protein of 42.6 kDa, or the proteasome subunit beta type-6 of 32.7 kDa. The latter may be of interest since a recent study (Leann Tilley; MMV Meeting Oktober 2017) found artemisinin to act against the proteasome.

We paid special attention to hits of about 50 kDa, since this was the size of the main band identified in *P. faclciparum* cultures treated with OZ277 and labeled with monoclonal antibodies against the adamantane portion of the ozonides (Jourdan et al., 2015). Three candidates were identified (Table 2): V-type proton ATPase subunit H (53.8 kDa), ATP-dependent RNA helicase UAP56 (52.2 kDa) and plasmepsin III (51.7 kDa). Plasmepsin III is a likely target since it localizes to the food vacuole and was suggested to digest hemoglobin (Moura et al., 2009). Further proteins of the parasite's food vacuole that were alkylated by the peroxidic probes were aminopeptidase P, also involved in hemoglobin degradation (Rhageb et al., 2009), and multidrug resistance protein 1, a transporter in the digestive vacuolar membrane (Ding et al., 2011; Valderramos et al., 2006; Duraisingh et al., 2005). Only one of the 45 identified alkylation targets (Table 2) was present in all the peroxide-treated cultures, the EMP-like protein PFNF54_01699, a parasite protein thought to be exported to the membrane of the erythrocyte and to associate with its cytoskeleton (Pei et al., 2007; Senczuk et al., 2001).

In conclusion, we show that the designed peroxide probes alkylate *P. falciparum* proteins in a peroxide-dependent manner, and that they possess alkylation signatures of distinct Western blot

bands and relatively small sets of target proteins. There was an overlap between the alkylation signatures of artemisinin and ozonides, but to a lesser degree than observed in a previous study (Ismail et al., 2016b). The presence of food vacuolar proteins among the alkylated targets, plasmepsin III in particular, supports the iron-dependent alkylation model. While we cannot presently say to what degree the observed protein alkylation signatures contribute to the antimalarial activity of the peroxides, we think that the identified sets of target proteins may hold a key to a better understanding of the mode of action of artemisinin and synthetic peroxides.

Material and Methods

Parasite cultivation and drug sensitivity testing

Asexual blood stages of *P. falciparum* strain NF54 (Origin: Airport, Netherlands; Provider MR4, MRA-1000) were cultivated in human erythrocytes by standard methods described previously (Dorn et al., 1995; Trager et al., 1976), maintained in humidified modular chambers in an atmosphere of 93% N_2, 4% CO_2 and 3% O_2 at 37 °C. The medium consisted of RPMI 1640 supplemented with 0.5% ALBUMAX® II, 25 mM Hepes, 25 mM $NaHCO_3$ (pH 7.3), 0.36 mM hypoxanthine and 100 µg/mL neomycin (Snyder et al., 2007). The 50% inhibitory concentrations (IC_{50}) were determined *in vitro* by quantifying the multiplication of *P. falciparum* via the incorporation of [^{3}H]-hypoxanthine as described (Desjardins et al., 1979).

Protein isolation

Parasite cultures at 5% (v/v) hematocrit and 7-12.5% parasitemia were treated for 4 h with 1 µg/mL or 0.1 µg/mL OZ726, OZ727, AA2, OZ03, carbaOZ727, deoxyAA2 or artemisinin. As control, parasites were treated with 0.1% DMSO. Cells were harvested by centrifugation and the cell pellets were resuspended in 0.3% saponin in PBS and incubated for 10 min on ice. The cells were washed three times with PBS and lysed in either 1.2% SDS plus EDTA-free protease inhibitor mixture tablets (Roche Applied Science) or in 50 µL of complete Ripa-Lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP40, 2 mM EDTA, EDTA-free protease inhibitor mixture tablets (Roche Applied Science)). After brief sonication, the samples were centrifuged and extracts normalized for protein content using a BCA protein assay kit (Merck Millipore).

Click chemistry

Azide-alkyne Huisgen cycloaddition reactions were performed using the Click-iT® Plus Alexa Fluor 488 Picolyl Azide Toolkit (Life Technologies), using 1.25 µM biotin azide (Thermo Fisher Scientific) instead of the Alexa Fluor 488 azide. 150 µL Click-iT Plus reaction cocktail were added to 35 µL protein extract and incubated for 30 min. Proteins were precipitated with four volumes of cold acetone (-20°C), incubated at -20°C for 1h, and centrifuged for 10 min at 13148 g. Pellets were air-dried for 15 min and resuspended in 1 ml 0.1% SDS in PBS with sonication.

