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Sensitivity of artemisinin towards different stages of development of the malaria parasite, *Plasmodium falciparum*

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Abstract. Artemisinin-based combination therapies (ACTs) serve as a first line of defence against malaria infection. The success of malaria treatment depends closely on the timing of action and the target of this drug. The early inhibitory effect of artemisinin associated with the integrity and pH of the digestive vacuole (DV) of the malaria parasite was investigated. Using the malaria SYBR Green I based-fluorescence assay, artemisinin showed activity against the chloroquine-sensitive strains of 3D7 and D10 higher than that against the chloroquine-resistant strain of Dd2. A significant inhibition of parasite growth with marked changes in parasite morphology was seen following treatment with 0.2-60 times the IC_{50} value of artemisinin for a period of 4 hours was observed at mid ring, late trophozoite and late schizont stages. The drug had no obvious alterations in the distribution of a pH-sensitive probe, LysoSensor Blue labelling of the DV even at the highest concentration examined. Using a ratiometric pH probe, SNARF-1-dextran, the DV pH of treated trophozoites remained acidic, suggesting the loss of DV pH is probably not the mode of action of artemisinin that causes parasite killing.

INTRODUCTION

Malaria treatment currently relies on artemisinin-based combination therapies (ACTs) to kill *P. falciparum*, which caused about 429 million deaths in 2015 (World Health Organization, World Malaria Report, 2016). Artemisinin and its derivatives clear *P. falciparum* infections rapidly and are active against parasites resistant to quinoline and antifolate classes of antimalarial drugs (Dondorp *et al.*, 2010). There is no significant toxicity observed in the field (Rijken *et al.*, 2008), despite the fact that neurotoxicity (Nontprasert *et al.*, 2000) and embryo lethality or malfunction (Clark *et al.*, 2004) were demonstrated in animals treated with higher doses of artemisinin.

Despite their high efficacies, artemisinin and its derivatives have short half-lives *in vivo* (~1-4 hours) (Eastman & Fidock, 2009), which have an impact on their therapeutic profile and clinical use. Artemisinin is associated with a high risk of recrudescence when used for 3 days in monotherapy treatments and therefore has been used with a longer half-life drug to increase compliance and reduce treatment time (Nosten & White, 2007). Therefore, greater understanding of the mechanism of action of artemisinin will aid the rational design of more potent analogues (Muraleedharan & Avery, 2009) and allow the prediction of potential resistance mechanism (Dondorp *et al.*, 2009).

The precise mechanism of action of artemisinin is however still debated. The activation of its endoperoxide pharmacophore, which generates radical intermediates that react with susceptible parasite targets, has been proposed as the initial key step in the mechanism of action of artemisinin (O'Neill *et al.*, 2010). Previous

studies suggested the catalytic role of hemoglobin-derived heme (Meunier & Robert, 2010) or ferrous iron (Abu Bakar, 2016; Haynes *et al.*, 2007), suggesting the parasite ingestion and digestion of hemoglobin are necessary for artemisinin activity. Klonis *et al.* (2011) showed the timing of action of artemisinin coincides with the onset of hemoglobin ingestion during the late ring or early trophozoite stage. The digestive vacuole (DV), which is the major site of hemoglobin digestion, has been implicated as a potential target of artemisinin (del pilar Crespo *et al.*, 2008; Krishna *et al.*, 2004). Other studies reported the iron-independent activation of artemisinin (Haynes *et al.*, 2010) by directly inhibiting the parasite calcium ATPase (*Pf*ATP6) (Krishna *et al.*, 2010). Components of the mitochondrial electron transport chain of *Saccharomyces cerevisiae* had also been shown to be susceptible to artemisinin (Li *et al.*, 2005).

In this study, we sought to investigate the timing of action of artemisinin by determining the inhibitory effect of this drug on intraerythrocytic development of the malaria parasite. *In vitro* fluorescence-based assays were performed using a nucleic acid intercalating dye, SYBR Green I that detects the presence of malarial DNA in infected erythrocytes as a measure of parasite growth and inhibition by the drug. High molecular weight dextran-linked fluorescent markers in resealed erythrocytes were employed that permitted simultaneously analysis of the effects of artemisinin on hemoglobin uptake (TMR-dextran) and pH of endocytic structures and DV (SNARF-1-dextran or LysoSensor Blue DND-192).

MATERIALS AND METHODS

Materials

Artemisinin was purchased from Sigma-Aldrich. SYBR Green I (10,000x in DMSO), tetramethylrhodamine-dextran (TMR-dextran, 10 kDa), 5'(and 6')-carboxy-10-dimethylamino-3-hydroxy-spiro[7H-benzo[c] xanthene-7,1'(3H)-isobenzofuran]-3'-one-dextran (SNARF-1-dextran, 10 kDa) and LysoSensor Blue DND-192 were from Invitrogen, Life Technologies.

