Pharmacokinetics of the Antischistosomal Lead Ozonide OZ418 in Uninfected Mice Determined by Liquid Chromatography-Tandem Mass Spectrometry

Anna Leonidova,^{a,b} Mireille Vargas,^{a,b} Jörg Huwyler,^c Jennifer Keiser^{a,b}

Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Basel, Switzerland^a; University of Basel, Basel, Switzerland^b; Department of Pharmaceutical Sciences, Division of Pharmaceutical Technology, University of Basel, Basel, Switzerland^c

One of the major neglected tropical diseases, schistosomiasis, is currently treated and controlled with a single drug, praziquantel. The quest for an alternative drug is fueled by the lack of activity of praziquantel against juvenile *Schistosoma* worms and the fear of emerging resistance. The synthetic ozonide OZ418 has shown high activity against *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum* *in vivo*, but its drug disposition remains unknown. To bridge this gap, our study determined the basic pharmacokinetic (PK) parameters of a single oral dose (400 mg/kg of body weight) of OZ418 in uninfected mice. First, a simple liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to quantify OZ418 concentrations in mouse plasma was successfully developed and validated according to U.S. FDA guidelines. This method proved to be selective, accurate (93 to 103%), precise (5 to 16%), and devoid of significant matrix effects (90 to 102%) and provided excellent recovery (101 to 102%). A median peak concentration of 190 (range, 185 to 231) $\mu\text{g/ml}$ was reached at 2 h (2 to 3 h) posttreatment. A naive pooled noncompartmental PK analysis estimated a mean area under the plasma concentration-versus-time curve (AUC) of 9,303 $\mu\text{g h/ml}$ (7,039.2 to 11,908.5 $\mu\text{g h/ml}$) and a half-life of 38.7 h (20 to 64.6 h). Thus, the OZ418 level in plasma remained well above its *in vitro* 50% inhibitory concentrations (IC_{50}s) of 27.4 $\mu\text{g/ml}$ (adult *S. mansoni* worms at 72 h) for at least 75 h. Consistently, OZ418 degraded little in plasma at 37°C (<20% in 121 h) and weakly inhibited cytochrome P450 (CYP450) metabolism (IC_{50} of 37 to 144 μM). Our results provide a first insight into the disposition of OZ418, paving the way for further studies of its biological fate and effect.

Adult trematodes of the genus *Schistosoma* parasitize blood vessels of the intestine (e.g., *Schistosoma mansoni* and *S. japonicum*) and bladder (*S. haematobium*), causing a chronic condition known as schistosomiasis (1). Being one of the major neglected tropical diseases, schistosomiasis affects over 200 million people and leads to chronic morbidity and, in some cases, mortality (1–5). Moreover, almost 800 million people, mostly in rural sub-Saharan Africa, are estimated to be at risk for *Schistosoma* infection (6). No vaccine is yet available, and changing human behavior and eliminating the intermediate host (snail) remain challenging (7–9). Thus, the current strategy relies on praziquantel for the treatment and control of schistosomiasis (10–12). This drug is safe, relatively inexpensive, and easy to distribute (13, 14). However, it lacks efficacy against developing juvenile stages of *Schistosoma*, and its widespread use raises grave concerns about emerging resistance (15–20). In addition, praziquantel's mechanism of action still requires further elucidation to intelligently design active derivatives (21–26). Therefore, new antischistosomal pharmacophores have been greatly sought after in recent years (10, 27, 28).

Inspired by the peroxide pharmacophore of the artemisinin drug family, synthetic 1,2,4-trioxolanes (ozonides [OZs]) have been initially developed as potent antimalarial drug candidates (29), one of which (OZ439) is currently entering phase IIb clinical trials (Fig. 1). Compared with the artemisinins, synthetic ozonides possess a simplified structure and easy synthesis that gives access to a large library of compounds with tunable properties and potentially viable industrial production. Most importantly, they often offer improved bioavailability, pharmacokinetic (PK) properties, and antiparasitic activity (29–31). The artemis-

inins and ozonides have also shown promising efficacy against several parasitic worms, including schistosomes (27, 32–40). In more detail, the first screening of seven structurally diverse synthetic ozonides identified OZ288 as a possible antischistosomal compound. This *cis*-8'-phenyl ozonide had significant activity against juvenile *S. mansoni* infection (95.4% worm burden reduction) and a moderate effect on adult *S. mansoni* infection (52%) in mice (35). Follow-up studies of nine different *cis*-8'-phenyl ozonides *in vivo* revealed OZ418 as a potential lead candidate (32). A single oral dose (200 mg/kg of body weight for juvenile and 400 mg/kg for adult infections) of OZ418 reduced the total worm burden by 100, 80, and 86% in mice infected with juvenile *S. mansoni*, adult *S. mansoni*, and adult *S. haematobium*, respectively (32). Recently, *in vivo* assays with OZ418 in *S. japonicum*-infected mice demonstrated 70 to 84% total adult (200-mg/kg single oral dose) and 27 to 76% total juvenile (400-mg/kg single oral dose)

