Rapid kill of malaria parasites by artemisinin and semi-synthetic endoperoxides involves ROS-dependent depolarization of the membrane potential

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Objectives: Artemisinin and artemisinin semi-synthetic derivatives (collectively known as endoperoxides) are first-line antimalarials for the treatment of uncomplicated and severe malaria. Endoperoxides display very fast killing rates and are generally recalcitrant to parasite resistance development. These key pharmacodynamic features are a result of a complex mechanism of action, the details of which lack consensus. Here, we report on the primary physiological events leading to parasite death.

Methods: Parasite mitochondrial (∆Ψₘ) and plasma membrane (∆Ψₚ) electrochemical potentials were measured using real-time single-cell imaging following exposure to pharmacologically relevant concentrations of endoperoxides (artemisinin, dihydroartemisinin, artesunate and the synthetic tetraoxane RKA182). In addition, mitochondrial electron transport chain components NADH:quinone oxidoreductase (alternative complex I), bc₁ (complex III) and cytochrome oxidase (complex IV) were investigated to determine their functional sensitivity to the various endoperoxides.

Results: Parasite exposure to endoperoxides resulted in rapid depolarization of parasite ∆Ψₘ and ∆Ψₚ. The rate of depolarization was decreased in the presence of a reactive oxygen species (ROS) scavenger and Fe³⁺ chelators. Depolarization of ∆Ψₘ by endoperoxides is not believed to be through the inhibition of mitochondrial electron transport chain components, owing to the lack of significant inhibition when assayed directly.

Conclusions: The depolarization of ∆Ψₘ and ∆Ψₚ is shown to be mediated via the generation of ROS that are initiated by iron bioactivation of endoperoxides and/or catalysed by iron-dependent oxidative stress. These data are discussed in the context of current hypotheses concerning the mode of action of endoperoxides.

Keywords: Plasmodium, mitochondria, iron, haem, lipid peroxidation, free radicals, oxidative damage

Introduction

Artemisinin is a tetracyclic 1,2,4-trioxane containing an endoperoxide bridge (C–O–O–C; Figure 1), the key pharmacophore of the drug.¹ To improve the solubility and pharmacological activity of artemisinin, a first series of semi-synthetic compounds were synthesized with a similar backbone but with modifications at the C₁₀ position, generating hemi-acetal, ether or ester derivatives such as dihydroartemisinin, arteether and the synthetic tetraxane RKA182 (Figure 1). Artemisinins possess potent antimalarial activity and the WHO recommends the use of artemisinin combination therapy for first-line therapy of Plasmodium falciparum malaria worldwide. Based on the structure of the endoperoxide bridge, extensive studies have been devoted to the synthesis of fully synthetic endoperoxides, some of which are currently in clinical and pre-clinical development (e.g. OZ439, an ozonide designed to provide a single-dose oral antimalarial cure in humans, and the tetraxane RKA182; Figure 1).² ³

The mechanism(s) of activation and subsequent biological target(s) of endoperoxides continue to be debated.⁴ The antimalarial activity of the artemisinins and related bioactive endoperoxides is believed to be mediated by activation of the endoperoxide bridge. Located in the core of the structure, its cleavage generates short-lived cytotoxic oxyradicals in the presence of haem iron or free iron Fe²⁺.⁵ ⁶ From this premise of ‘endoperoxide bioactivation’, two different mechanisms have been proposed. The first, proposed by the Posner laboratory using ¹⁸O-labelled trioxane analogues, hypothesizes that the oxygen-centred radicals produced are rearranged to more stable carbon-centred radicals.⁷ ⁸ In this reductive
An alternative pathway of artemisinin bioactivation has been suggested via electron transport chain (ETC) components causing oxidative membrane damage.27,28 By using radio- or fluorescent-labelled artemisinins and microscopy, two studies contest the accumulation of endoperoxide drugs in the food vacuole and their possible interaction with haem.29,30 Additionally, Haynes et al.31,32 have proposed that artemisinins do not inhibit haemoglobin formation and cannot react with haem according to conventional chemistry models. Several studies have shown that iron chelation, selective for non-haem iron, antagonizes the accumulation and antimalarial activity of endoperoxide antimalarials in vitro and can prevent their toxic effects in mice.5,30,33

