

# Monoclonal Antibodies That Recognize the Alkylation Signature of Antimalarial Ozonides OZ277 (Arterolane) and OZ439 (Artefenomel)

Joëlle Jourdan,<sup>†,‡</sup> Hugues Matile,<sup>§</sup> Ellen Reift,<sup>†,‡</sup> Oliver Biehler,<sup>#</sup> Yuxiang Dong,<sup>⊥</sup> Xiaofang Wang,<sup>⊥</sup> Pascal Mäser,<sup>†,‡</sup> Jonathan L. Vennerstrom,<sup>⊥</sup> and Sergio Wittlin<sup>\*,†,‡</sup>

<sup>†</sup>Swiss Tropical and Public Health Institute, Socinstrasse 57, CH-4002 Basel, Switzerland

<sup>‡</sup>University of Basel, CH-4003 Basel, Switzerland

<sup>§</sup>F. Hoffmann-La Roche Ltd., CH-4070 Basel, Switzerland

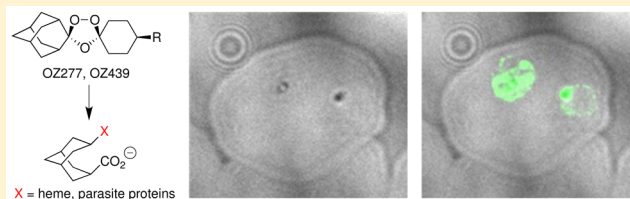
<sup>#</sup>Imaging Core Facility, Biozentrum, University of Basel, CH-4003 Basel, Switzerland

<sup>⊥</sup>College of Pharmacy, University of Nebraska Medical Center, 986025 Nebraska Medical Center, Omaha, Nebraska 68198, United States

## Supporting Information

**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 (arteftenomel). 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, this paper now describes 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 *Plasmodium 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 the authors' knowledge, this shows for the first time that antimalarial ozonides, such as the artemisinins, alkylate proteins in *P. falciparum*.

**KEYWORDS:** alkylation, artemisinin, immunofluorescence, monoclonal antibody, ozonide, *Plasmodium falciparum*



The discovery of artemisinin (ART) from *Artemisia annua*<sup>1</sup> 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<sup>2</sup> (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.<sup>3–6</sup> One of these, ozonide (1,2,4-trioxolane) OZ277,<sup>7</sup> also known as arterolane maleate, was introduced in 2012 to the Indian market as a combination product with piperazine phosphate (Synriam).<sup>8–10</sup> More recently, the “next generation” ozonide OZ439 (arteftenomel)<sup>11,12</sup> has progressed to phase IIb trials (Figure 1).

The peroxide bond in ART and antimalarial synthetic peroxides is essential for antiplasmodial activity,<sup>6,13</sup> suggesting a chemistry-driven mechanism of action. A considerable amount of data<sup>4,14–24</sup> demonstrates that the activity of antimalarial

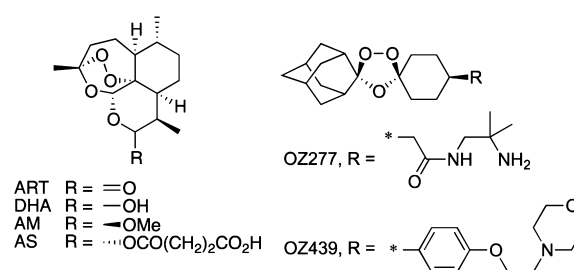
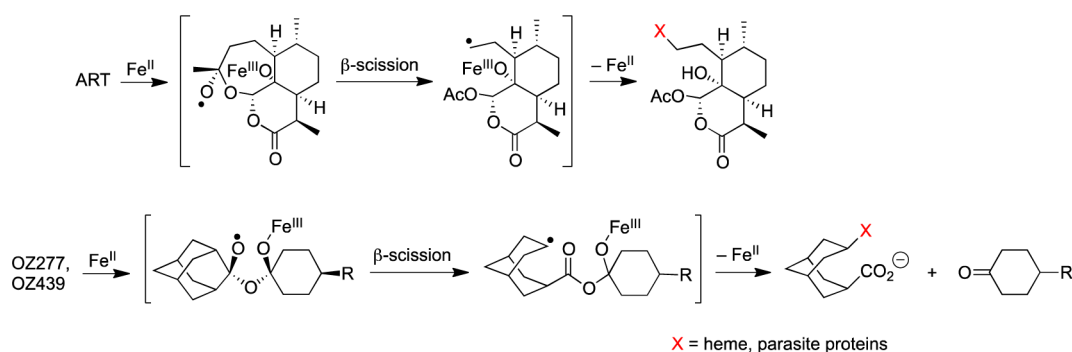