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Address correspondence to Jennifer Keiser, Jennifer.keiser@unibas.ch.

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FIG 1 Structures of artemisinins (ART, artemisinin; DHA, dihydroartemisinin; AM, artemether; AS, artesunate) and synthetic ozonides (OZs).

worm burden reductions (39). The latter varied widely with the age of juvenile schistosomes.

The broad-spectrum efficacy of the artemisinins and ozonides most likely arises from a redox/radical mechanism triggered by degraded hemoglobin, which is on the menu of both *Plasmodium* and many trematode parasites (41–46). More specifically, hemoglobin-derived iron(II) reduces the peroxide bond of the drug and thereby converts it into radicals that can attack the parasite by alkylation (47–49). Certain ozonides, however, have been reported to act against *S. mansoni* and *S. japonicum* in the absence of hemin *in vitro*, indicating a possible iron-independent pathway (35, 39, 40).

Interestingly, data from *in vivo* studies suggest that acid functional groups can significantly boost the antischistosomal effect of the ozonides compared to neutral and weak basic substituents (32). Besides OZ418, an acid substituent is also present in another synthetic ozonide with strong trematocidal activity, OZ78 (33, 50). Although OZ78 has failed to affect adult *S. mansoni* in mice (35), it has shown a significant effect against both juvenile and adult *S. mansoni* in hamsters (35) as well as juvenile and adult *S. japonicum* in hamsters (36) and mice (40) and adult *S. japonicum* in rabbits (40). The role of acid functionality is not yet fully clear, but it might be an advantage in the low-pH environment of the parasite gut (38). The *cis*-8'-phenyl substituent, on the other hand, is thought to stabilize ozonides against blood-mediated degradation, most likely via steric interaction (30, 51, 52). As a result, the PK parameters of aryl ozonides are often greatly improved compared to those of their alkyl analogues (30). Being an essential step in drug development, PK studies have already been performed for several antimalarial ozonides (29–31) and OZ78 (53–55).

To further characterize and understand the *in vivo* behavior of the antischistosomal drug candidate OZ418, this study aimed to determine its basic PK parameters in uninfected mice. To this end, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) analytical method was developed and validated to quantify OZ418 levels in mouse plasma. Additionally, to deepen our understanding of OZ418 disposition and activity, we evaluated its *in vitro* effect on adult *S. mansoni*, cytochrome P450 (CYP450) metabolism inhibition, plasma stability, and reactivity toward Fe(II).

MATERIALS AND METHODS

Chemicals and reagents. OZ418, OZ352 (internal standard [IS]), and OZ78 were provided by J. L. Vennerstrom of the College of Pharmacy, University of Nebraska Medical Center (Omaha, NE, USA). Acetonitrile of LC-MS grade (Rotisolv) was purchased from Carl Roth GmbH (Karlsruhe, Germany). Ultrapure water was produced with a Millipore Milli-Q water purification system. Dimethyl sulfoxide (DMSO), FeSO₄·

7H₂O, and formic acid (LC-MS grade) were purchased from Sigma-Aldrich (Buchs, Switzerland). Blank mouse plasma was obtained from Dunn Labortechnik GmbH (Asbach, Germany) or prepared by centrifugation (9,000 rpm [$4,980 \times g$] for 10 min) of fresh whole blood collected by cardiac puncture from NMRI mice (Charles River, Germany and Janvier Labs, France) into Li-heparin-coated tubes. Vivid CYP450 screening kits were provided by Life Technologies (Carlsbad, CA, USA).