Once activated, endoperoxide antimalarials have been reported to disrupt a number of parasite functions and enzymes, including the haem detoxification pathway,33 the translationally controlled tumour protein (PTCTP),34 the sarco/endoplasmic reticulum membrane calcium P-type ATPase6 and the parasite mitochondrion. Specific targeting of parasite mitochondria by endoperoxides was reported initially based on morphological changes to mitochondria following exposure to artemether.35,36,37 Zhao et al.37 reported a specific inhibitory effect by artemether against Plasmodium cytochrome c oxidase (complex IV) that was later also reported by Krungkrai et al.38 based on measurements of O2 consumption. Li et al.39 hypothesized a role for parasite mitochondria, specifically the type II NADH:quinone oxidoreductase (PfNDH2),39,40 by acting
both as a target and as an activator of endoperoxides via electron donation by the ETC. Conflicting data have since emerged, with Crespo et al., only observing mitochondrial dysfunction (as determined by rhodamine 123 fluorescence) upon exposure to artemisinin after 8 h but not after 4 h, interpreting this as a downstream effect rather than an initiator of killing. Conversely, Wang et al. reported depolarization of the membrane potential of isolated parasite mitochondria by artemisinin following 2 h of incubation.

Studies of artemisinin toxicity in human cells indicate that respiring mitochondria play an essential role in endoperoxide-induced cytotoxicity via the generation of ROS; however, using HeLa \(\rho^0\) cells, which are devoid of a functioning ETC, it was demonstrated that the ETC does not have any role in the reductive activation of the endoperoxide to cytoxic carbon-centred radicals.

We have previously described the use of a real-time single-cell imaging method for monitoring malaria parasite mitochondrial (\(\Delta \Psi_m\)) and plasma membrane potential (\(\Delta \Psi_p\)) and reported malaria parasite ETC activities including PfNDH2. Using these approaches, we have re-examined the effect of endoperoxides on parasite bioenergetic functions and discuss our data in the context of the current hypotheses.

**Materials and methods**

**Parasites, culture and drug sensitivity testing**

\(P. falciparum\) (3D7 strain) cultures consisted of a 2% (w/v) suspension of O+ erythrocytes in RPMI 1640 medium (R8758, glutamine and NaHCO\(_3\)) supplemented with 10% pooled human AB+ serum, 25 mM HEPES (pH 7.4) and 20 \(\mu\)M gentamicin sulphate. Cultures were grown under a gaseous headspace of (in v/v) 4% O\(_2\) and 3% CO\(_2\) in N\(_2\) at 37°C. Parasite growth was synchronized by treatment with sorbitol. Drug susceptibilities were determined with an inoculum size of 0.5% parasitaemia (ring stage) and 1% haematocrit and were assessed by the measurement of fluorescent fluorescence upon exposure to artemisinin following 2 h of incubation.

**Preparation of decylubiquinol**

Decylubiquinol was dissolved in 100 \(\mu\)L of 96% ethanol (acidified with 10 mM HCl) and stored in aliquots at \(-80^\circ\)C. Decylubiquinol concentration \(c\) was determined spectrophotometrically from absolute spectra, using \(\epsilon_{288-320} = 4.14 \text{mM}^{-1} \text{cm}^{-1}\).

**Measurement of bc\(_1\) protein and complex IV activities**

Decylubiquinol:cytochrome c oxidoreductase (bc\(_1\) protein) and cytochrome c oxidase (complex IV) activity were assayed in a Cary 4000 spectrophotometer (Varian Inc., USA). The bc\(_1\) reaction buffer consisted of 50 mM potassium phosphate (pH 7.5), 2 mM EDTA, 10 mM KCN (1 M stock solution, pH adjusted to 7.5) and 30 \(\mu\)M horse heart cytochrome c (oxidized) (Sigma). The complex IV mixture is composed of 50 mM potassium phosphate (pH 7.5), 2 mM EDTA and 5 mM antimycin. Inhibitors were added prior to the addition of substrate. The reaction volume was 700 \(\mu\)L and assays were performed at room temperature. \(P. falciparum\) bc\(_1\) and complex IV were assayed from cell-free \(P. falciparum\) extract at a total protein concentration of 30–60 \(\mu\)g/mL. Cytochrome c reductase (bc\(_1\)) and oxidase (complex IV) activities were initiated, respectively, by the addition of 50 \(\mu\)M decylubiquinol (dHQ2) and 30 \(\mu\)M horse heart cytochrome c (reduced). The horse heart cytochrome c was reduced by sodium dithionite and then passed through a PD-10 desalting column (Pharmacia, Piscataway, NJ, USA). Activities were measured by monitoring the cytochrome c (reduced) concentration at 550–542 nm (\(e_{340-342} = 18.1 \text{mM}^{-1} \text{cm}^{-1}\)).