Western blot

Resuspended protein samples were diluted 1:2 with 2x LDS sample buffer (50% 4x LDS sample buffer, Invitrogen; 20% mercaptoethanol; 30% H_2O). Samples were heated at 95 °C for 10 min and loaded onto polyacrylamide gels (NuPAGE 4-12% bis-tris-polyacrylamide gels, Invitrogen). Gels were run in 1x MES SDS running buffer (Invitrogen) and transferred onto nitrocellulose membranes (0.2 µm pore size, Invitrogen). After blocking for 1 h in 3% milk powder in TBS-Tween pH 8.0, streptavidin-coupled horseradish peroxidase (Pierce, Thermo Fisher Scientific), diluted 1/500 in blocking solution (0.5% milk powder in TBS-Tween pH 8.0), was added and incubated for 1 h at RT. Membranes were washed four times with TBS-Tween, and Super Signal West Pico chemiluminescent substrate (Thermo Fisher Scientific) was added. Signals were detected after 5 min on Carestream Kodak Biomax light film (Sigma Aldrich).

Sample preparation for mass spectometry

Samples were prepared according to a recently published protocol (Turriziani et al., 2014). In brief, EDTA-free protease inhibitor mixture tablets (Roche Applied Science) were added to the samples and samples were incubated with 50 µL Pierce™ NeutrAvidin™ Agarose (Thermo Fisher Scientific; Product No. 29201) with rotation overnight at 4 °C. Samples were centrifuged and washed 3 times

with 1 ml 1% SDS in PBS, once with 0.1% SDS in PBS, once with 6 M Urea in ddH_2O, once with PBS and once with ddH_2O. Then, samples were washed 3 times with 700 µL 100 mM ammoniumbicarbonate. Next 100 µL elution buffer 1 (1.6 M urea, 100 mM ammoniumbicabonate, 5 µg/ml trypsin) was added, samples were vortexed and incubated for 30 min on a thermomixer at 27 °C at 1200 rpm. Samples were centrifuged and the supernatant was collected into a fresh tube. Then, 40 µL elution buffer 2 (1.6 M urea, 100 mM Ammoniumbicabonate, 1 mM tris(2-carboxyethyl)phosphine) was added, samples were vortexed, centrifuged and the supernatant was added to the first eluate. This washing step was repeated once. Next, the tubes containing the supernatants were vortexed and incubated overnight at RT. Then, 40 µl of an aqueous iodoacetamide solution (5 mg/ml) was added, samples were vortexed and incubated in the dark for 30 min. Subsequently, samples were acidified with 170 µL 5% trifluoroacetic acid to a pH below 2. Next, peptides were cleaned up using C18 microspin columns (Harvard Apparatus, SEM SS18V) following the manufacturer's instructions. Finally, peptides were dried under vacuum and stored at -80°C until further processing.

Mass spectrometry and statistical analyses

1 ug of peptides of each sample were subjected to LC–MS analysis using a dual pressure LTQ-Orbitrap Elite mass spectrometer connected to an electrospray ion source (both Thermo Fisher Scientific) as described recently (Ahrné et al., 2016) with a few modifications. In brief, peptide separation was carried out using an EASY nLC-1000 system (Thermo Fisher Scientific) equipped with a RP-HPLC column (75 µm × 30 cm) packed in-house with C18 resin (ReproSil-Pur C18–AQ, 1.9 µm resin; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) using a linear gradient from 95% solvent A (0.15% formic acid, 2% acetonitrile) and 5% solvent B (98% acetonitrile, 0.15% formic acid) to 28% solvent B over 90 min at a flow rate of 0.2 µl/min. The data acquisition mode was set to obtain one high resolution MS scan in the FT part of the mass spectrometer at a resolution

of 120,000 full width at half-maximum (at m/z 400) followed by MS/MS scans in the linear ion trap of the 20 most intense ions using rapid scan speed. The charged state screening modus was enabled to exclude unassigned and singly charged ions and the dynamic exclusion duration was set to 30s. The ion accumulation time was set to 300 ms (MS) and 25 ms (MS/MS).

For label-free quantification, the generated raw files were imported into the Progenesis QI software (Nonlinear Dynamics (Waters), Version 2.0) and analyzed using the default parameter settings. MS/MS-data were exported directly from Progenesis QI in mgf format and searched against a decoy database the forward and reverse sequences of the predicted proteome from *Plasmodium falciparum* (isolate NF54, UniProt, download date: 12/10/2015, total of 12,376 entries) using MASCOT (version 2.4.1). The search criteria were set as follows: full tryptic specificity was required (cleavage after lysine or arginine residues); 3 missed cleavages were allowed; carbamidomethylation (C) was set as fixed modification; oxidation (M) as variable modification. The mass tolerance was set to 10 ppm for precursor ions and 0.6 Da for fragment ions. Results from the database search were imported into Progenesis QI and the final peptide measurement list containing the peak areas of all identified peptides, respectively, was exported. This list was further processed and statically analyzed using our in-house developed SafeQuant R script (Ahrné et al., 2016). The peptide and protein false discovery rate (FDR) was set to 1% using the number of reverse hits in the dataset.

