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In vitro assessment of the pharmacodynamic properties and the partitioning of OZ277/RBx-11160 in cultures of *Plasmodium falciparum*

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***Objectives*: Using synchronous cultures of *Plasmodium falciparum* malaria, the stage sensitivity of the parasite to OZ277 (RBx-11160), the first fully synthetic antimalarial peroxide that has entered Phase II clinical trials, was investigated *in vitro* over a concentration range of 1× to 100× the IC_{50}. Secondly, partitioning of OZ277 into *P. falciparum*-infected red blood cells (RBCs) and uninfected RBCs was studied *in vitro* by measuring its distribution between RBCs and plasma (R/P).**

***Methods*: The effects of timed *in vitro* exposure (1, 6, 12 or 24 h) to OZ277 were monitored by incorporation of [^{3}H]hypoxanthine into parasite nucleic acids and by light-microscopic analysis of parasite morphology. Partitioning studies were performed with radiolabelled [^{14}C]OZ277.**

***Results*: After 1 h of exposure to OZ277 at the highest concentration (100× the IC_{50}) followed by removal of the compound, the hypoxanthine assay showed that growth of mature stages of *P. falciparum* was reduced to below 20%. Young ring forms were slightly less sensitive (43% growth). Similar stage-specific profiles were found for the antimalarial reference compounds artemether and chloroquine. Strong inhibition (≤6% growth) of all parasite stages was observed when the parasites were exposed to each of the three compounds for 6 h or longer. After removal of the compounds, the parasites did not recover, indicating that the observed growth inhibitions were cytotoxic rather than cytostatic. Pyrimethamine was confirmed to be active exclusively against young schizonts. Light-microscopic analysis also demonstrated the specificity of pyrimethamine against the schizont forms and showed that OZ277, artemether and chloroquine attenuated parasite growth more rapidly than did pyrimethamine. The R/P for OZ277 was 1.5 for uninfected RBCs and up to 270 for infected RBCs.**

***Conclusions*: The present study indicates similar stage-specific profiles for OZ277 and for the more well-established antimalarial agents artemether and chloroquine. Secondly, the study describes a significant accumulation of radiolabelled OZ277 in *P. falciparum*-infected RBCs.**

Keywords: stage specificity, uptake, peroxides, antimalarials

Introduction

The sesquiterpene lactone artemisinin was isolated by Chinese scientists in 1971 by extraction of *Artemisia annua* (the sweet wormwood plant) into diethyl ether at low temperature. The 'active principle' was subsequently shown to cure mice infected with *Plasmodium berghei*. In 1972, further work culminated in the isolation of a crystalline compound that was named qinghaosu, or artemisinin, after the generic name of the plant[1] and demonstrated that its peroxide bond is essential for antimalarial activity.[2,3] Artemisinin can be converted into its semisynthetic derivatives, artemether and artesunate, which are more active than the parent molecule.[4] However, the isolation of artemisinin from the plant makes it and its semisynthetic derivatives several fold more expensive than the relatively inexpensive standard antimalarials chloroquine and pyrimethamine/sulfadoxine.

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The first total synthesis (13 steps, 5% overall yield) of artemisinin was reported in 1983.[5] Since then, several groups have reported different pathways for the synthesis of artemisinin, but all require numerous steps and have low yields.[6–8] Thus, none of these complex syntheses provides a viable method for large-scale production of the molecule.[9] Although the semisynthetic artemisinins (in combination with longer acting antimalarials) are currently the drugs of choice to treat multidrug-resistant malaria, access to these agents in disease endemic countries has been limited mainly due to their cost and availability. Recently, Vennerstrom *et al.*[10] published the discovery of a new synthetic peroxide antimalarial called OZ277 (RBx-11160). OZ277, currently in Phase II clinical trials, exhibits structural simplicity, an economically feasible and scalable synthesis, superior antimalarial activity and a better biopharmaceutical profile than artemisinin and its semisynthetic derivatives. Furthermore, OZ277 is fully synthetic and structurally different from the artemisinin drug class. Artemisinins contain a six-membered 1,2,4-trioxane heterocycle whereas OZ277 contains a five-membered 1,2,4-trioxolane, more commonly known as a secondary ozonide. The present study describes for the first time the pharmacodynamic effects of this novel drug development candidate on *Plasmodium falciparum* cultures by assessing its stage specificity and rate of action in comparison with three standard antimalarial drugs at clinically relevant concentrations. The partitioning of OZ277 into infected and non-infected red blood cells (RBCs) was also examined.