**Measurement of PfNDH2 activity**

PfNDH2 enzyme activity was determined based on a modification of the NADH:quinone oxidoreductase assay previously described. Enzyme activity was measured in a buffered solution (final volume 0.7 mL) containing 50 mM potassium phosphate (pH 7.5), 2 mM EDTA, 10 mM KCN and 50 \(\mu\)M coenzyme Q\(_1\) \(Q_1\) at room temperature. Recombinant PfNDH2 enzyme was added as an Escherichia coli crude membrane preparation at a total protein concentration of 10 and 20 \(\mu\)g/mL. Inhibitors were added before initiation of the reaction by addition of 200 \(\mu\)M NADH. PfNDH2 activity was measured spectrophotometrically by monitoring the decrease of NADH concentration at 340 nm (\(e_{340} = 6.22 \text{mM}^{-1} \text{cm}^{-1}\)) and Q\(_1\) concentration at 283 nm (\(e_{283} = 8.1 \text{mM}^{-1} \text{cm}^{-1}\)).

**Real-time single-cell monitoring of membrane potential**

The rhodamine derivative tetramethyl rhodamine ethyl ester (TMRE) was used to monitor the membrane potential of the cytoplasm and mitochondria from malaria-infected red blood cells. TMRE is cationic and reversibly

**Table 1. Inhibitory profiles of endoperoxide compounds for three major components of the ETC of \(P. falciparum\) (3D7 strain)**

| Inhibitor | PfNDH2 \(\pm \text{SEM}\) | bc\(_1\) protein (complex III) \(\pm \text{SEM}\) | complex IV \(\pm \text{SEM}\) |
|-----------|---------------------|---------------------------------|---------------------|
| Artemisinin (1 \(\mu\)M) | 17.1 ± 4.2 | 3.2 ± 3.7 | 18.4 ± 4.3 |
| Tetraoxane (1 \(\mu\)M) | 12.3 ± 4.9 | 23.2 ± 4.2 | 19.1 ± 2.0 |
| Dihydroartemisinin (1 \(\mu\)M) | 5.0 ± 4.3 | 6.7 ± 4.1 | 21.1 ± 7.9 |
| Artesunate (1 \(\mu\)M) | 3.7 ± 2.9 | 2.3 ± 0.4 | 21.1 ± 3.9 |
| Artemether (1 \(\mu\)M) | 4.1 ± 3.3 | 1.8 ± 1.9 | 23.0 ± 2.0 |
| HDQ (100 nM) | 88.1 ± 0.5 | ND | ND |
| Atovaquone (50 nM) | ND | 89.0 ± 1.0 | ND |
| Cyanide (15 mM) | ND | ND | 100.0 ± 0.0 |

Specific inhibitors of PfNDH2 [1-hydroxy-2-dodecyl-1(1H)quinolone; HDQ], bc\(_1\) protein (atovaquone) and complex IV (cyanide) were used as positive controls. Direct activity assays were performed on recombinant PfNDH2 enzymes, whereas bc\(_1\) protein and complex IV were assayed with cell-free parasite extracts.

Inhibitor concentrations used are indicated in brackets.