Figure 1. Artemisinin and ozonide structures.

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

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

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.<sup>25–27</sup> This mechanism accounts not only for the high antiparasitodal 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.<sup>13,28,29</sup>

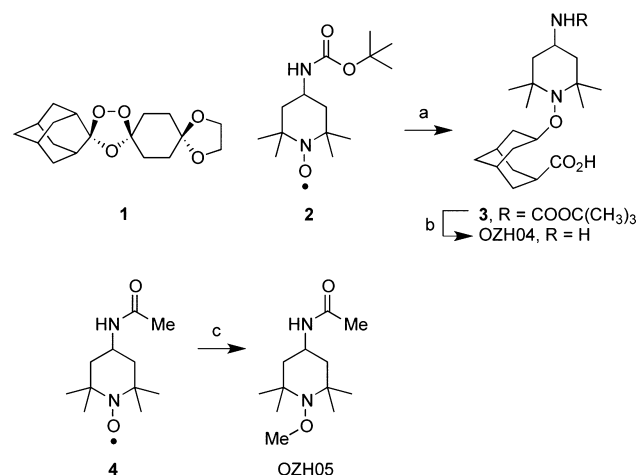
Electron transfer from heme to the peroxide bond antibonding  $\sigma^*$  orbitals of ART and antimalarial ozonides produces short-lived alkoxy radicals (Figure 2). For ART, rearrangement via  $\beta$ -scission forms a primary carbon-centered radical; for OZ277 and OZ439, rearrangement via  $\beta$ -scission 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—with heme or with proteins. Because we had good success in capturing the ozonide-derived secondary carbon-centered radical with the stable nitroxide radical TEMPO and its analogues,<sup>7,22,30</sup> 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,<sup>30</sup> 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 aqueous hydrogen peroxide according to the method of Dichtl et al.<sup>31</sup>

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 keyhole limpet hemocyanin (KLH). 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

## Scheme 1. Synthesis of *N*-Alkoxyamines OZH04 and OZH05<sup>a</sup>



<sup>a</sup>(a) Fe(OAc)<sub>2</sub>, 1:1 CH<sub>2</sub>Cl<sub>2</sub>/CH<sub>3</sub>CN, Fe(OAc)<sub>2</sub>, 35 °C, 24 h; (b) 6 N HCl, THF, room temperature, 12 h; (c) 50% H<sub>2</sub>O<sub>2</sub>, CuCl, acetone, room temperature, 12 h.

immune responses were selected for fusion to PAI myeloma cells.

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 two top rows). No immunofluorescence signals were detected with DHA-treated

**Table 1. Immunofluorescence Experiments with *P. falciparum* Cultures Treated with 10  $\mu$ g/mL OZ277, 10  $\mu$ g/mL OZ439, 10  $\mu$ g/mL DHA, or 0.1% DMSO for 2 h<sup>a</sup>**

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

<sup>a</sup>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.

parasites, 0.1% DMSO, or an unrelated IgG1 control antibody. An antibody raised against the cytosolic protein GAPDH served as a positive control.

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 OZ277 and OZ439 (Table 2).

**Table 2. Immunofluorescence Experiments with *P. falciparum* Cultures Treated with 10  $\mu\text{g}/\text{mL}$  OZ277 or 0.1% DMSO for 2 h<sup>a</sup>**

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

<sup>a</sup>For competition, 33  $\mu\text{M}$  hapten OZH04 or OZH05 was combined with the primary antibody OZH04-2/2 (0.33  $\mu\text{M}$ ) or OZH04-1/8 (0.33  $\mu\text{M}$ ) and incubated at room temperature for 1 h. The secondary antibody was goat anti-mouse Alexa 488. + indicates fluorescence, and – indicates no fluorescence.