Data availability

All mass spectrometry raw data files have been deposited to the ProteomeXchange Consortium (accession code PXDxxx, http://proteomecentral.proteomexchange.org, reviewer login: username: xxx, password: xxx) via the PRIDE partner repository (Vizcaino et al., 2013).

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Conflict of Interest

The use of ozonides as antimalarials has been patented by the University of Nebraska (US 20040039008 A1 and US 20080125411 A1).

Author Contribution

Chemical syntheses were performed by XW, YD and JLV, all other experiments by JJ. Planning of experiments and data analysis were mainly done by JJ, with helpful suggestions from SW, JLV, HM, AS, RSS and PM. JJ and PM wrote most of the manuscript, with contributions from all authors.

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Table 2. All proteins that were significantly enriched in *P. falciparum* cultures treated with 100 ng/ml or 1000 ng/ml OZ726, OZ727 or AA2 over the DMSO-treated controls, minus the proteins that were also identified in the parent drug controls (artemisinin and OZ03) or in the nonperoxidic controls (deoxyAA2 and carbaOZ727). For unknown reasons, there were no significant hits in the 1000 ng/ml sample of OZ727 in comparison to the DMSO control (Table S1).

UniProt ID	Protein description	MW [kDa]	AA2 100	OZ726 100	OZ727 100	AA2 1000	OZ726 1000
PFNF54_01699	Conserved *Plasmodium* protein, unknown function	196.6	x	x	x	x	x
PFNF54_01242	Eukaryotic translation initiation factor 3 subunit E, putative	61.4	x	x			
PFNF54_02052	GTPase-activating protein, putative	56.5	x	x			
PFNF54_00777	Glideosome associated protein with multiple membrane spans 2	42.6	x	x			
PFNF54_04517	T-complex protein 1 subunit delta	58.0		x	x		
PFNF54_01390	T-complex protein 1 subunit zeta	61.5		x	x		
PFNF54_02843	Chromodomain-helicase-DNA-binding protein 1 homolog, putative	381.3		x	x		
PFNF54_04470	Nuclear movement protein, putative	46.0		x	x		
PFNF54_05640	Thioredoxin peroxidase 1	21.8		x	x	x	x
PFNF54_02310	Nucleoside transporter 2	67.6	x		x		
PFNF54_03725	Conserved *Plasmodium* protein, unknown function	122.8		x			x
PFNF54_04135	V-type proton ATPase subunit H, putative	53.8		x			
PFNF54_05670	FACT complex subunit SSRP1, putative	58.8		x			
PFNF54_02736	RNA-binding protein, putative	106.3		x			
PFNF54_02383	Hsp70-like protein	75.1		x			
PFNF54_02720	Conserved *Plasmodium* protein, unknown function	14.5		x			
PFNF54_01130	FACT complex subunit SPT16, putative	132.7		x			x
PFNF54_05278	Nascent polypeptide-associated complex subunit beta	19.4		x			
PFNF54_03483	Dynamin-like protein	96.4		x			
PFNF54_04789	Thioredoxin-related protein, putative	24.0	x			x	x
PFNF54_00257	ATP-dependent RNA helicase UAP56	52.2	x				x

PFNF54_02120	Histone S-adenosyl methyltransferase, putative	129.2	x			x	
PFNF54_05599	Conserved protein, unknown function	32.6	x				
PFNF54_03298	T-complex protein 1 subunit alpha	60.3	x				
PFNF54_01342	Conserved *Plasmodium* protein, unknown function	334.4			x		
PFNF54_01696	Importin-7, putative	145.5				x	x
PFNF54_03261	Spermidine synthase	36.6				x	x
PFNF54_03012	Casein kinase II subunit beta	28.4				x	x
PFNF54_03252	Prefoldin subunit alpha, putative	29.1				x	x
PFNF54_03153	Insulinase, putative	173.6				x	x
PFNF54_00668	V-type proton ATPase subunit B	55.8				x	x
PFNF54_00014	Nucleoside transporter 4	50.0					x
PFNF54_04551	Conserved *Plasmodium* protein, unknown function	291.0					x
PFNF54_03586	Eukaryotic translation initiation factor 5A	17.6					x
PFNF54_02629	Proteasome subunit beta type-6, putative	32.6					x
PFNF54_05054	Aminopeptidase P	90.1					x
PFNF54_01342	Conserved *Plasmodium* protein, unknown function	334.4					x
PFNF54_01192	Multidrug resistance protein	162.3					x
PFNF54_04908	Plasmepsin III	51.7					x
PFNF54_05266	60S Ribosomal protein L7-3, putative	32.7					x
PFNF54_03239	Protein phosphatase, putative	34.5					x
PFNF54_04191	V-type proton ATPase catalytic subunit A	68.6					x
PFNF54_00552	T-complex protein 1 subunit epsilon	59.2					x
PFNF54_02838	60S Ribosomal protein L30e, putative	11.8					x
PFNF54_02025	Glutamate dehydrogenase, putative	160.4				x	

Table 3. Hits identified herein that are also present in the published datasets by Wang et al. (2015) and Ismail et al. (2016a, 2016b).