Values are means ± SEM; \(n\) = 3 independent experiments. ND, not determined.
Rapid ROS-dependent membrane depolarization by endoperoxides

Endoperoxides have a minimal inhibitory effect on the major mitochondrial respiratory chain components

As described, there is conflicting evidence on the role of the parasite ETC in the activation and/or resultant mitochondrial dysfunction following the exposure of parasites to endoperoxides. To determine whether there is a direct inhibitory effect of endoperoxides on ETC components, activities from three of the main ETC enzymes, PfNDH2, bc1 complex, and cytochrome c oxidase, were measured directly in the presence of a number of endoperoxides. As described in the Materials and methods section, the bc1 complex (complex III) and cytochrome c oxidase (complex IV) were measured directly from parasite cell-free extracts, whilst PfNDH2 activity was measured from membrane preparations of a heterologous expression system described previously. No or relatively weak (~20%) inhibition of the individual respiratory components was observed for all the endoperoxides tested at comparatively high fixed doses (1 μM final concentration; Table 1). Positive controls using selective inhibitors of the individual respiratory components were consistent with previously reported inhibitory values (Table 1).

Endoperoxides collapse membrane potential-dependent accumulation of TMRE in P. falciparum-infected erythrocytes

To determine the effect of endoperoxides on membrane potential, a real-time single-cell imaging approach was used. The measurement is based on the accumulation of the cationic fluorescence probe TMRE according to the Nernst equation. Due to dynamic fluorescence measurements, the probe is subject to photobleaching. To minimize this, several parameters of the confocal laser scanning microscope (laser power, scan speed, pinhole diameter, number of scan sweeps and degree of magnification) were optimized before recording each experiment. Upon addition of TMRE to P. falciparum-infected erythrocytes, a strong fluorescence signal was observed from the whole cytosol (except the food vacuole), corresponding to the addition of plasma and mitochondrial membrane potential. For all assays, the fluorescence dynamic range was set up so that untreated TMRE-loaded cells were regarded as having complete fluorescence (100%), whereas the baseline (0%) was set by the addition of 10 μM H+ ionophore carbonyl cyanide p-(trifluoromethyl)phenylhydrazone (FCCP).

The endoperoxide compounds used in this study were shown to inhibit parasite growth in the low nanomolar range, consistent with previous studies (Figure 1). Addition of endoperoxides to trophozoite-stage parasites (100 nM) resulted in a 55%–60% reduction of total membrane potential-dependent fluorescence within <3 min (Figure 2c–f). Using the same conditions, atovaquone, the selective bc1 complex inhibitor, decreased the total membrane potential-dependent fluorescence by 30% (Figure 2a), consistent with previous observations of the mitochondrial contribution to fluorescence. In contrast, addition of deoxyartemisinin, which lacks the endoperoxide bridge (Figure 1), resulted in the minimal loss of membrane potential-dependent fluorescence, consistent with its poor antimalarial activity (Figure 2b).

Although these experiments demonstrate for the first time the rapid depolarization of membrane potential upon exposure to endoperoxides, since there is a contribution from both plasma (ΔΨp) and mitochondrial membrane (ΔΨm) potentials to TMRE accumulation, further experiments were performed to measure ΔΨp and ΔΨm independently. Depolarization of the mitochondrial membrane potential by endoperoxides

To evaluate the impact of endoperoxides on ΔΨm, cells were pre-treated with concanamycin A, a V-type H+-ATPase inhibitor. Upon addition of concanamycin A (200 nM), the fluorescence intensity from the cytosol decreases ~70%–80%, leaving a local and strong signal originating from the parasite mitochondrion, as demonstrated in Figure 3(b). To measure ΔΨm-dependent fluorescence, concanamycin A-treated parasites were normalized to 100% and the baseline (0%) was set by FCCP addition (10 μM). Atovaquone addition rapidly (~3 min) reduced concanamycin-independent TMRE fluorescence by 70% (Figures 3 and 4a). Similarly, both artesunate and tetrathione decreased the concanamycin-independent TMRE fluorescence by 60% and 50%, respectively (Figures 3 and 4b and c).

Depolarization of the plasma membrane potential by endoperoxides

The ΔΨm was demonstrated to represent between 20% and 30% of the total cellular TMRE fluorescence, leaving a homogeneous cytosolic signal originating from the plasma membrane only. To evaluate the ΔΨp-dependent fluorescence, parasites were pre-treated with 100 nM atovaquone for 5 min before addition of the endoperoxide inhibitor. For these experiments, atovaquone-treated parasites were normalized to 100% and the baseline (0%) was set by