In co-localization studies, synchronized *P. falciparum* trophozoites were treated with 10  $\mu\text{g}/\text{mL}$  OZ277 or DMSO and directly labeled 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 labeled with Alexa, but was instead incubated with a secondary antibody containing the label Alexa 488 or Alexa 594, resulted in much more pronounced signals in the food vacuole (dark round structure) (Figure 3b,c). The explanation for this phenomenon is unclear. One possibility is that the fluorescence signal of the OZ antibody directly labeled with Alexa dye is quenched in the food vacuole by hemozoin, a biocrystallized form of hemozoin, the latter being a well-known acceptor for energy transfer.<sup>32</sup>

Previously, ultrastructural autoradiographic studies of [<sup>3</sup>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.<sup>15</sup> Other work indicated that a fluorescent TAMRA OZ277 conjugate was associated with the food vacuole and the parasite endoplasmic reticulum,<sup>33</sup> although in this study the 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 versus 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 IC<sub>99</sub> (concentration at which 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, when false-positive signals can become an issue. On this basis, 10000 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)<sup>13</sup> or when uninfected erythrocytes were used (Supporting Information Figure 3). These data indicate that the antibody binding was specific for alkylated proteins.

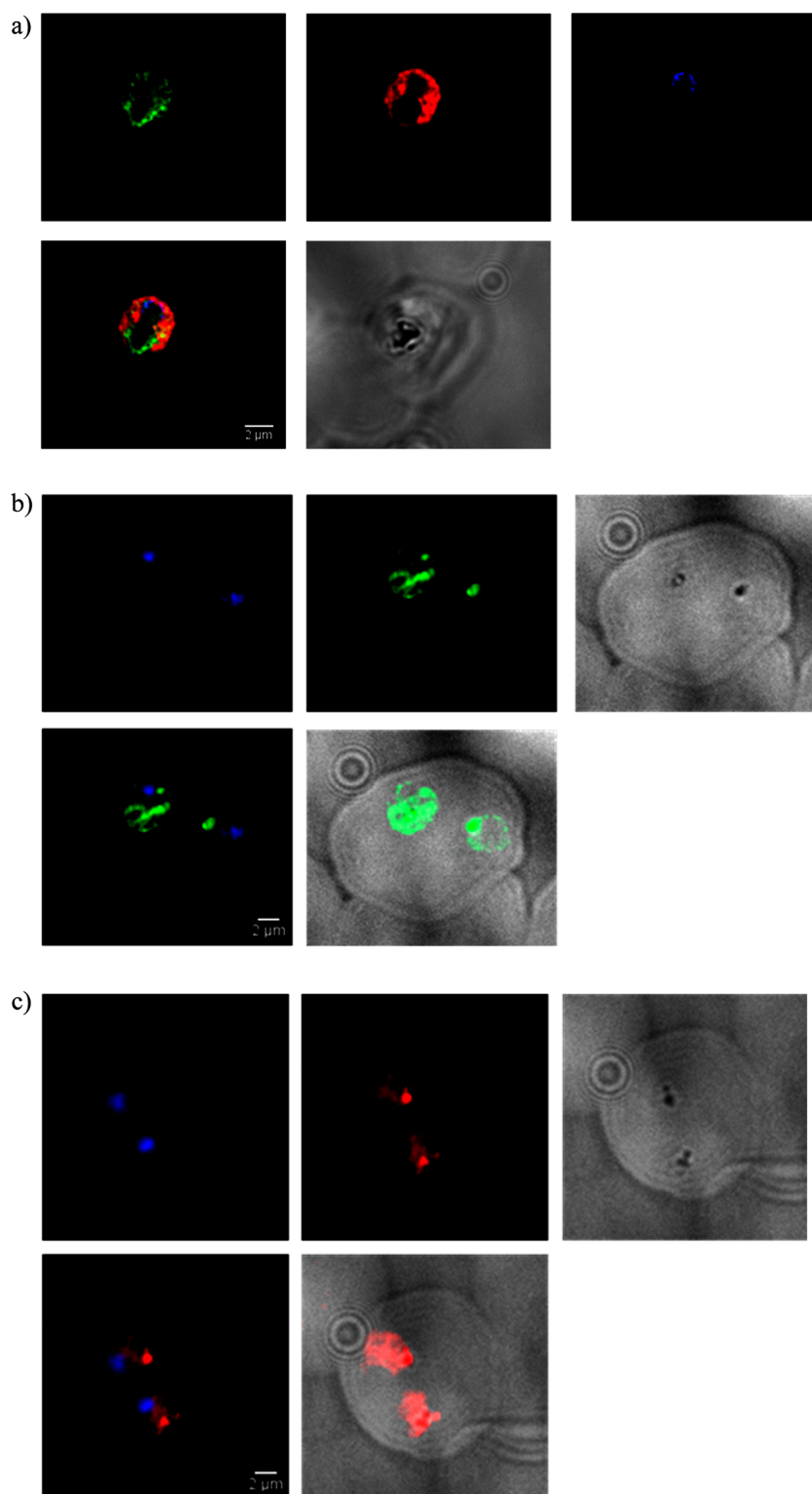
Additionally, no protein bands were detected in Western blot experiments performed with lysates from ozonide-treated *Babesia divergens* (Supporting Information Figure 4), parasites that also proliferate inside erythrocytes but, in contrast to *P. falciparum*, do not catabolize hemoglobin.<sup>34</sup>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