UniProt ID	Protein description	AA2 100	OZ726 100	OZ727 100	AA2 1000	OZ726 1000	Wang 2015	Ismail 2016a,b
PFNF54_04789	Thioredoxin-related protein, putative	x			x	x	x	
PFNF54_00257	ATP-dependent RNA helicase UAP56	x				x	x	
PFNF54_02310	Nucleoside transporter 2	x		x			x	
PFNF54_05640	Thioredoxin peroxidase 1		x	x	x	x	x	
PFNF54_01696	Importin-7, putative				x	x	x	
PFNF54_03261	Spermidine synthase				x	x	x	
PFNF54_03153	Insulinase, putative				x	x	x	
PFNF54_02025	Glutamate dehydrogenase, putative				x		x	
PFNF54_04517	T-complex protein 1 subunit delta		x	x			x	
PFNF54_01390	T-complex protein 1 subunit zeta		x	x			x	
PFNF54_04908	Plasmepsin III					x	x	
PFNF54_02838	60S ribosomal protein L30e, putative					x	x	
PFNF54_01192	Multidrug resistance protein					x	x	x
PFNF54_04191	V-type proton ATPase catalytic subunit A					x	x	x
PFNF54_00668	V-type proton ATPase subunit B				x	x		x

Supplementary Material

Table S1. All significant hits obtained with chemical probes as compared to DMSO-treated *P. falciparum* control cultures.

UniProt ID	Protein description	MW [kDa]	AA2 100	AA2 1000	ART 100	ART 1000	deoxyAA2 100	deoxyAA2 1000	OZ726 100	OZ726 1000	OZ727 100	OZ727 1000	OZ03 100	OZ03 1000	carbaOZ727 100	carbaOZ727 1000
PFNF54_01699	conserved Plasmodium protein, unknown function	196.6	X	X					X	X	X					
PFNF54_01242	eukaryotic translation initiation factor 3 subunit E, putative	61.4	X						X							
PFNF54_02052	GTPase-activating protein, putative	56.5	X						X							
PFNF54_00777	glideosome associated protein with multiple membrane spans 2	42.6	X						X							
PFNF54_04517	T-complex protein 1 subunit delta	58.0							X		X					
PFNF54_01390	T-complex protein 1 subunit zeta	61.5							X		X					
PFNF54_02843	chromodomain-helicase-DNA-binding protein 1 homolog, putative	381.3							X		X					
PFNF54_04470	nuclear movement protein, putative	46.0							X		X					
PFNF54_05640	thioredoxin peroxidase 1	21.8		X					X	X	X					
PFNF54_02310	nucleoside transporter 2	67.6	X								X					
PFNF54_03725	conserved Plasmodium protein, unknown function	122.8							X	X						
PFNF54_04135	V-type proton ATPase subunit H, putative	53.8							X							
PFNF54_05670	FACT complex subunit SSRP1, putative	58.8							X							
PFNF54_02736	RNA-binding protein, putative	106.3							X							
PFNF54_02383	heat shock protein 70	75.1							X							
PFNF54_02720	conserved Plasmodium protein, unknown function	14.5							X							
PFNF54_01130	FACT complex subunit SPT16, putative	132.7							X	X						
PFNF54_05278	basic transcription factor 3b, putative	19.4							X							
PFNF54_03483	dynamin-like protein	96.4							X							
PFNF54_04789	thioredoxin-related protein, putative	24.0	X	X						X						
PFNF54_00257	ATP-dependent RNA helicase UAP56	52.2	X							X						
PFNF54_02120	histone S-adenosyl methyltransferase, putative	129.2	X	X												
PFNF54_05599	conserved protein, unknown function	32.6	X													
PFNF54_03298	T-complex protein 1 subunit alpha	60.3	X													
PFNF54_01342	conserved Plasmodium protein, unknown function	334.4									X					
PFNF54_01696	importin-7, putative	145.5		X						X						
PFNF54_03261	spermidine synthase	36.6		X						X						
PFNF54_03012	casein kinase II beta chain	28.4		X						X						