Figure 2. Effect of endoperoxide antimalarials on both the plasma and mitochondrial membrane potential of P. falciparum. Time course of TMRE-dependent fluorescence of P. falciparum-infected erythrocytes after the addition of (a) 100 nM atovaquone, (b) 100 nM deoxyartemisinin, (c) 100 nM artesunate, (d) 100 nM tetrathione (compound RKA182), (e) 100 nM atovaquone and (f) 100 nM dihydroartemisinin. The data were normalized to 100% in untreated cells and to 0% in FCCP (10 μM)-treated cells. Graphs show means from experiments performed independently ± standard errors (n ≥ 7).
FCCP addition (10 μM). The vacuolar H^+-ATPase is involved in transforming the energy of ATP hydrolysis to generate the electrochemical potential at the surface of the malaria parasite through the transport of H^+ across the plasma membrane. As expected, the addition of concanamycin A, a well-known V-type ATPase inhibitor, rapidly decreased DC_p-dependent fluorescence by 60% (Figure 5a). In a similar manner, artemisinin and tetraoxane were observed to decrease the atovaquone-independent fluorescence signal by 40% and 60%, respectively (Figure 5b and c).

The described data confirm that both DC_m and DC_p are rapidly depolarized upon exposure to physiological concentrations of endoperoxides. The next set of experiments was performed to ascertain whether the rapid depolarization would be affected by chelation of free Fe^{3+}, as previously hypothesized by other studies.

**Effect of Fe^{3+} chelators desferrioxamine (DFO) and deferipone (DFP) on the membrane potential depolarization by artemisinin and tetraoxane**

DFO and DFP, two chelating agents selective for non-haem Fe^{3+}, were used at a fixed dose to determine the effect of free iron on the rapid endoperoxide-induced, parasite total (ΔΨ_m and ΔΨ_p) membrane potential depolarization.

Growth inhibition studies (48 h IC_{50}) using both iron chelators revealed moderate antimalarial activity against 3D7 P. falciparum of 17.3 ± 2 μM (DFO) and 111.8 ± 2 μM (DFP). However, during the short time period of the single-cell assays (<6 min), the addition of 100 μM DFO or 100 μM DFP to the perfusate did not result in a decrease of parasite ΔΨ_m and ΔΨ_p-dependent fluorescence signal. However, addition of DFO and DFP (100 μM) to the perfusate was observed to significantly minimize the depolarization caused by either artemisinin (100 nM; Figure 6a) or tetraoxane (100 nM; Figure 6b), relative to chelator-free controls. For both endoperoxides, DFO was observed to confer greater protection than DFP.

Next, it was investigated whether ROS scavengers would also confer a protective effect against endoperoxides as postulated in previous studies.

**Effect of the superoxide scavenger Tiron on the membrane potential depolarization induced by artemisinin and tetraoxane**

Tiron, a cell membrane-permeable superoxide scavenger, was added (100 μM) to the perfusate and endoperoxide-induced ΔΨ_m and ΔΨ_p depolarization was measured as described previously. Tiron was clearly observed to have a significant protective effect against endoperoxide-induced membrane potential depolarization, decreasing the rate of depolarization by >50% for both artemisinin and tetraoxane (Figure 7). In control experiments, Tiron alone had no
Discussion

ETC components are not a direct target for endoperoxides

Specific inhibition by endoperoxides has previously been reported for *Plasmodium* cytochrome c oxidase (complex IV) \(^{37,38}\) and type II NADH:quinone oxidoreductase (*PfNDH2*). \(^ {16}\) In agreement with previous studies, we also noted inhibition of complex IV but only to a maximum of 20% at relatively high concentrations (1 \(\mu\)M) of a range of endoperoxides (Table 1). Little or no inhibition was observed, however, for either recombinant *PfNDH2* or *bc\(_1\)* (Table 1). It should be noted that all enzymatic assays were performed using either cell-free extracts or *E. coli* membrane preparations and would be expected to have contained trace Fe\(^{2+}\) and, for the parasite extract, haem. These data indicate that ETC components are not direct targets for endoperoxide inhibition in *Plasmodium*. Previous studies reporting inhibition of *PfNDH2* by endoperoxides \(^ {16}\) did not measure the enzyme directly, but rather showed increased sensitivity to endoperoxides in yeast transfected with *PfNDH2*. It is likely, therefore, that *PfNDH2* in the yeast system increased sensitivity to endoperoxides via an indirect effect, possibly by serving as a source of ROS by electron transfer from the *PfNDH2* flavin to O\(_2\) or potentially directly to the endoperoxides.