In vitro IC₅₀ studies with adult *S. mansoni*. For *in vitro* studies of adult *S. mansoni*, schistosomes were collected from the hepatic portal and mesenteric veins of infected mice at 7 to 8 weeks postinfection. Animal studies were performed according to Swiss legislation on animal welfare (permit no. 2070). Mice (female NMRI, 3 weeks old; Charles River, Sulzfeld, Germany) were allowed to adapt under controlled conditions (temperature of ca. 22°C, ca. 50% humidity, 12-h light-and-dark cycle, and free access to rodent diet and water) for 1 week. *S. mansoni* cercariae harvested from infected snails were then subcutaneously injected into mice (~100 cercariae per mouse) (56). Adult schistosomes harvested from infected mice were placed into RPMI culture medium, quickly rinsed, and maintained at 37°C in 5% CO₂. Culture medium for adult worms was prepared by supplementing RPMI 1640 medium with 5% heat-inactivated fetal calf serum (iFCS), penicillin (100 U/ml), and streptomycin (100 µg/ml). The 50% inhibitory concentrations (IC₅₀s) of OZ418 were assessed under two different conditions: RPMI medium and RPMI medium plus red blood cells. The 2% red blood cell suspension in RPMI medium was prepared by using a red blood cell concentrate (blood group A rhesus positive). An OZ418 stock solution (10 mg/ml) was diluted in these media to obtain dilution series (30, 10, 3.3, and 1 µg/ml) in a 24-well flat-bottom plate. At least three schistosomes of both sexes were placed into each well. Schistosomes incubated with the highest concentration of DMSO served as controls. Plates were incubated for 24, 48, 72, 96, and 120 h at 37°C in 5% CO₂ and then examined phenotypically with an inverse microscope by using a motility scale described previously (57). Measurements were performed in duplicates. IC₅₀s were calculated by using Prism 7.01 (2016; GraphPad Software), plotting the average for each measurement.

***In vivo* pharmacokinetic studies.** Female mice (26 to 34 g and 6 weeks old) were acquired from Charles River. The animals were maintained as described above. For oral application, a solution of OZ418 (47 mg/ml) was prepared with 7% Tween 80 and 3% (vol/vol/vol) ethanol in water. Mice were treated with single 400-mg/kg oral doses of OZ418. The study followed a batch design where several samples were taken from each mouse but not at all the time points. The first batch of mice (3 mice) was sampled at 3, 5, 7, and 24 h posttreatment. The second batch (4 mice) was sampled at 1, 10, 12, and 18 h posttreatment. The third batch (4 mice) was sampled at 0.5, 2, 48, and 96 h posttreatment. At the first three time points, blood samples were collected by using sodium heparin-coated capillaries (60 μ l; Marienfeld, Germany). Samples were then centrifuged at 12,000 rpm ($12,074 \times g$) for 5 min. At the last time point, the mice were sacrificed (CO_2), and blood samples were withdrawn from the heart by using a 1-ml syringe. After being collected in Li-heparin-coated tubes, these samples were centrifuged at 9,000 rpm ($4,980 \times g$) for 10 min. The obtained plasma samples were stored at -20°C prior to analysis.

LC-MS/MS equipment and conditions. The high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) included two LC-20AD pumps, an online DG-3310 degasser (Sanwa Tsusho, Tokyo, Japan), a CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland), and a 6-port injection valve (Vici Valco Instruments, Schenkon, Switzerland) with a 5- μ l loop. Chromatographic separation was achieved at 20°C with an 0.8-ml/min flow rate with a Phenomenex Luna 5- μ m C₈(2) 50- by 2.0-mm column (Brebhüler AG, Schlieren, Switzerland), using 0.2% HCOOH in water (solvent A) and 0.2% HCOOH in acetonitrile (solvent B). The solvent gradient was defined as follows: 20% solvent B at 0 min, 20% solvent B at 0.38 min, 40% solvent B at 0.75 min, 95% solvent B at 2.06 min, 100% solvent B at 2.25 min, 100% solvent B at 3.75 min, 20% solvent B at 4.00 min, and 20% solvent B at 4.5 min. To limit the contamination of the mass spectrometer, the flow from the HPLC column was diverted (10-port valve; Vici Valco Instruments, Schenkon, Switzerland) to the mass spectrometer only during 1.9 to 2.3 min, when OZ352 and OZ418 peaks were eluted. The compounds were ionized in the negative mode and detected by multiple-reaction monitoring (m/z 413.2/355.2 for OZ418 and m/z 355.2/169.1 for OZ352) by using an API 3200 instrument (AB Sciex, Framingham, MA, USA) equipped with a turbo ion spray source. The MS/MS parameters were optimized to maximize OZ418 and OZ352 signals (see Table SA1 in the supplemental material). The concentration of OZ78 was quantified by using a previously reported analytical method revalidated for mouse blood plasma (55).