PFNF54_03252	prefoldin subunit 5, putative	29.1		X						X						
PFNF54_03153	insulinase, putative	173.6		X						X						
PFNF54_00668	V-type proton ATPase subunit B	55.8		X						X						
PFNF54_00014	nucleoside transporter 4	50.0								X						
PFNF54_04551	conserved Plasmodium protein, unknown function	291.0								X						
PFNF54_03586	eukaryotic translation initiation factor 5A	17.6								X						
PFNF54_02629	proteasome subunit beta type-6, putative	32.6								X						
PFNF54_05054	aminopeptidase P	90.1								X						
PFNF54_01342	conserved Plasmodium protein, unknown function	334.4								X						
PFNF54_01192	multidrug resistance protein 1	162.3								X						
PFNF54_04908	plasmepsin III	51.7								X						
PFNF54_05266	60S ribosomal protein L7-3, putative	32.7								X						
PFNF54_03239	protein phosphatase, putative	34.5								X						
PFNF54_04191	V-type proton ATPase catalytic subunit A	68.6								X						
PFNF54_00552	T-complex protein 1 subunit epsilon	59.2								X						
PFNF54_02838	60S ribosomal protein L30e, putative	11.8								X						
PFNF54_02025	glutamate dehydrogenase, putative	160.4		X												
PFNF54_02028	1-cys peroxiredoxin	25.2					X									
PFNF54_03743	cell cycle associated protein, putative	324.1							X	X	X		X			
PFNF54_01079	bifunctional dihydrofolate reductase-thymidylate synthase	371.7	X	X	X		X		X	X	X		X	X		
PFNF54_05172	60S ribosomal protein L44	12.2	X				X		X	X	X		X	X		
PFNF54_03313	antigen UB05	13.7									X		X			
PFNF54_03023	histone H4	11.5											X			
PFNF54_01156	conserved Plasmodium protein, unknown function	22.7							X		X		X			
PFNF54_01149	40S ribosomal protein S24	15.4											X			
PFNF54_00991	deoxyribodipyrimidine photo-lyase, putative	129.2	X						X				X			
PFNF54_03121	conserved Plasmodium protein, unknown function	33.9	X	X					X	X	X		X			
PFNF54_01036	40S ribosomal protein S9, putative	22.1											X			
PFNF54_03241	tRNA (guanine-N(7)-)-methyltransferase, putative	35.6					X				X					
PFNF54_04551	conserved Plasmodium protein, unknown function	291.0	X				X									
PFNF54_00516	40S ribosomal protein S15A, putative	14.9					X				X					
59			**15**	**13**	**1**	**0**	**6**	**0**	**24**	**29**	**16**	**0**	**10**	**2**	**0**	**0**

Table S2. Summary of Table S1, indicating the overlaps between the sets of obtained hits.

	AA2 100	AA2 1000	ART 100	ART 1000	deoxyAA2 100	deoxyAA2 1000	OZ726 100	OZ726 1000	OZ727 100	OZ727 1000	OZ03 100	OZ03 1000	carbaOZ727 100	carbaOZ727 1000
AA2 100	**15**	5	1	0	3	0	8	6	5	0	4	2	0	0
AA2 1000		**13**	1	0	1	0	4	11	4	0	2	1	0	0
ART 100			**1**	0	1	0	1	1	1	0	1	1	0	0
ART 1000				**0**	0	0	0	0	0	0	0	0	0	0
deoxyAA2 100					**6**	0	2	2	4	0	2	2	0	0
deoxyAA2 1000						**0**	0	0	0	0	0	0	0	0
OZ726 100							**24**	8	11	0	6	2	0	0
OZ726 1000								**29**	6	0	4	2	0	0
OZ727 100									**16**	0	6	2	0	0
OZ727 1000										**0**	0	0	0	0
OZ03 100											**10**	2	0	0
OZ03 1000												**2**	0	0
carbaOZ727 100													**0**	0
carbaOZ727 1000														**0**

Figure S1. Optimization of assay parameters as determined by Western blot. A) Different incubation times of *P. falciparum* cultures with 1000 ng/ml ozonide alkyne, exposure 10 s; B) Different concentrations of ozonide alkyne at 4 h drug incubation, exposure 30 s. Parasite cultures were lysed with Ripa-Lysis buffer (see Material and Methods for details).