Materials and methods

Chemicals and materials

Chemicals and materials were from Sigma, Perkin-Elmer and GIBCO Invitrogen except for OZ277 tosylate (J. L. Vennerstrom, Nebraska, USA), artemether (Kunming Pharmaceuticals Corporation, China), pyrimethamine (Roche, Basel, Switzerland) and [8-^{3}H]hypoxanthine (Amersham Bioscience, UK). Antimalarial compounds were dissolved in dimethylsulfoxide (DMSO) at 10 mg/mL. The stock solutions were kept at 4°C for not more than 6 months. Two [^{14}C]OZ277 hydrogen maleate salts (508 g/mol) were used—one was labelled in the adamantane ring (OZ277[L], Moravek Biochemicals; specific activity: 31 mCi/mmol) and the other in the side chain (OZ277[R], a gift from F. Hoffmann-La Roche Ltd; specific activity: 42 mCi/mmol, Figure 1). The compounds were prepared as 10 mg/mL stock solutions in toluene and stored at –80°C.

Parasite cultivation

NF54, a drug-sensitive isolate of *P. falciparum*, was maintained in 10 cm Petri dishes and cultured by standard methods[11] in an atmosphere of 93% N_2, 4% CO_2, 3% O_2 at 37°C. The culture medium was RPMI 1640 10.44 g/L, supplemented with Hepes 5.94 g/L, Albumax II 5 g/L, hypoxanthine 50 mg/L, sodium bicarbonate 2.1 g/L and neomycin 100 mg/L. When required, parasites were synchronized twice with 5% D-sorbitol.[12] The second treatment was 7–8 h after the first. This procedure provided in most cases a parasite culture containing ≥80% young trophozoites (20 h old). Initial parasitaemias varied between 3% and 11% in all studies.

Growth inhibition assay and washing procedure

P. falciparum growth was assessed by measuring incorporation of the nucleic acid precursor [^{3}H]hypoxanthine.[13] IC_{50} values were found to be 0.91 ± 0.12 ng/mL for OZ277, 1.2 ± 0.1 ng/mL for artemether, 5.1 ± 0.8 ng/mL for chloroquine[10] and 5.6 ± 0.5 ng/mL for pyrimethamine. Synchronized cultures of young NF54 trophozoites (20 h) with parasite counts of 0.15% and a haematocrit of 5% were divided into three 10 cm Petri dishes. Two dishes were further incubated for 16 or 32 h at 37°C for maturation into early schizonts (36 h) or early ring stages (4 h). The third dish with the early trophozoites was used immediately for exposure for a 1, 6, 12 or 24 h period to the following four antimalarial compounds: OZ277 and artemether (final concentrations 100, 13, 1.6 ng/mL), chloroquine and pyrimethamine (final concentrations 500, 63, 8 ng/mL). After the respective incubation times for the parasite–compound mixture, the plates were washed four times resulting in a 1280-fold dilution of the free compound. After another incubation period of 24 h at 37°C in the atmosphere described above and in the presence of [^{3}H]hypoxanthine, the plates were frozen at –20°C. For the IC_{50} determination, plates were thawed and harvested with a Betaplate cell harvester (1295-004 Betaplate; Wallac Perkin-Elmer) onto glass filters. The dried filters were inserted into a plastic foil with 10 mL of scintillation fluid and counted in a Betaplate liquid scintillation counter (1205 Betaplate; Wallac Perkin-Elmer). The results of each well were recorded as counts per min and expressed as a percentage of the untreated controls. Suspensions of uninfected erythrocytes were used for background subtraction. For morphological analysis of antimalarial action, synchronized cultures were treated with compounds (100× IC_{50}) in 10 cm Petri dishes. Light-microscopic evaluation of Giemsa-stained thin blood smears was performed every hour by using an oil-immersion lens (1000×). Changes in parasite morphology were compared with compound-free control cultures.