Sample preparation. Stock 10-mg/ml solutions of OZ418 and OZ352 (IS) were prepared in DMSO. Three working OZ418 solutions (1,000, 100, and 10 μ g/ml) were then prepared by serial dilution in DMSO. OZ418 plasma calibration and quality control (QC) samples were obtained by spiking blank mouse plasma with either stock or working solutions of OZ418. The maximum final DMSO concentration was 2.5% (vol/vol). Nine calibration (1-, 5-, 10-, 25-, 50-, 100-, 150-, 200-, and 250- μ g/ml) samples and six replicates (using plasma from 6 different mice) of four QC samples (1, 2.5, 75, and 225 μ g/ml) were thus prepared. The IS solution of OZ352 was made by diluting the OZ352 stock to 10 μ g/ml with 0.2% HCOOH in acetonitrile.

Samples (5 μ l) were diluted (250 $\times$) with the IS solution, briefly vortex mixed, shaken for 10 min at 25°C at a shaking speed of 1,400 rpm, and centrifuged for 10 min at 15,000 rpm (15,093 $\times$ g) at 20°C (5415R centrifuge; Eppendorf, Hamburg, Germany). The supernatant was then transferred into 96-multiwell plates (deep well, 500 μ l; Eppendorf) sealed with SeptraSeal presplit caps (Thermo Scientific). During analysis, samples were maintained at 10°C in the autosampler rack. The sample injection volume was 5 μ l. All mouse plasma samples from the PK study were prepared and analyzed three times.

Method validation procedure. The LC-MS/MS method was validated in compliance with U.S. Food and Drug Administration guidelines (58). To this end, selectivity, linearity, precision, accuracy, recovery, matrix effects, and sample stability were evaluated. For successful validation, at least 4 out of 6 QC replicates (originating from 6 different mice) had to correspond to the criteria described below.

The selectivity of the method was verified by screening 18 blank mouse plasma samples (originating from 6 different mice [6 per day on 3 different days]) for potential interfering signals from endogenous substances. The lower limit of quantification (LLOQ) was chosen so that its signal-to-noise ratio would be >5 and the carryover from the upper limit of quantification (ULOQ) sample would amount to $<20\%$ of the LLOQ signal.

Calibration curves were obtained by plotting the OZ418 peak area (normalized to the IS peak area) versus the OZ418 nominal concentration. The curves were then fitted with linear regression ($1/x^2$ weighing). The accuracy and precision of calibration and QC samples were considered acceptable within a range of 85 to 115% (80 to 120% for the LLOQ).

To quantify matrix effects and sample recovery, three different sets of three QC samples (1, 75, and 225 μ g/ml; six replicates, each originating from six different mice) were prepared. The first QC set was prepared by spiking blank mouse plasma with stock and working solutions of OZ418.

For the second QC set, pure water was used instead of mouse plasma. A portion of the second set was later used for the third QC set (see below). The two QC sets were then diluted with the IS solution, vortex mixed, shaken, and centrifuged as described above. To obtain the third QC set, blank mouse plasma was first diluted with the IS acetonitrile solution and then vortex mixed, shaken, and centrifuged under the same conditions as those for the other two sets. The resulting supernatant was then spiked with OZ418 to obtain the same final concentrations as the other two QC sets. To calculate the matrix effect, QC set 3 was compared with QC set 2. To determine recovery, QC set 3 was compared to QC set 1. The matrix effects were accepted within a range of 85 to 115% (80 to 120% for the LLOQ), provided that the effects did not vary more than $\pm 15\%$ (standard deviation [SD]) along the concentration range. Sample recovery was considered acceptable if it did not deviate more than $\pm 15\%$ ($\pm 20\%$ for the LLOQ) (SD) among the six replicates for each QC concentration and along the concentration range.

The stability of OZ418 was also evaluated under experimental conditions of sample storage, preparation, and analysis. More specifically, the bench stability of freshly prepared sets of QC samples was verified after 5 h at room temperature, and the autosampler stability was checked after 48 h at 10°C. Freeze-thaw stability was tested by remeasuring a set of QC samples after three consecutive freeze-thaw cycles ($-20^\circ\text{C}/+25^\circ\text{C}$). To confirm freezer stability, samples were analyzed after 3 months at -20°C .