Rapid loss of parasite vitality upon exposure to endoperoxides

In unicellular organisms, life can be defined as the maintenance of an electrochemical potential across the organism’s outer membrane. The potential (\(\Delta\Psi_m\)) is generated by the activity of the electron transport chain (ETC) and is critical for maintaining the integrity of the parasite's plasma membrane (PM) and for its metabolism. The ETC, which is composed of a series of redox proteins, converts the energy of electrons extracted from food molecules into a membrane potential across the plasma membrane. This potential drives the production of ATP, a key energy currency for the cell, and is also crucial for maintaining intracellular pH, ion gradients, and the integrity of the plasma membrane.

The activity of the ETC is regulated by a variety of factors, including the availability of oxygen and the presence of oxygen-damaging agents such as endoperoxides. Endoperoxides are compounds that are produced by the body as a defense mechanism against pathogens and are known to be cytotoxic. They act by forming free radicals, which can cause oxidative stress and disrupt cellular functions.

The effect of endoperoxides on the ETC components of *Plasmodium* was studied in this research. The authors observed that endoperoxides had little or no direct inhibitory effect on the ETC components. Instead, they noted a rapid ROS-dependent membrane depolarization upon exposure to endoperoxides. This was evidenced by the loss of mitochondrial membrane potential (TMRE fluorescence) and plasma membrane potential (TMRE-concanamycin fluorescence) (Figure 4).

Figure 4. Effect of artemisinin and tetraoxane on mitochondrial membrane potential only. Plasma membrane potential is depolarized by treatment of *P. falciparum*-infected erythrocytes with 200 nM concanamycin A before addition of inhibitors. Time course of TMRE-plasma independent fluorescence is followed after addition of (a) 10 nM atovaquone, (b) 100 nM artemisinin and (c) 100 nM tetraoxane. Data were normalized to 100% in untreated cells and to 0% in FCCP (10 \(\mu\)M)-treated cells. Graphs show means from experiments performed independently ± standard errors (n ≥ 7).

Rapid ROS-dependent membrane depolarization by endoperoxides

In unicellular organisms, life can be defined as the maintenance of an electrochemical potential across the organism's outer membrane. This potential is critical for the cell's viability and is generated by the electron transport chain (ETC). The ETC is a series of redox proteins that convert the energy of electrons extracted from food molecules into a membrane potential across the plasma membrane. This potential drives the production of ATP, a key energy currency for the cell, and is also crucial for maintaining intracellular pH, ion gradients, and the integrity of the plasma membrane.

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membrane (negative entropy).\textsuperscript{53} Plasmodia have a proton potential across the plasma membrane ($\Delta V_p$) of $\approx$ 95 mV.\textsuperscript{51,54} It is generated by V-type H\textsuperscript{+}-ATPases, which transfer protons out of the parasite cytosol.\textsuperscript{55,56} Localized in the plasma membrane, the V-type H\textsuperscript{+}-ATPase also plays a role in regulating a neutral cytosolic pH (near 7.3), crucial for enzyme function, incorporation of vitamin B5 and generation of a pH gradient across membranes of internal organelles.\textsuperscript{56,57} The maintenance of a $\Delta V_p$ is also important in mediating the influx of K\textsuperscript{+} in the parasite cytosol and the uptake of nutrients such as choline.\textsuperscript{54,58,59} The direct inhibition of the V-type H\textsuperscript{+}-ATPase by specific inhibitors such as bafilomycin A\textsubscript{1} or concanamycin A has been demonstrated to depolarize the plasma membrane potential and disrupt the physiology of the parasite, leading to its death.\textsuperscript{53,55,57} The parasite mitochondrial membrane potential ($\Delta V_m$) is generated via the ETC, through the activity of the bc\textsubscript{1} complex and cytochrome c oxidase.\textsuperscript{53,60}

An essential function of the mitochondrion for parasite survival during the intraerythrocytic stages is the provision of orotate for pyrimidine biosynthesis through the activity of dihydroorotate dehydrogenase.\textsuperscript{51,62}