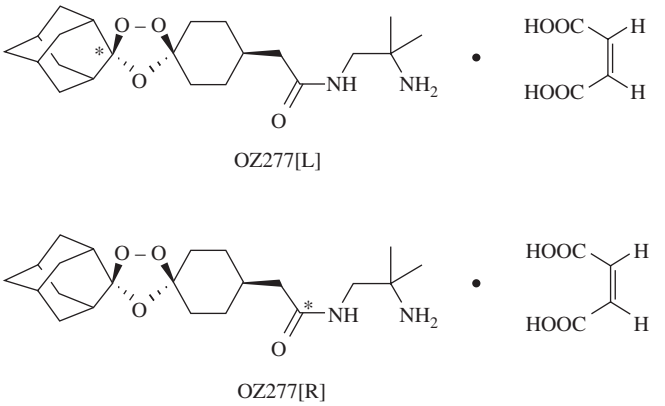

Figure 1. Structure of the two [^{14}C]OZ277 molecules. One was labelled in the adamantane ring (OZ277[L], Moravek Biochemicals) and the other in the side chain (OZ277[R], a gift from F. Hoffmann-LaRoche Ltd). The asterisk denotes the position of the ^{14}C label.

Time-course of uptake into RBCs

Samples containing 230 µL of infected erythrocytes and 470 µL of plasma at a final OZ277 concentration of 77 ng/mL (as either the R or L radiolabelled material, Figure 1) were incubated in a water bath at 37°C for various time periods ranging from 10 min up to 3 h. At the end of the incubation period, tubes were centrifuged at 600 **g** for 5 min to form RBC pellets; 230 µL of each supernatant was transferred to a new tube. The rest of the supernatant was discarded and the pellets (230 µL) were kept for further processing. In addition, all experiments included the following two controls: (i) 700 µL of plasma (no RBCs) containing a final [^{14}C]OZ277 concentration of 77 ng/mL was incubated for 20 min and 2 h; and (ii) a mixture

of 470 μL of plasma (containing no [^{14}C]OZ277) and 230 μL of uninfected RBCs was incubated for 1 h. From tube (i) 230 μL of the drug mixture and from the background-control (ii) 230 μL of the supernatant were also transferred to new tubes. Then, to each of the tubes containing the various controls, test supernatants and pellets, 1 mL of a 50:50 mixture of isopropanol and soluene-350 was added, followed by incubation at 60°C for 1 h in a water bath. The samples were then bleached with 3.2 mL of 30% hydrogen peroxide and again incubated at 60°C for 30 min in a water bath. Finally, 15 mL of OptiPhase 'Super Mix' scintillation cocktail was added. The contents of the tubes were mixed, 4 mL transferred to scintillation vials and counted after 3–4 h using a liquid scintillation counter (1450 Microbeta plus; Wallac Perkin-Elmer). To confirm mass balance and the absence of a significant contribution of extracellular fluid on the measured counts for the pellet, the counts for the total test-vial sample volume were calculated from the measured counts for the plasma supernatant and the RBC pellet. This value was then compared with the total counts for the plasma control (i). Results were not considered if the measured counts of OZ277 for the plasma control (i) differed by more than 10% from that calculated for the test-vial (containing RBCs and plasma). For the vast majority of the experiments, total counts for the control and test vials differed by <10%. Results of the background-control (ii) were used for background subtraction. Where uptake ratios (R/P; i.e. gradient between RBCs and plasma) were calculated, they represented the calculated ratio of radioactivity in 230 μL of 100% infected RBCs to the same volume of plasma collected at the end of incubation. The uptake into non-infected RBCs was accounted for by using the following equation:

$$\mathrm{dpm_I} = \frac{\mathrm{dpm_R} - \mathrm{dpm_N}\left(1-\frac{x}{100}\right)}{x} * 100$$

where $\mathrm{dpm_I}$ = dpm in 230 μL of 100% infected erythrocytes, $\mathrm{dpm_R}$ = dpm in 230 μL of infected cell pellet (i.e. containing infected and non-infected RBCs), $\mathrm{dpm_N}$ = dpm in 230 μL of non-infected cell pellet (i.e. containing only non-infected RBCs) and x = % parasitaemia.

R/P was then calculated by dividing $\mathrm{dpm_I}$ by dpm in 230 μL of plasma collected at the end of incubation.

Effect of compound concentration on RBC uptake

Infected or uninfected erythrocytes (230 μL) were mixed with 420 μL of plasma and 50 μL of [^{14}C]OZ277[L] in plasma at concentrations ranging from 40 to 64 000 ng/mL. All samples, including controls (i) and (ii), were incubated for 1 h at 37°C. Extraction of the samples was performed as described above.