Stability in the presence of Fe(II), mouse blood plasma, and mouse whole blood. The degradation of OZ418 and OZ78 in the presence of FeSO₄ at 37°C was monitored as previously described (51) but with increased concentrations of both ozonides (0.3 mM) and FeSO₄ (30 mM). Mouse blood was collected into Li-heparin tubes by cardiac puncture (12 different mice). Half of these samples were then centrifuged (9,000 rpm for 10 min) to obtain blank mouse plasma. Both whole blood and plasma samples were spiked with stock solutions (10 mg/ml in DMSO) of either OZ418 or OZ78 to reach an initial concentration of 100 μ g/ml. Samples were maintained at 37°C, and aliquots were periodically taken. Whole-blood aliquots were immediately centrifuged (9,000 rpm for 10 min) to recover plasma. All samples were stored at -20°C until analysis. OZ418 and OZ78 concentrations were quantified as described above.

CYP450 inhibition screening. *In vitro* studies to evaluate the CYP450 inhibition potential of OZ78 and OZ418 were performed by using commercially available fluorogenic human recombinant Vivid CYP450 screening kits (CYP1A2 EOMCC, CYP3A4 BOMR, CYP2C19 EOMCC, and CYP2D6 MOBFC) according to the kit's user guide (59). The following known CYP450 inhibitors were used as positive controls: propranolol for CYP1A2, omeprazole for CYP2C19, quinidine for CYP2D6, and ketoconazole for CYP3A4. Drug stock solutions (10 mM) were prepared in DMSO. Working dilution series (0, 0.69, 2.06, 6.17, 18.52, 55.56, 166.67, and 500 μ M for ozonides; 0, 0.34, 1.03, 3.08, 9.26, 27.78, 83.33, and 250 μ M for propranolol and omeprazole; and 0, 0.0034, 0.0103, 0.0309, 0.0926, 0.2778, 0.8333, and 2.5 μ M for quinidine and ketoconazole) were then prepared from stock solutions by using Vivid reaction buffers. The solutions were then added (40 μ l/well) to the corresponding wells on Costar 96-well black polystyrene plates (Corning, USA) containing baculosomes and the Vivid regeneration system in Vivid reaction buffer (50 μ l/well). After a 10-min incubation period, the fluorogenic Vivid substrate mixed with NADP⁺ in Vivid reaction buffer (10 μ l/well) was added to wells (final volume, 100 μ l/well). Assays were run in the kinetic mode, measuring the fluorescence signal every minute for 20 min (SpectraMax M2 and Softmax version 5.4.1; Molecular Devices). The assays were run in duplicate and performed twice.

Estimation of passive intestinal absorption. Physicochemical descriptors for OZ418 and OZ78 (see Table SA8 in the supplemental material) were calculated by using the Chemical Development Kit (CDK) open-source software (cdk-1.5.13 [see <http://cdk.sourceforge.net/>]). Chemical structures were encoded as SMILES (simplified molecular input line entry specification). Effective intestinal permeability (P_{eff}) was predicted by the model $\log P_{eff} = -2.546 - 0.011 \times \text{PSA} - 0.278 \times \text{HBD}$

TABLE 1 Intra- and interassay accuracy and precision^b

Nominal concn (μg/ml)	Intra-assay ^a (<i>n</i> _{min} = 12)			Interassay (<i>n</i> _{min} = 36)		
	Mean concn (μg/ml)	Accuracy (%)	RSD (%)	Mean concn (μg/ml)	Accuracy (%)	RSD (%)
1	0.95	95	14	0.97	97	14
2.5	2.46	99	10	2.49	100	8
75	77.5	103	7	76.3	102	6
225	218.8	97	8	211.0	94	7

^a Data for only one out of three intra-assays are shown (see Tables SA3 to SA5 in the supplemental material for all the intra-assay data).

^b *n*_{min} is the minimal number of replicate measurements used for each mean concentration calculation. RSD, relative standard deviation.

(60), where PSA is the polar surface area and HBD is the number of hydrogen bond donors.

Pharmacokinetics and statistical analysis. Integration of LC-MS/MS signals, linear fitting of the calibration line, determination of concentrations in QC and mouse samples, and calculation of accuracies and precisions were carried out by using Analyst 1.6.2 software (AB Sciex). A naive pooled noncompartmental PK analysis was run by using the PK R language package (61) for the median, lowest, and highest concentrations at each sampling time point. PK parameters were determined by using a noncompartmental heteroscedastic regression model (62). The area under the plasma concentration-versus-time curve (AUC) was calculated by using the linear trapezoidal rule. The half-life (*t*_{1/2}) was estimated by a linear regression on semi-log-transformed data. The maximum concentration of the drug in plasma (*C*_{max}) and the time to reach *C*_{max} (*T*_{max}) were taken directly from the observed data and are represented as medians (ranges).