Exposure of *P. falciparum*-infected erythrocytes to pharmacologically relevant concentrations of endoperoxides resulted in a rapid loss of membrane potential-dependent accumulation of TMRE (Figure 2). On account of the comparatively high contribution by the $\Delta V_p$ (relative to mammalian cells), it was not possible from these experiments to distinguish whether the depolarization was on account of an effect on the $\Delta V_p$ alone and/or the $\Delta V_m$. Inhibition of the parasite V-type H\textsuperscript{+}-ATPase by addition of concanamycin A to the perfusate ‘unmasked’ the parasite to reveal the $\Delta V_m$-dependent accumulation of TMRE (Figure 3). With this manipulation, it was possible to determine that for all the endoperoxide classes tested, $\Delta V_m$ was rapidly depolarized (Figure 4).

**Figure 5.** Effect of artemisinin and tetraoxane on plasma membrane potential only. Mitochondrial membrane potential is depolarized by treatment of *P. falciparum*-infected erythrocytes with 100 nM atovaquone before addition of inhibitors. Time course of TMRE-mitochondrial independent fluorescence is followed after addition of (a) 200 nM concanamycin A, (b) 100 nM artemisinin and (c) 100 nM tetraoxane. Data were normalized to 100% in untreated cells and to 0% in FCCP (10 $\mu$M)-treated cells. Graphs show means from experiments performed independently $\pm$ standard errors ($n \geq 7$).
data are consistent with the findings by Wang et al., who reported depolarization of the membrane potential of isolated parasite mitochondria by artemisinin following 2 h of incubation. Crespo et al. reported mitochondrial dysfunction following exposure to artemisinin only after 8 h but not after 4 h, interpreting this as a downstream effect. We note, however, that the Crespo et al. study did not distinguish between DCp-o or DCm-dependent rhodamine accumulation and that a washing step occurred in between rhodamine staining and microscopy. As rhodamine is a cationic fluorophore, there is a balance between the concentration gradient of the probe and the total \( \Delta \Psi \) according to the Nernst equation: \( \Psi = RT/F \ln(P_{in}/P_{out}) \), where \( R, T, F, D_{in} \), and \( D_{out} \) represent the universal gas constant, the absolute temperature, the Faraday constant and the intra- and extracellular probe concentrations, respectively. The washing step would therefore affect the distribution of the probe in a time-dependent manner and may account for the

![Figure 6](image1.png)

**Figure 6.** Effect of artemisinin and tetraoxane on the membrane potential in the presence of iron chelators. Time course of TMRE-dependent fluorescence of *P. falciparum*-infected erythrocytes after addition of (a) 100 nM artemisinin and (b) 100 nM tetraoxane. Cells are not treated (open circles) or subjected to iron chelator treatment with 100 \( \mu \)M DFO (filled circles) or 100 \( \mu \)M DFP (filled diamonds). Data were normalized to 100% in untreated cells and to 0% in FCCP (10 \( \mu \)M)-treated cells. Graphs show means from experiments performed independently \( \pm \) standard errors (\( n \geq 7 \)).

![Figure 7](image2.png)

**Figure 7.** Effect of artemisinin and tetraoxane on the membrane potential in the presence of ROS scavenger. Time course of TMRE-dependent fluorescence of *P. falciparum*-infected erythrocytes after addition of (a) 100 nM artemisinin and (b) 100 nM tetraoxane. Cells are not treated (open circles) or subjected to ROS scavenger treatment with 100 \( \mu \)M Tiron (filled circles). Data were normalized to 100% in untreated cells and to 0% in FCCP (10 \( \mu \)M)-treated cells. Graphs show means from experiments performed independently \( \pm \) standard errors (\( n \geq 7 \)).
discord between the data of Crespo et al. and those presented here and by Wang et al. Pretreatment of parasites with atovaquone in the perfusate further allowed the measurement of $\Delta \Psi_p$ alone. Exposure to endoperoxides resulted in the rapid depolarization of parasite $\Delta \Psi_p$ (Figure 5). The rapid onset of $\Delta \Psi_p$ depolarization exposed to pharmacologically relevant concentrations of endoperoxides indicates that this is a primary pharmacodynamic event leading to parasite death and is consistent with in vitro and in vivo studies reporting the rapid killing rate of the endoperoxide class. The rapid onset of $\Delta \Psi_p$ depolarization by endoperoxides is also consistent with studies demonstrating that short pulses of artemisinins (1–6 h) are sufficient for parasite kill, albeit with stage-dependent differences. It is also noteworthy that disruption of the parasite transmembrane pH gradient via inhibition of the V-type ATPase has been reported to drop the cytosolic pH by 0.4 pH units in <3 min and 0.5–0.6 units within 20 min, leading to the inhibition of parasite growth within 30 min to 4 h (depending on inhibitor concentration).