Results

Pharmacodynamic studies

Stage-specific effects of 1, 6, 12 or 24 h of compound exposure were investigated using the hypoxanthine incorporation assay with highly synchronous cultures of *P. falciparum* NF54 over a concentration range of 1× to 100× the IC_{50} of the four compounds OZ277, artemether, chloroquine and pyrimethamine. The washing procedure following the respective incubation periods was performed in a way that the unbound compound was diluted at least 1000-fold. Using this procedure, the viability of parasites during the subsequent [^{3}H]hypoxanthine-incubation period of 24 h could be assessed in the absence of unbound compound. Parasitic stages evaluated were young rings (4 h), young trophozoites (20 h) and young schizonts (36 h). Figure 2 shows parasite growth plotted against incubation time for three selected compound concentrations: ~1× IC_{50}, ~10× IC_{50} and ~100× IC_{50}. After 1 h of exposure to OZ277, artemether or chloroquine at the highest concentration (100× the IC_{50}), ring forms were slightly more resistant to the three compounds than trophozoites and schizonts. From 6 up to 24 h of compound incubation, all stages reacted similarly, with strong inhibition (≤6% growth) at 100× IC_{50}. Interestingly, at the lowest OZ277 concentration (~1× IC_{50}), schizonts underwent a steady decrease of growth after 6 h, then an increase at 12 h and a decrease at 24 h. The marked increase in growth after 12 h was repeated and confirmed in three independent experiments. The growth inhibition of ring and trophozoite populations remained relatively stable from 6 h onwards. At the lowest artemether concentration tested (~1× IC_{50}), a minimal inhibitory effect was observed against all parasite forms over the entire period investigated, whereas at the lowest chloroquine concentration a relatively linear decrease of growth within 24 h was noted for all stages. Stage-specific analysis with pyrimethamine demonstrated that growth of the ring and trophozoite stages was not affected, even after 24 h at the highest concentration. The only pyrimethamine-sensitive parasite form was the schizont. Growth inhibition of 20–40% was observed after 6 and 12 h at medium to high pyrimethamine concentrations. The 24 h incubation period clearly resulted in the strongest schizont growth inhibition, even at the lowest concentration. To determine the morphological changes of *P. falciparum* by light microscopy, synchronous cultures of NF54 were exposed to OZ277, artemether, chloroquine and pyrimethamine, respectively, at fixed compound concentrations of ~100× IC_{50}. Thin, Giemsa-stained blood smears were taken hourly from 1 to 9 h and after 24 h. The morphological changes observed were very similar for OZ277, artemether and chloroquine. In young ring forms, the nucleus was most affected in the first 1–2 h. In schizont and trophozoite forms, the first changes were observed usually after 1 and 3 h, respectively. Morphology changes were clumping of crystals, a paler disorganized cytoplasm and vacuolation (data not shown). In contrast to OZ277, artemether and chloroquine, most of the pyrimethamine-treated ring and trophozoite stages developed further to their next stage, even up to the end of the monitoring period (24 h). Only the cytoplasm of schizonts became paler and crystals started to clump (8–9 h) until the parasites actually stopped developing or disintegrated (24 h).

Time-course of uptake into RBCs

Table 1 shows the accumulation of [^{14}C]OZ277[L] by uninfected erythrocytes or erythrocytes infected with young and mature parasites over a time period of 10 min up to 3 h at a compound concentration of 77 ng/mL. Mature parasites accumulated the compound more quickly and at a higher average ratio (30 min, R/P = 214) than ring forms (2 h, R/P = 53). Based on the R/P ratio at 3 h, concentrations of the radiolabelled compound in infected RBCs of both mature and young parasite stages declined to about half the maximum value. Under these same conditions, uninfected erythrocytes showed an uptake ratio of about 1.5, which was maintained throughout the 3 h incubation period. Figure 3 shows the time-course of the two different radiolabelled OZ277 molecules [L] and [R]. Young ring stages tested at 10 min showed comparable uptake of both radiolabelled