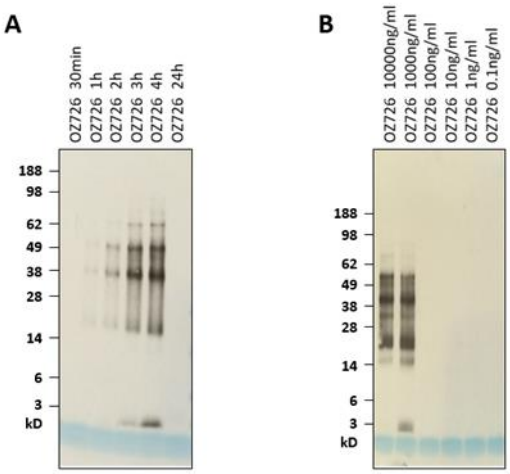


Figure S2. Optimization of washing conditions of the neutravidin-coupled agarose beads after reaction with biotinylated peptides as determined by silver stain. *P. falciparum* cultures were incubated with 100 ng/ml AA2 or 0.1% DMSO for 4 h. Protein extracts were prepared with 1.2% SDS in PBS.

	Sample	Wash
1	Beads only	7x
2	No treatment	7x
3	Artemisinin alkyne 2	7x
4	DMSO	7x
5	DMSO, beads preincubated with BSA	7x
6	DMSO, beads preincubated and incubated with BSA	7x
7	DMSO, beads preincubated with milk powder	7x
8	DMSO, beads preincubated and incubated with milk powder	7x
9	DMSO	5x

5x wash steps:

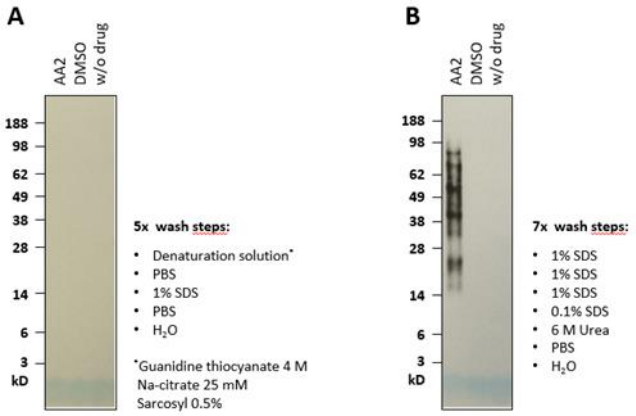
- Denaturation solution*
- PBS
- 1% SDS
- PBS
- H_2O

*Guanidine thiocyanate 4 M
Na-citrate 25 mM
Sarcosyl 0.5%

7x wash steps:

- 1% SDS
- 1% SDS
- 1% SDS
- 0.1% SDS
- 6 M Urea
- PBS
- H_2O

Figure S3. Optimization of washing conditions of the neutravidin-coupled agarose beads after reaction with biotinylated peptides as determined by Western blot. *P. falciparum* cultures were treated with 100 ng/ml AA2 or 0.1% DMSO and extracts were prepared with 1.2% SDS in PBS. Exposure 1 min.



5. Discussion

Artemisinin is an antimalarial of highest potency and tolerability. However, it also has drawbacks: As natural products, artemisinin and it derivatives suffer from poor availability and high costs. Furthermore, they have short *in vivo* half-lifes and are difficult to chemically modify and improve (White, 2008; Tilley et al., 2016). Vennerstrom reported in 2004 the development of synthetic peroxides, which should overcome the drawbacks of artemisinins (Vennerstrom et al., 2004). The next-generation ozonide OZ439 showed promising properties in clinical trials with a rapid parasite clearance, good safety profiles and a prolonged elimination half-life of 46-62 hours, in contrast to the first-generation ozonide OZ277 (Phyo et al., 2016; Möhrle et al., 2012). This raises hope for a single-dose treatment of OZ439 in combination with another drug to become an important new treatment for *P. falciparum* infections (Phyo et al., 2016; Möhrle et al., 2012; Mäser et al., 2010; Phillips et al., 2017). However, a prerequisite for any new antimalarial is activity against artemisinin-resistant *P. falciparum* isolates, which have been reported from South-East Asia since 2006 and are the cause of the phenomenon of delayed parasite clearance after artemisinin treatment (Noedl et al., 2008; Dondorp et al., 2009). Several different mutations in the K13 propeller domain were found to be major determinants of artemisinin resistance (Chapter 1). Thereby individual point mutations in K13 were sufficient to induce enhanced parasite clearance half-lives of >5h after artemisinin treatment, indicating artemisinin resistance in *P. falciparum* isolates (Blasco et al., 2017; Straimer et al., 2015). It is suggested that ring stages become refractory to drug action and that these ring stages have an enhanced capacity to regulate cellular stress response to counteract the protein damage caused by artemisinin. Thereby the unfolded protein response or the ubiquitin/proteasome system, which serves as a regulated protein degradation system, are involved. But the exact function and mechanism of K13 mutations protecting ring-stages in not known (Blasco et al., 2017; Tilley et al., 2016, Wang et al., 2017).