RESULTS AND DISCUSSION

Analytical method development and validation. As synthetic ozonides have been intensively researched as antimalarials, several LC-MS methods for their analytical quantification in rat and human blood plasma can be found in the literature (29–31, 63). In addition, analytical methods have been developed to quantify OZ78 concentrations in rat and sheep plasma and bile (54, 55, 64). However, the lipophilicity and the MS responsiveness of synthetic ozonides can vary considerably with different substituents on the cyclohexane ring. For instance, while most antimalarial ozonides could be ionized by positive electrospray ionization (ESI), carboxylic acid ozonides such as OZ78 can be well detected only in the negative mode (64). Thus, existing analytical methods might require substantial readjustments before they could be applied to a different ozonide.

As previously reported for carboxylic acid ozonides, *m/z* 413.2 [*M* – H][–] ions were observed in the negative ESI spectra of OZ418 (55, 64). The major fragment monitored for OZ418 was at *m/z* 355.2, corresponding to [*M* – CH₂C(O)OH][–]. The structural similarity between OZ78 and OZ418 allowed us to use previously reported LC-MS/MS methods for OZ78 (55, 64) as a starting point to develop a method to monitor OZ418 in mouse plasma. Besides the eluent composition and gradient, the calibration line range and sample preparation had to be modified as well due to the differences in lipophilicity and *pK*_a between OZ78 and OZ418. While OZ78 can be analyzed by using both *C*₈ and *C*₁₈ phase columns (55, 64), OZ418 is difficult to elute off a *C*₁₈ phase column. In addition, the OZ418 responsiveness with negative ESI-MS appeared to largely exceed that of OZ78, most probably

TABLE 2 Absolute recovery and matrix effects for OZ418 and the internal standard OZ352

Analyte	Nominal concn (μg/ml)	Mean absolute recovery ± RSD (%)	Mean matrix effect ± RSD (%)
OZ418	1	101 ± 16	102 ± 12
	75	101 ± 6	100 ± 15
	225	102 ± 5	90 ± 5
OZ352 (IS)	10	94 ± 5	103 ± 3

due to the higher lipophilicity of OZ418 (65). Concentrations of OZ418 as low as 1 ng/ml could be easily quantified, while the OZ78 LLOQ after sample preparation was about 40 ng/ml (55). The linear dynamic range of the OZ418 calibration curve, however, extended up to 1 μg/ml only. Since the preliminary analysis of mouse plasma samples confirmed 10- to 100-fold-higher concentrations of OZ418 (as expected from the PK data for the other ozonides) (30, 31, 53–55, 64), dilution of samples was incorporated into sample preparation. The latter consisted of a simple protein precipitation with acetonitrile. The OZ418 LC-MS/MS analytical method was then validated for a concentration range of 1 to 250 μg/ml by using the same IS, OZ352, as the one used for OZ78 quantification (see Fig. SA1 in the supplemental material). Note that the sensitivity of the method can easily be increased down to 10 ng/ml by modifying the sample preparation.

The calibration curves for OZ418 were linear over a concentration range of 1 to 250 μg/ml in mouse plasma, with a coefficient of variation (*r*²) above 0.99. Accuracy and precision were calculated for 9 batches of QC samples (3 batches per day on 3 different days) at 4 concentrations (1, 2.5, 75, and 225 μg/ml). As shown in Table 1 (see also Tables SA3 to SA5 in the supplemental material), the intraday (*n* = 3) and interday (*n* = 9) batch precisions were 5 to 16% and 7 to 14%, respectively, with 91 to 103% accuracies. Extraction recoveries at three different concentrations (1, 75, and 225 μg/ml) were 101 to 102% (±5 to ±16%) (Table 2). The matrix effect varied from 90 to 102% (±5 to ±15%). No interfering signals were observed at the retention time of OZ418 (see Fig. SA1 and Table SA6 in the supplemental material). Carryover from the ULOQ sample was observed (13% ± 3%), but it was limited to <20% of the LLOQ signal (see Table SA6 in the supplemental material). OZ418 also remained stable under the conditions of sample storage, preparation, and analysis (see Table SA7 in the supplemental material). All above-mentioned values were within the range of ±15% (±20% for the LLOQ) recommended by the U.S. FDA for bioanalytical methods (58). The LC-MS/MS method presented in this paper thereby provides sufficient selectivity, accuracy, and precision as well as excellent analyte recovery without significant carryover and matrix effects. In addition, the analytical run time was reduced from 11 to 4.5 min compared to the existing OZ78 LC-MS/MS method (55).