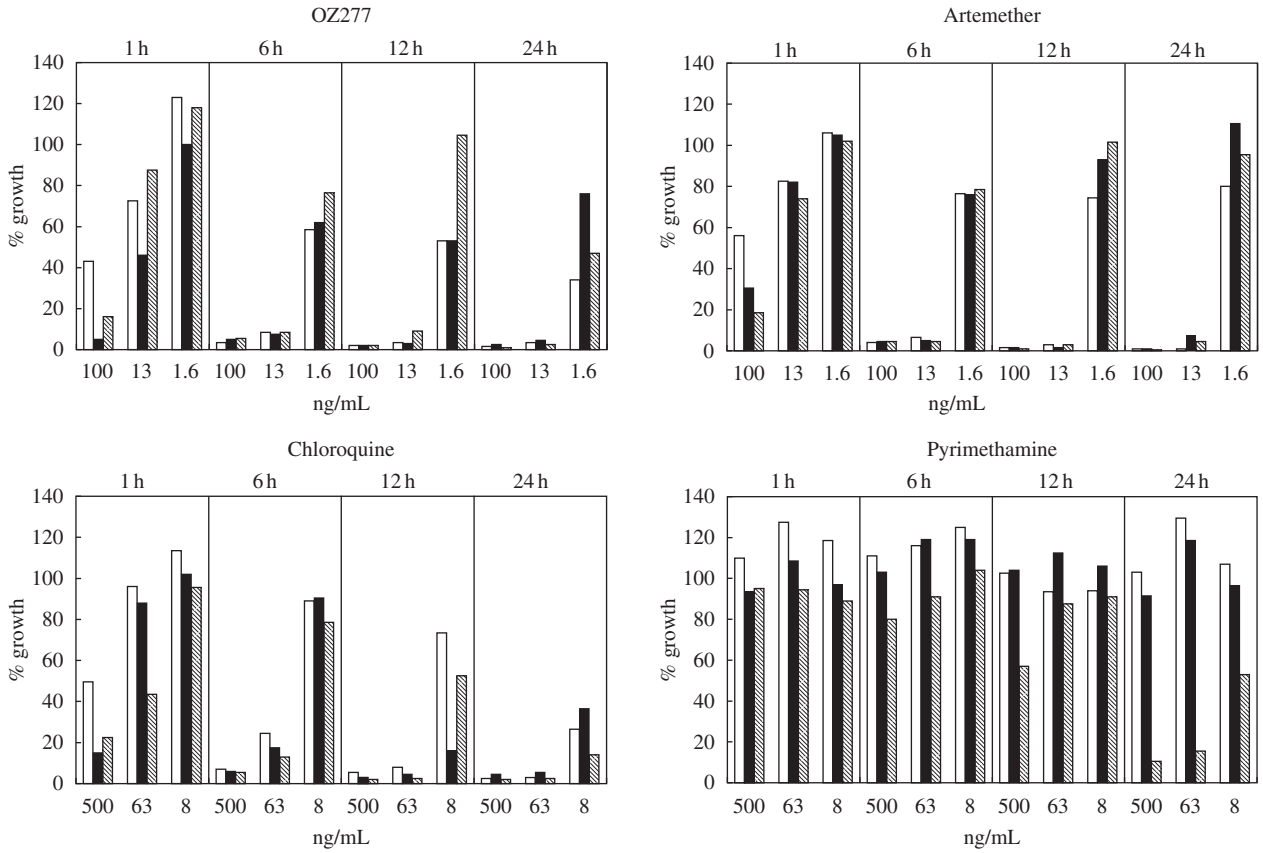

Figure 2. Stage-dependent effects of OZ277, artemether, chloroquine and pyrimethamine (~1×, ~10× and ~100× the IC_{50}) on [^{3}H]hypoxanthine incorporation in synchronous cultures of *P. falciparum* strain NF54. Compounds were added for 1, 6, 12 or 24 h. After removal of the compounds, parasites were incubated for another 24 h in the presence of [^{3}H]hypoxanthine. Compound effects are expressed as the percentage of growth of the respective development stage relative to untreated control. White bars, ring stage; black bars, trophozoite stage; hatched bars, schizont stage. Each bar represents the mean of $n = 2$ independent experiments. Variation of individual values was low except for OZ277 ring stage at 1 h (±65% at 13 ng/mL and ±46% at 1.6 ng/mL) and pyrimethamine ring stage at 1 h (±31% at 63 ng/mL and ±55% at 8 ng/mL). Pyrimethamine 6 h experiment was only conducted once.

compounds. However after 10 min, the concentration of the [R] molecules in the infected RBCs decreased steadily compared with the [L] molecules. Uninfected RBCs incubated with OZ277[R] showed similar uptake ratios compared with OZ277[L].