The fear of cross-resistance of artemisinin-resistant parasites against synthetic peroxides overshadowed the superior pharmacological properties of OZ439, since both, artemisinins and synthetic peroxides, share the peroxide bridge essential for drug action (Kaiser et al., 2007). Hence, we undertook *in vitro* studies to test for potential cross-resistance of an artemisinin-resistant *P. falciparum* clinical isolate from Battambang (Cambodia) carrying a K13 mutation (Cam3.I^{R539T}) against synthetic peroxides (Chapter 3). The mutation R539T was found to confer increased *in vitro* survival rates and delayed parasite clearance in patients after artemisinin treatment (Ariey et al., 2014; Ménard et al., 2016; Straimer et al., 2015). In Chapter 3 we showed that the artemisinin-resistant clinical isolate had a 45-fold lower survival rate *in vitro* when exposed to OZ439 than to dihydroartemisinin, indicating no cross-resistance between the two antimalarial peroxides (Chapter 3). Our finding was supported by other *in vitro* studies showing no cross-resistance of the clinical isolate Cam3.II^{R539T} and another clinical isolate carrying a C580Y mutation (Cam3.II^{C580Y}) against OZ439 (Straimer et al., 2017; Siriwardana et al., 2016). In addition, Yang et al. found that synthetic peroxides are more effective than dihydroartemisinin against early-ring stages when exposed for physiologically relevant periods (Yang et al., 2016). The ability of OZ439 to overcome artemisinin resistance was further supported by the finding that the median parasite clearance half-life in patients infected with *P. falciparum* parasites carrying mutations in K13 was only one hour longer than in patients with *P. falciparum* parasites lacking K13 mutations (Phyo et al., 2016). Together, all these facts raise hope that OZ439 is a promising drug candidate to overcome artemisinin resistance in combination with a partner drug.

It was shown that the mode of action of synthetic peroxides involves the formation of carbon-centered radicals, predominantly on the adamantane-portion of the ozonides (Vennerstrom et al., 2004; Maerki et al., 2006). Therefore, the production of monoclonal antibodies raised against this adamantane-portion offered us the unique opportunity to investigate the alkylation signature of the synthetic peroxides OZ277 and OZ439. In Chapter 2 I showed that both monoclonal antibodies used

in this study bind specifically to the alkylation targets of OZ277 and OZ439 (Chapter 2). Since no protein bands were observed in a Western blot experiment using *Babesia divergens* treated with synthetic peroxides, an intraerythrocytic protozoan parasite that does not digest hemoglobin and therefore does not produce hemozoin, we could demonstrate that digestion of hemoglobin is essential for the activity of ozonides (Chapter 2; Zintl et al., 2003; Richier et al., 2006). This finding was in agreement with Kaiser et al., who showed that OZ277 was not active *in vitro* against *B. divergens* (Kaiser et al., 2007).

In Chapter 4, I analysed the alkylation signatures of the newly synthesized alkyne OZ726 by mass spectrometry and identified aminopeptidase P and plasmepsin III, both suggested to be involved in hemoglobin catabolism, further supporting the iron-dependent alkylation hypothesis (Chapter 4). I could also show that the peroxide bridge in synthetic peroxides is essential for drug action, since carbaOZ727 and deoxyAA2 showed no activity *in vitro* against *P. falciparum* NF54 and no protein alkylation was found in Western blot experiments with the nonperoxidic analogues OZ727 and AA2 (Chapter 4). Further, mass spectrometry with carbaOZ727 and deoxyAA2 identified only four hits with deoxyAA2, while no hit was found in parasite cultures treated with carbaOZ727 (Chapter 4).

In addition to the alkylation of heme which was described by Creek (Creek et al., 2008), I showed here that several other parasite proteins were alkylated by OZ277 and OZ439 (Chapter 2). This alkylation signature was further investigated by the establishment and application of the click chemistry approach using novel synthesized alkynes of artemisinin and synthetic peroxides. I identified 47 alkylated proteins in *P. falciparum* after treatement with OZ726, OZ727 or AA2 for 4 h. One of these proteins, PFNF54_01699, was found to be most promising, since it was found in all samples (except 1000 ng/ml OZ727, which did not return a single hit). This protein is suggested to be an erythrocyte membrane protein (EMP)-like protein, a parasite protein thought to be exported to the RBC membrane (Pei et al., 2007; Senczuk et al., 2001). It appears there at the later stages of the parasite life cycle (Pei et al., 2007; Senczuk et al., 2001). *P. falciparum* EMP 1 mediates the

adhesion of the infected RBCs to the human vascular endothelium and is therefore important in the host-pathogen interaction (Pei et al., 2007; Senczuk et al., 2001; Sampaio et al., 2017). But further studies will be needed to characterize this alkylated protein.