Heme has been shown to be an alkylation target of the antimalarial ozonides.<sup>17</sup> 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, such as 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<sup>15,35</sup> and also proteins. Among the identified target proteins was the *P. falciparum* translationally controlled tumor protein (TCTP) homologue.<sup>16</sup> Parasite redox-active flavoenzymes,<sup>36</sup> mitochondrial reductive activation,<sup>37</sup> 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.<sup>38</sup> 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 antibody 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.

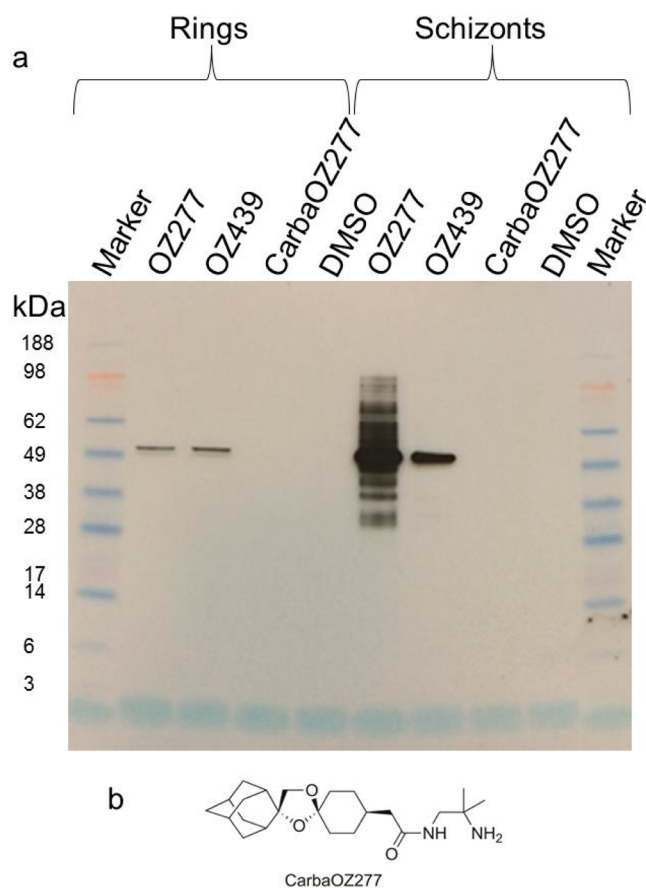
## METHODS

**Monoclonal Antibody Preparation.** Hapten OZH04 was coupled to keyhole limpet hemocyanin (KLH; Thermo Scientific 77600 Imject mKLH) and bovine serum albumin (BSA; Thermo Scientific 77110 Imject BSA), respectively, by