Effect of different compound concentrations on RBC uptake

The results from incubations conducted at concentrations of 38–64 000 ng/mL of radiolabelled OZ277[L] and analysed at a single time-point of 1 h showed that at higher concentrations the uptake ratios reached a constant value of ~23 (Figure 4). Uninfected RBCs had a steady uptake ratio of about 2 (data not shown) over this same incubation period.

Discussion

Relatively little is known about the rate of action and selective toxicity of antimalarials against the morphologically distinguishable different blood stages of *P. falciparum*.[14] These pharmacodynamic factors may be important determinants of immediate antimalarial drug efficacy in severe malaria and could provide important clues about the mechanism of action. Another reason for the selective toxicity of antimalarials such as chloroquine and the artemisinin family of drugs can be attributed to the increased accumulation in parasitized RBCs.[15–18] Given these considerations, we assessed *in vitro* the pharmacodynamic properties and the partitioning of OZ277, the first fully synthetic antimalarial peroxide that has entered Phase II clinical trials, in cultures of *P. falciparum*.

Pharmacodynamic studies indicated that OZ277, artemether and chloroquine influence growth of all parasite stages in a similar way (Figure 2). An exposure time of ≥1 h at a high compound concentration range was sufficient to achieve substantial growth inhibition and morphological changes of all parasite stages. However, at a relatively low compound concentration of 1× IC_{50}, parasite growth was reduced only marginally, and after 24 h, chloroquine showed the strongest growth reduction effect followed by OZ277 and artemether. These observations are consistent with microscopic studies performed by Alin *et al.*[19] and Ye *et al.*[20], who reported that an artemisinin concentration of ~4 ng/mL for 48 h had no appreciable effect on the parasites. In related studies, Ter Kuile *et al.*[21] measured the antimalarial effects of artemisinin, artelinic acid and other compounds by inhibition of incorporation of [^{3}H]hypoxanthine as an indicator of nucleic acid synthesis, [^{3}H]isoleucine as an indicator of protein synthesis and lactate production as an indicator of parasite glycolysis. These authors stated that the trophozoite and schizont stages were considerably more sensitive to artemisinin and

Table 1 Selective accumulation of [^{14}C]OZ277[L] by uninfected RBCs and by RBCs infected with *Plasmodium falciparum* strain NF54

	Total parasitaemia (%)	Ring (%)	Troph (%)	Schizont (%)	R/P 10 min	R/P 20 min	R/P 30 min	R/P 1 h	R/P 2 h	R/P 3 h
Uninfected RBCs Exp. 1					1.1	1.0	1.5	2.1	1.3	1.8
Uninfected RBCs Exp. 2					1.6	1.5	1.6	2.2	1.6	1.2
Mean of all 12 time points							1.5			
Infected RBCs Exp. 1	3.8	0.1	0.5	3.2	80	124	270	165	91	95
Infected RBCs Exp. 2	4.8	0.2	1.4	3.2	87	162	157	173	108	77
Mean of each time point					84	143	214	169	100	86
Infected RBCs Exp. 3	8.3	6.8	1.5	–	19	22	44	47	64	17
Infected RBCs Exp. 4	11.3	10.8	0.5	–	19	21	31	39	41	32
Mean of each time point					19	22	38	43	53	25

The initial concentration of the drug was 77 ng/mL. R/P is the uptake ratio obtained from the disintegrations per minute (dpm) in 230 µL of pure parasitized RBCs (correction made for 100% parasitaemia) divided by the dpm in 230 µL of plasma from the same tube.