TABLE 3 *In vitro* activity of OZ418 against adult *S. mansoni*

Medium	IC ₅₀ (μg/ml)				
	24 h	48 h	72 h	96 h	120 h
RPMI	30.2	29.2	27.4	28.2	26.9
RPMI + red blood cells (2%)	35.7	22.8	18.3	13.5	9.8

FIG 2 Plasma concentrations of OZ418 in healthy mice following a single oral dose of 400 mg/kg. Mice were separated into three batches, with each mouse being sampled at four different time points (batch 1 at 3, 5, 7, and 24 h; batch 2 at 1, 10, 12, and 18 h; and batch 3 at 0.5, 2, 48, and 96 h posttreatment).

OZ418 activity *in vitro*. While the antischistosomal activity of OZ418 in *in vivo* *S. mansoni*, *S. haematobium*, and *S. japonicum* infection models has already been demonstrated (32, 39), its *in vitro* activity has been studied in *S. japonicum* only. The data shown in Table 3 reveal a nonnegligible effect of OZ418 on adult *S. mansoni* (IC_{50} of 18.3 to 27.4 µg/ml or 44.2 to 66.2 µM after 72 h), similar to that reported previously for juvenile and adult *S. japonicum* (IC_{50} of 16 µg/ml after 72 h) (39). However, these values are less impressive than the IC_{50} s of praziquantel (0.08 to 0.16 µM) in similar assays reported previously (66, 67). As synthetic ozonides are suspected to act via an iron-dependent mechanism, we also evaluated the *in vitro* activity of OZ418 in the presence of red blood cells. A previous study of *S. japonicum* indeed reported a significant effect of hemin on the antischistosomal activity of OZ418 (39). In the case of *S. mansoni*, although an iron source appears to slightly increase the impact of OZ418 (Table 3; and see Fig. SA4 and SA5 in the supplemental material), our preliminary data do not allow us to attribute any statistical significance to it. Interestingly, the peroxide moiety is essential for the antiparasitic activity of synthetic peroxides, yet the effect of iron sources on their *in vitro* activity varies widely (35, 40, 68). Thus, an additional iron-independent mechanism of action remains an open possibility for this type of compound.

OZ418 pharmacokinetics. Using our validated method, concentrations of OZ418 were determined in plasma samples from uninfected mice treated with single 400-mg/kg oral doses of OZ418. The resulting concentration-versus-time curves are shown in Fig. 2, and the PK parameters estimated with a naive pooled noncompartmental analysis are summarized in Table 4.