**Figure 3.** Immunofluorescence studies with *P. falciparum* trophozoites that were treated with 10  $\mu\text{g}/\text{mL}$  OZ277 for 2 h. (a) In immunofluorescence colocalization 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 labeled 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 the bottom row right, the reference image (exposure of 1 s). Scale bar = 2  $\mu\text{m}$ . (b) Same as (a), except that unlabeled primary antibodies OZH04-2/2 were used. After six washes with PBS, 20  $\mu\text{g}/\text{mL}$  of the secondary antibody goat anti-mouse Alexa 488 (green signal) was incubated for 1 h at room temperature. 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  $\mu\text{m}$ . (c) Same as (b), except that the secondary antibody used was goat anti-mouse Alexa 594 with an exposure of 1 s (red signal).





**Figure 4.** Western blot with *P. falciparum* cultures synchronized for rings and schizonts. Cultures were treated with 10  $\mu\text{g}/\text{mL}$  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.

cross-linking with glutaraldehyde on the basis of the method of Onica et al.<sup>38</sup>

OZH04 (0.1 mL) dissolved at 57.5 mg/mL in DMSO was mixed with 20 mg of the respective carrier protein in 0.2 M  $\text{Na}_2\text{HPO}_4$ , pH 8.0, and 1 mL of 0.2% glutaraldehyde in 0.2 M  $\text{Na}_2\text{HPO}_4$  was then slowly added with agitation. After 1 h of incubation at room temperature, the reaction was stopped by adding 0.25 mL of 1 M glycine, pH 8.0, and dialyzed against PBS overnight.

Naval Medical Research Institute (NMRI) mice were immunized with 100  $\mu\text{g}$  subcutaneous injections of KLH-conjugated OZH04 emulsified in aluminum hydroxide gel (Alhydrogel-2%, Brenntag Biosector) containing CPGOGN as previously described.<sup>39</sup> 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.<sup>40</sup>

**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,<sup>41–43</sup> consisting of RPMI 1640 supplemented with 0.5% ALBUMAX II, 25 mM Hepes, 25 mM  $\text{NaHCO}_3$ , pH 7.3, 0.36 mM hypoxanthine, and 100  $\mu\text{g}/\text{mL}$  neomycin. Human erythrocytes served as host cells. Cultures were maintained at 37  $^\circ\text{C}$  in an atmosphere of 3%  $\text{O}_2$ , 4%  $\text{CO}_2$ , and 93%  $\text{N}_2$  in humidified modular chambers. Inhibitory

concentrations of 50, 90, and 99% ( $\text{IC}_{50}$ ,  $\text{IC}_{90}$ , and  $\text{IC}_{99}$ ) of OZ277 in nanograms per milliliter against unsynchronized NF54 parasites were 0.91, 1.7, and 2.7 when determined at the standard parasitemia of 0.3%.<sup>7</sup> At about 10–30 times higher parasitemia, the inhibitory concentrations were found to be 10–30 times higher, which is consistent with the so-called “inoculum 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.<sup>44,45</sup> Also, OZ277 has been shown to partition into *P. falciparum*-infected red blood cells.<sup>46</sup>

**Immunofluorescence.** *P. falciparum* NF54 cultures (5% (v/v) hematocrit, 8–10% parasitemia) were either synchronized with 5% D-sorbitol for trophozoites<sup>47</sup> prior to treatment or used as mixed cultures. The cultures were treated with 10  $\mu\text{g}/\text{mL}$  OZ277, 10  $\mu\text{g}/\text{mL}$  OZ439, DHA, 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 ( $-20$   $^\circ\text{C}$ ; 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 room temperature with concentrations of 50  $\mu\text{g}/\text{mL}$ . After six washes with PBS, the secondary antibodies goat anti-mouse Alexa 488 (Invitrogen) and goat anti-rabbit Alexa 594 (Invitrogen) were incubated with concentrations of 20  $\mu\text{g}/\text{mL}$  for 1 h at room temperature. Control experiments, performed with secondary antibody only, resulted in no detectable immunofluorescence signals (not shown).