Another protein of the alkylation signature of OZ726 was the multidrug resistance protein 1 (MDR1), an ABC transporter located in the digestive vacuole membrane (Blasco et al., 2017; Rohrbach et al., 2006). PfMDR1 was found to be linked to the sensitivity of *P. falciparum* to several antimalarial drugs (Ding et al., 2010). Thereby, partial but insufficient associations between mutations in PfMDR1 and chloroquine resistance were found (Duraising et al., 2005, Valderramos et al., 2006). Further, it was shown that increased copy numbers of PfMDR1 resulted in decreased *in vitro* susceptibility to mefloquine, quinine, halofantrine and artemisinin (Valderramos et al., 2006; Sidhu et al., 2006).

In 1994 Asawamahasakda identified four major protein bands (25kDa, 50kDa, 65kDa and >200kDa) and two minor ones (32kDa and 42kDa) with radiolabelled dihydroartemisinin. I have observed similar protein bands, except for the >200kDa band, when using the newly synthesized alkyne AA2 in Western blots. The same pattern was also observed with either OZ726 or OZ727 (Chapter 4). The antibody studies also revealed a prominent protein band at 50 kDa in rings and schizonts treated with either OZ277 or OZ439 (Chapter 2). All these findings support the data of Asawamahasakda (Asawamahasakda et al., 1994). In addition, I found different proteins with the same identified masses than Asawamahasakda (Asawamahasakda et al., 1994) in the mass spectometry experiments when identifying the alkylation signatures of the synthetic peroxides and artemisinin-alkynes (as discussed in Chapter 4).

Next, we could show that protein alkylation by OZ277 takes place in the cytoplasm and other structures such as the nucleus and the food vacuole (Chapter 2). This alkylation signature was similar to the ultrastructural autoradiographic studies performed by Maeno, where radiolabeled dihydroartemisinin and artemisinin located to the food vacuole, mitochondria and nuclear

membranes (Maeno et al., 1993). Other studies showed that TAMRA-labeled OZ277 was found in the cytosol or in the food vacuole (Uhlemann et al., 2007). In addition, it was found that artemisinin causes the disruption of the digestive vacuole membrane and that the food vacuole is an important site of synthetic peroxide action (Crespo et al., 2008). By analysing alkylation signatures of synthetic peroxides and artemisinin with mass spectrometry, we identified various proteins located to the cytoplasm, nucleus or food vacuole (Chapter 4).

When we compared our identified alkylation signatures of synthetic peroxides and artemisinin to the recently observed data of Wang, who had also used a click chemistry approach with newly synthesized, but structurally slightly different artemisinin alkyne, we obtained 14 identical proteins, 6 of which previously proposed as antimalarial drug targets (Wang et al., 2015). In contrast, click chemistry studies with synthetic peroxides and artemisinin performed by Ismail et al. did not show such a high degree of overlap (Ismail et al., 2016a and 2016b). This could be due to the use of trophozoites only compared to mixed parasite cultures used in our experiments, or because of prolonged drug incubation time (6 h in Ismail et al., 2016a and 2016b compared to 4 h in Chapter 4). To further investigate the variation of identified alkylation signatures in different studies (Wang et al., 2015, Ismail et al., 2016a and 2016b; Chapter 4), one should use more stringent criteria, such as the use of highly synchronized parasite stages (rings, trophozoites or schizonts), identical drug incubation times and identical parasitemia.

We demonstrated an overlap between the alkylation signatures of synthetic peroxides and artemisinin, but we did not observe such a high identity, i.e. 90%, as Ismail (Ismail et al., 2016b; Chapter 4). Our results strengthen the hypothesis that artemisinins and synthetic peroxides have a similar, but not identical mode of action (Vennerstrom et al., 2004; Jefford, 2001; Kaiser et al., 2007). Since our approach using alkynes with click chemistry worked well, a next step could be the investigation of alkylation signatures of synthetic peroxides and artemisinin in artemisinin-resistant *P. falciparum* strains compared to artemisinin-susceptible strains. Thereby one could identify the

differences in alkylation patterns in relation to artemisinin resistance and possibly shed light on the mechanism of artemisinin resistance.

6. Conclusion

In conclusion, I could show that OZ277 and OZ439 localize to the cytoplasm of *P. falciparum* and other structures such as the nucleus and the food vacuole. I have identified the alkylation signatures of both, artemisinin and synthetic peroxides by using a click chemistry approach with newly synthesized alkynes. Further we could demonstrate that there is no cross-resistance between artemisinin and OZ439 in a Kelch-13 mutant *P. falciparum* field isolate. Further studies will be needed to investigate the importance of the most prominent identified alkylation target (PFNF54_01699) for drug action and identify the mechanism of artemisinin resistance to ensure that OZ439 remains a promising drug candidate that will overcome artemisinin resistance in combination with a partner drug.

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