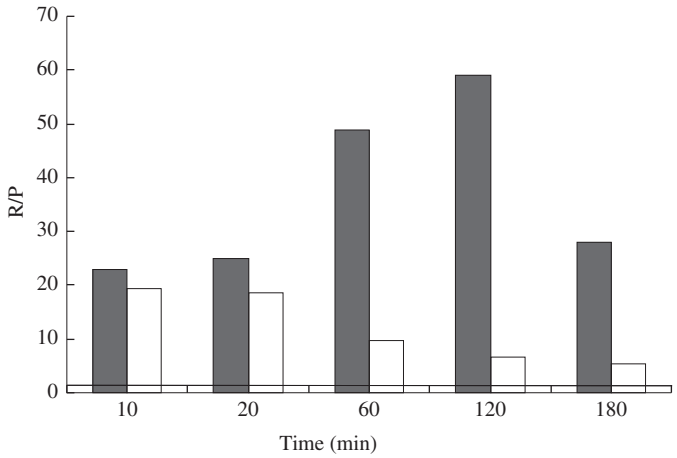

Figure 3. Time-course of uptake of [^{14}C]OZ277[L] (black bars) and [^{14}C]OZ277[R] (white bars) from 10 min to 3 h by *P. falciparum* strain NF54 (ring stage). The OZ277 concentration was 77 ng/mL. One representative experiment is shown for each compound. The horizontal line indicates the drug accumulation in uninfected RBCs (R/P = 1.5).

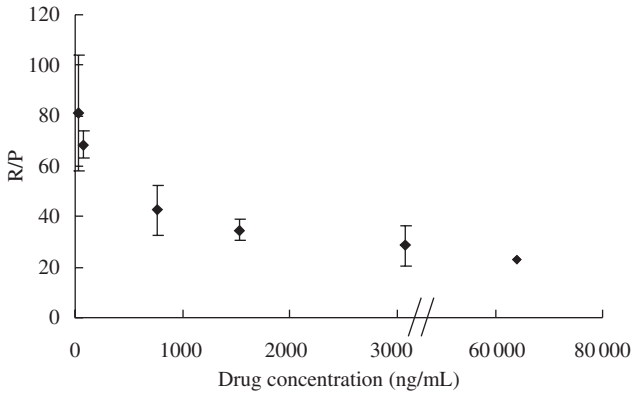

Figure 4. Effect of initial external concentration of [^{14}C]OZ277[L] on the distribution between RBCs infected with *P. falciparum* strain NF54 and plasma (R/P). Data points represent the mean of $n = 3$ experiments (±SE) or the mean of $n = 2$ with individual values differing by <10%. Each experiment was performed with unsynchronized parasite cultures incubated for 1 h in the presence of radiolabelled compound.

artelinic acid than were the young ring stages. They also reported that the inhibition was dose-dependent. Our results with artemether at the 1 h time-point (1× up to 100× IC_{50}) (Figure 2) are in good agreement with these reports. Studies performed by Geary *et al.*[22] using the [^{3}H]hypoxanthine assay also showed a similar stage specificity for artemisinin. This is encouraging, especially since a direct comparison of our results with those of Ter Kuile *et al.*[21] is difficult, because of the different methodologies used in the two studies. Ter Kuile *et al.*[21] used a higher parasitaemia of 0.4–0.8% and a two cycle as well as a same cycle experiment, factors that could have altered parasite growth, and thereby efficacy profiles. Our data are also consistent with data reported by Skinner *et al.*[23] and Alin *et al.*[19] Skinner *et al.* showed that an IC_{90} concentration of dihydroartemisinin (DHA) is rapidly effective against all three stages of the parasite. In these experiments, a [^{3}H]hypoxanthine assay was used to show that young trophozoites (20–24 h) and young schizonts (36 h) achieve ~95% growth inhibition after 2 h of incubation. They also observed ring forms to be more resistant, achieving ~95% growth inhibition only after 6 h. Surprisingly, in this same work, the group demonstrated that artemether and artemisinin, unlike DHA, showed relatively little activity against trophozoites, while a more DHA-like effect was found against the other two forms. The authors explained the discrepancy of their trophozoite result with the relatively low compound concentration (IC_{90}) used in their experiments. Alin *et al.*[19] performed microscopic counting experiments with unsynchronized parasites that had been incubated for 1 h with an artemisinin concentration range of 40–4000 ng/mL. They reported that 48 h after removal of the compound, the predominant parasite stage that remained viable in culture was rings. In contrast, after ≥6 h in the presence of compound, the remaining viable stages were evenly distributed, suggesting that ≤6 h is enough to affect all stages to the same extent.