For competition experiments, 33  $\mu\text{M}$  OZH04 or OZH05 was combined with the primary antibody OZH04-1/8 (0.33  $\mu\text{M}$ ) or OZH04-2/2 (0.33  $\mu\text{M}$ ) and incubated at room temperature for 1 h. As secondary antibody, goat anti-mouse Alexa 488 (20  $\mu\text{g}/\text{mL}$ ) was incubated for 1 h at room temperature.

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 labeled with Alexa Fluor 488 succinidyl ester (Life Technologies) and Alexa Fluor 594 succinidyl ester (Life Technologies), respectively. OZH04-2/2 (50  $\mu\text{g}/\text{mL}$ ) directly labeled with Alexa 488 was incubated for 1 h at room temperature. After six washings with PBS, 50  $\mu\text{g}/\text{mL}$  GAPDH directly labeled with Alexa 594 was incubated for 1 h at room temperature. Vectashield Hard Set with DAPI (Vector Laboratories) was added to all slides after six washings with PBS. The slides were analyzed with a Widefield Delta Vision core microscope based on an Olympus IX71 stand, using a 60 $\times$ /1.42NA oil objective. Basic image analysis (e.g., contrast and brightness adjustments) were done with ImageJ Fiji, and images were analyzed 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.<sup>47</sup> Cultures were treated with 10  $\mu\text{g}/\text{mL}$  OZ277, 10  $\mu\text{g}/\text{mL}$  OZ439, 10  $\mu\text{g}/\text{mL}$  CarbaOZ277, or 0.1% DMSO for 2 h. After centrifugation, pellets of samples were resuspended in 1.5 mL of 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  $\mu$ L 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)) 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 2 $\times$  LDS sample buffer (50% 4 $\times$  LDS sample buffer, Invitrogen; 20% mercaptoethanol; 30% ddH<sub>2</sub>O), heated for 10 min at 70  $^{\circ}$ C, loaded onto polyacrylamide gels (10  $\mu$ L of sample; 4–12% Bis-Tris-polyacrylamide Gels, Invitrogen), and run for 35 min (120 mA; 200 V) using 1 $\times$  MES SDS Running Buffer (20 $\times$  MES DS Running Buffer, Invitrogen) as running buffer. Gels were transferred onto nitrocellulose membranes (0.2  $\mu$ m 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 consists of 20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH adjusted to 8.0 with HCl). OZH04-2/2 antibody (1  $\mu$ g/mL), diluted in 0.5% milk powder blocking solution, was added to the membrane and incubated for 1 h at room temperature. Membranes were washed three 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 room temperature. Membranes were washed four times for 5 min with TBS-Tween buffer. Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) was added to the membrane and incubated for 5 min, and the Western blot was developed using a Carestream Kodak Biomax light film (Sigma).

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsinfecdis.5b00090](https://doi.org/10.1021/acsinfecdis.5b00090).

Information on the synthesis and characterization of OZH04 and OZH05; in addition, immunofluorescence experiments with *P. falciparum* rings 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 (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

\*(S.W.) Mail: Swiss Tropical and Public Health Institute, Socinstrasse 57, CH-4002 Basel, Switzerland. Phone: 41 61 284 8136. E-mail: [sergio.wittlin@unibas.ch](mailto:sergio.wittlin@unibas.ch).

### Author Contributions

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, and J.J., H.M., P.M., J.L.V., and S.W. wrote the paper.

## Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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## ■ 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 dehydrogenase; IC, inhibitory concentration; KLH, keyhole limpet hemocyanin; NF54, *P. falciparum* strain (Netherlands Airport strain); NMRI, Naval Medical Research Institute mice; OZ277, artemolane; 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; TEMPO, 2,2,6,6-tetramethylpiperidine 1-oxyl;  $\alpha$ -CRT, chloroquine resistance transporter

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