The area under the plasma concentration-versus-time curve extrapolated to infinity ($AUC_{0-\infty}$) was estimated to be 11,282.4 µg h/ml (range, 7,132.9 to 18,809 µg h/ml). The AUC to the last observed time point (AUC_{0-t}) of 9,303.8 µg h/ml (7,039.2 to 11,908.5 µg h/ml) thus constitutes >80% of the $AUC_{0-\infty}$ estimate, confirming its reliability. Following oral administration, the OZ418 plasma level peaked by ~2 h (2 to 3 h) after administration, similarly to OZ78 and OZ439. A secondary peak suggesting a possibility of enterohepatic recirculation was observed at 10 h. However, this peak could also be an artifact originating from the incomplete batch design and small sample size used in this study, so additional investigation would be required to confirm it. So far, no enterohepatic recirculation has been reported for synthetic ozonides. A special feature of the OZ418 PK profile is its relatively long elimination half-life of 38.7 h (20.0 to 64.6 h), one of the longest half-lives reported for synthetic ozonides (29–31, 53–55). For comparison, the first generation of ozonides had a half-life of 1 to 2 h only, an improvement over the extremely short 0.5-h half-life of artesunate (29). Despite a certain structural similarity between OZ78 and OZ418, their half-lives are as strikingly different, an effect previously observed for the antimalarial drug candidates OZ277 (1 h) and OZ439 (23 h) in healthy rats (30). Calculated physicochemical parameters for OZ78 and OZ418 (see Table SA8 in the supplemental material) predict high ($\log P_{eff} > -4$; PSA, <140 Å) passive intestinal permeability for both compounds (60, 69). Thus, the prolonged circulation of OZ418 is most likely due to its reduced clearance rate and possibly an increased volume of distribution. Previous studies of OZ277 have shown that although CYP450 metabolism contributed significantly to OZ277 elimination (30, 70), the major clearance mechanism most probably proceeded via peroxide bond reduction by endogenous Fe(II) sources in blood and tissues (30, 31). Our data on OZ78 and OZ418 reactivity toward Fe(II) at 37°C (see Fig. SA6 in the supplemental material) highlight an improved stability of OZ418 (apparent first-order degradation constant of $0.034 \pm 0.003 \text{ h}^{-1}$ versus $0.242 \pm 0.005 \text{ h}^{-1}$ for OZ78 [best-fit values $\pm$ standard errors {SE}]). Consistently, the mouse blood plasma stability of OZ418 at 37°C also exceeded that of OZ78 (Fig. 3A). CYP450 inhibition was screened with the major drug-metabolizing isozymes CYP1A2, CYP3A4, CYP2D6, and CYP2C19. While no inhibition of any of the CYP450 enzymes was detected for OZ78 at the tested concentration range (up to 200 µM), IC_{50} s of 37 to 144 µM (15 to 47 µg/ml) were measured for OZ418 (Fig. 3B). Hence, OZ418 is a very weak inhibitor, which correlates with the long half-life observed *in vivo*.

The long circulation of OZ439 and OZ418 can then be at least partially attributed to their blood stability enhanced by the *cis*-8'-

TABLE 4 Pharmacokinetic parameters of OZ418 after oral administration of 400 mg/kg in uninfected mice^a

Treatment	Species	Dose ^b	C_{max} (µg/ml) (range or SD)	T_{max} (h) (range or SD)	AUC_{0-t} (µg h/ml) (range or SD)	$t_{1/2}$ (h) (range or SD)	Reference
OZ418	Mouse	400 mg/kg	190 (185–231)	2 (2–3)	9,303.8 (7,039.2–11,908.5)	38.7 (20–64.6)	
OZ439	Rat	3 mg/kg				23	30
OZ439	Human	1,200 mg (maximum, 20 mg/kg)	0.70 (0.15)	3 (2–6)	6.53 (0.17)	31.6 (46.9)	31
OZ78	Rat	50 mg/kg	70.1 (19.1)	1.6 (1.6)	496.6 (66.5)	2.5 (1.5)	54

^a For comparison, PK parameters reported previously for OZ439 and OZ78 are shown. Either standard deviations or observed ranges are indicated in parentheses. All noncompartmental analysis results for OZ418 are represented as medians (ranges).

^b The dose was a single oral dose. Shown are the drug concentrations measured in plasma.

FIG 3 (A) Stability of OZ418 and OZ78 in mouse blood plasma at 37°C (mean $\pm$ SD; $n = 3$). (B) OZ418 and OZ78 IC₅₀s for inhibition of CYP450 enzymes (mean $\pm$ SE; $n = 2$). Positive controls are propranolol (CYP1A2), ketoconazole (CYP3A4), omeprazole (CYP2C19), and quinidine (CYP2D6).

aryl substituent (30, 51, 52). Increases in the *in vivo* half-life and blood exposure can be particularly important to achieve a single-dose oral cure. Such prolonged exposure to OZ418 is most probably one of the foundations of its high antischistosomal activity observed *in vivo*. Indeed, after a single oral dose of 400 mg/kg, OZ418 plasma levels remained well above the *in vitro* IC₅₀s for adult *S. mansoni* (Table 3) for about 75 h.

In conclusion, we successfully derived an LC-MS/MS method to quantify OZ418 concentrations in mouse blood plasma, building on established analytical methods for OZ78. The new analytical method fully complied with U.S. FDA guidelines and allowed us to estimate the PK parameters of OZ418 in uninfected mice. It is important to keep in mind, however, that PK parameters can be strongly influenced by parasitic infection, as demonstrated, for example, for artesunate and dihydroartemisinin in *Fasciola hepatica*-infected rats or for mefloquine in *S. mansoni*-infected mice (71). Thus, further studies in mice harboring *Schistosoma* would be necessary to fully explore the PK properties of OZ418.

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