In terms of chloroquine, our stage dependency data were in good agreement with two studies using similar techniques.[21,24] Both groups reported that trophozoite and schizont stages were considerably more sensitive to the compound. Pyrimethamine is the only compound that was found to be strongly stage specific for young schizonts (Figure 2). However, this observation was

not unexpected, since similar data have been published previously by Dieckmann and Jung.[25] These authors showed that ring and trophozoite stages exposed to 10 nM (= ~2.5 ng/mL) pyrimethamine for 6 h grew normally after removal of the compound, which is consistent with our observations for these parasite stages. Of particular interest is that ring forms and trophozoites were resistant to the compound up to the extreme conditions tested (24 h at 500 ng/mL or 100× IC_{50}). Young schizonts, which had been exposed to 100× IC_{50} concentrations of pyrimethamine for 12 h, still grew to about 60%. However, a 24 h incubation period resulted in quite strong growth inhibition, even at relatively low pyrimethamine concentrations. Therefore, a long pyrimethamine exposure time seems to be more critical for schizont growth than a high compound concentration. This, and the pronounced stage specificity for schizonts, distinguishes pyrimethamine from the faster acting compounds OZ277, artemether and chloroquine.

A feature of the specific activity of artemether and chloroquine is assumed to be their enrichment in malaria-infected RBCs. Vyas *et al.*[18] as well as Gu *et al.*[16] found that [^{14}C]artemisinin and [^{3}H]DHA partition into *P. falciparum*-infected RBCs and are accumulated up to 300-fold. This degree of concentration also resembles that of chloroquine.[15,17] We have obtained similar results with [^{14}C]OZ277 (Table 1), which suggest that the accumulation of OZ277 by malaria-infected RBCs could be an important aspect of the selective toxicity of this compound. Preliminary studies have also suggested similar high accumulation of OZ277 in *P. berghei*-infected erythrocytes (S. A. Charman, unpublished data).

Time-course experiments performed with OZ277 radiolabelled either in the adamantane ring (OZ277[L]) or in the right side chain (OZ277[R]) showed that after 10 min, young ring forms take up comparable amounts of both molecules (Figure 3). However, after 10 min the concentration of the [R] molecules in the infected RBCs decreased steadily, whereas the [L] molecules accumulated continuously, reaching their maximum after 2 h. This data supports the mechanism of action of OZ277 as proposed by Vennerstrom *et al.*[10] in that carbon-centred radicals form predominantly on the adamantane ring. Ultimately, such adamantane radicals could alkylate parasite proteins, releasing the remaining unlabelled cleavage product for partitioning back into the supernatant. The fate of such products is unknown and will be further examined in subsequent experiments designed to assess the partitioning behaviour in a more comprehensive manner. Further studies to explore these processes will utilize specific analytical methods in order to characterize the nature of the species present within the erythrocyte and the supernatant.

Similar to DHA and chloroquine,[16,26] the maximum uptake of OZ277 into infected RBCs was reached faster with mature parasites (~30 min) than with young ring stages (~2 h) (Table 1). This result corresponds to our pharmacodynamic observations where after a 1 h compound exposure, OZ277 and artemether were more effective against more mature parasite stages (Figure 2). However, after having reached the maximum uptake, it can only be speculated as to why a steady-state phase was not observed with [^{14}C]OZ277[L] and [^{14}C]OZ277[R] (Figure 3). The relatively fast decrease in the R/P value observed with both radioactive OZ277 variants might be an indication of how rapidly the ring forms lose their viability, which in this specific experiment would be after the 2 h time-point. This observation is clearly different from the results reported for [^{3}H]DHA,[16] where using a comparable drug concentration, a stable R/P equilibrium could be observed for ~20 h.

In summary, the present study describes a significant accumulation of radiolabelled OZ277, a novel peroxide-based compound, in *P. falciparum*-infected RBCs. Similar stage-specific profiles were found for OZ277, artemether and chloroquine. The latter drugs are known to prevent sequestration in malaria patients significantly by attenuating the growth of young, asexual parasite forms.[14] Since in onset and recrudescence experiments using the *P. berghei* murine model, OZ277 has been shown to clear parasitaemia rapidly to below quantifiable limits,[10] the hopes are high that OZ277 will also show similar *in vivo* pharmacodynamics in malaria patients. Studies of this nature provide further understanding of the relationship between drug concentration and antimalarial effect which may ultimately help to optimize the assessment of antimalarial efficacy and the design of treatment regimens for malaria. Further research into the mechanisms responsible for uptake of OZ277 (and other antimalarials) into infected erythrocytes will be valuable in further defining its pharmacodynamic activity.

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Transparency declarations

None to declare.

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