Deoxyartemisinin did not have any depolarizing effect on either parasite $\Delta \Psi_p$ or $\Delta \Psi_m$ (Figure 2). This result is in line with previous studies and confirms the importance of the endoperoxide bond in mediating antimalarial activity.

**Rapid depolarization of the parasite $\Delta \Psi_p$ involves iron and ROS**

The parasite’s unique ability to digest haemoglobin is generally accepted to confer selective toxicity to endoperoxides (e.g. Klonis et al.) either directly, by activation of the endoperoxides by Fe$^{3+}$ and/or haem, or indirectly through the ability of Fe$^{3+}$ to oxidize cytosolic (reduced) cofactors. In this study, we used DFO and DFP, two known chelators of Fe$^{3+}$, to determine the effect on endoperoxide-induced $\Delta \Psi_p$ depolarization. Addition of the chelators to the perfusate was clearly observed to confer a protective effect to the parasite upon exposure to the endoperoxides (Figure 6). These data can be interpreted to suggest that Fe$^{3+}$ is involved in the depolarization of the parasite membrane. The iron-mediated hypothesis of endoperoxide activation identifies a role for Fe$^{3+}$ as the chelators have a much higher affinity for Fe$^{3+}$ compared with Fe$^{2+}$, such a mechanism would necessitate the additional presence of redox cycling activities, such as thiols (GSH $\leftrightarrow$ GSSG (reduced and oxidized glutathione, respectively)) and/or flavin enzymes. In previous experiments performed in situ using single-cell imaging, we observed that endoperoxide–acidine adducts could be completely washed out in the presence of DFO, but remained irreversibly bound in the absence of DFO, suggesting that the drugs were being activated by iron to form stable covalent adducts. It is therefore possible that the protective effect of the iron chelators observed in our studies here is a result of a decrease in the bioactivation of the endoperoxides. An alternative or additional explanation is that the iron chelators conferred a protective effect by decreasing the generation of ROS through Fe$^{3+}$-mediated oxidative stress such as the Fenton reaction, generating the highly membrane damaging hydroxyl radical (OH). It is also noteworthy that DFO has been reported to be able to directly scavenge ROS. It should also be noted that in the experiments presented here, intracellular haem concentrations cannot be selectively and rapidly manipulated; therefore, the potential role of haem in the depolarization of $\Delta \Psi_p$ by endoperoxides cannot be elucidated.

Although the exact protective mechanism for iron cannot be deduced from our data, a role for ROS is clearly demonstrated in experiments using Tiron, the superoxide anion ($O_2^·$) scavenger, showing a decreased rate of membrane depolarization following exposure to endoperoxides (Figure 7). The indiscriminate nature of the depolarization of both $\Delta \Psi_p$ or $\Delta \Psi_m$ is suggestive of a mechanism involving the general lipid peroxidation of the parasite membranes; however, in addition to this, a specific inhibition of key enzymes such as the V-type ATPase by ROS cannot be ruled out.

**Conclusions**

In summary, this study reports that rapid loss of $\Delta \Psi_p$ following exposure to endoperoxides is a primary physiological event leading to parasite death. A loss of $\Delta \Psi_p$ is also reported, but the lack of direct inhibition of ETC H$^+$-pumping complexes by endoperoxides and the indiscriminate nature of membrane depolarization is consistent with a non-ETC mode of action, as reported recently in mammalian cells. The rapid depolarization of $\Delta \Psi_p$ by endoperoxides involves ROS and iron, but it is not discernible from this study whether iron plays a role in endoperoxide bioactivation and/or iron-mediated oxidative stress.

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**Transparency declarations**

None to declare.

**Supplementary data**

Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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