P. falciparum cultures

Chloroquine-sensitive (3D7 and D10) and resistant strains (Dd2) of *P. falciparum* were cultured using O^+ type human erythrocytes and pooled serum in complete culture medium (CCM) containing GlutaMAX I and HEPES in RPMI 1640 (GIBCO BRL, Invitrogen) supplemented with 0.25% Albumax, hypoxanthine (0.21 mM, Sigma), 45% glucose (Sigma) and 50 mg/mL gentamicin (Duopharma) (Trager & Jensen, 1976). For synchronization, cultures (~5% parasitemia, mainly ring stage) were treated with 5% D-sorbital at room temperature for 10 minutes and then allowed to mature for 24 hours (Lambros & Vanderberg, 1979). Synchronized mature stage parasites were harvested on a VarioMACS magnetic separation system (Miltenyi Biotec) or on a Percoll gradient (Miao & Cui, 2011) to above 95% purity as assessed by counting Giemsa-stained thin blood smears.

Assessment of the antimalarial activity of artemisinin

In a standard 48-hour drug sensitivity assay, 96-well plates containing triplicates of two-fold serial dilutions of artemisinin in CCM were prepared before being added into plates containing synchronized cultures of ring stage parasites (2% hematocrit, 1% parasitemia) (Desjardin *et al.*, 1979). Parasites were incubated at 37°C in a box filled with malaria gas mix or in a candle jar for 24 hours. Parasites were supplemented with fresh CCM and appropriate drug concentrations, and incubated for additional 24 hours. Samples were collected for assessment of parasite growth and parasitemia by adding 20 µL of 20x SYBR Green I solution (Vossen *et al.*, 2010). After 30 minutes incubation, the fluorescence signal was measured using excitation and emission wavelengths of 485 and 530 nm, respectively by a microplate reader (Bio-Rad, USA).

Synchronized parasites (3D7) at different stages (2% hematocrit, 1% parasitemia) were treated with artemisinin at various

concentrations (4, 16, 163, 250 and 1000 nM) for 4 hours to mimic the *in vivo* effect of a short half-life artemisinin. The range of drug concentrations was based on the *in vitro* IC_{50} value of artemisinin obtained using a 48-hour drug sensitivity assay. The SYBR Green I profile (parasite growth) was monitored by comparing treated and untreated (controls) parasites. The morphology of treated and untreated parasites in Giemsa-stained thin blood smears was assessed by light microscopy.

Analysis of pH of the digestive vacuole

Resealed erythrocytes were prepared in the presence of 50 µM TMR-dextran or SNARF-1-dextran and inoculated with synchronized mature stage parasites (Abu Bakar *et al.*, 2010). Parasites (3D7) at different stages grown in resealed erythrocytes containing TMR-dextran were treated with artemisinin (1 µM) for 4 hours. Erythrocytes co-labelled with TMR-dextran and LysoSensor Blue DND-192 were imaged using the FITC and DAPI filter cubes in an epifluorescence microscope (Olympus BX41). The effect of artemisinin (16, 32 and 64 nM) on the pH of endocytic compartments and DV of the parasites (D10) in SNARF-1-dextran-containing resealed erythrocytes was also determined by confocal microscopy at 37°C. SNARF-1-dextran was excited at 514 nm and the yellow (580–635 nm) and red (>635 nm) fluorescence were detected using the avalanche photodiode detectors (APDs) of a Confocor3 system.

RESULTS AND DISCUSSION

The sensitivity of different parasite stages to artemisinin

The malaria SYBR Green I-based fluorescence (MSF) assay was used to examine the effect of artemisinin on *P. falciparum* growth at different intra-erythrocytic stages. Since mature erythrocytes do not contain DNA and cannot synthesize any RNA, the binding of SYBR Green I is specific for malarial DNA in intraerythrocytic stages of the parasite development (Bennet *et al.*, 2004). The method is accurate, reliable, requires less sophisticated equipment, and involves a single processing step than that of the radioactive substrate incorporation assay (Desjardin *et al.*, 1979; Elabbadi *et al.*, 1992) or the colorimetric enzyme-linked immuno-sorbent assay (ELISA) (Druilhe *et al.*, 2001; Noedl *et al.*, 2002).

In this study, the malaria parasites were treated with artemisinin and their respective 50% inhibitory concentrations (IC_{50}s) were determined by using the MSF assay. Artemisinin inhibited 3D7, D10 and Dd2 parasite growth with the IC_{50} values of 17.05±0.93, 15.00±4.00 and 25.01±2.33 nM, respectively (Figure 1). The value for the 3D7 strain is slightly lower than that reported previously for the same strain using the [^{3}H]-hypoxanthine incorporation-based assay (Duraisingh *et al.*, 2000). The previous study proved the MSF assay produced IC_{50} values for malaria laboratory strains treated with chloroquine, mefloquine and quinine comparable to the standard histidine-rich protein 2 capture ELISA method (Bacon *et al.*, 2007). The study also demonstrated the ability of the MSF assay to determine the drug IC_{50}s from fresh clinical isolates.

Next, we modeled parasite responses to drug exposure at levels that might be expected to be achieved in patients. Tightly synchronized 3D7 parasites at different stages were treated with 0.2–60 times the IC_{50} value of artemisinin for a period of 4 hours (the period of clinical exposure). The increasing level of DNA and RNA during parasite development allows the use of MSF assay to examine parasite growth. Changes were observed in the SYBR Green I labelling profile (Table 1), revealing the different sensitivity of the parasites towards artemisinin treatments at different stages. The drug exhibited its antimalarial action as early as at mid ring stage with markedly low SYBR Green I fluorescence intensity compared to controls, indicating the inhibition of parasite growth. Artemisinin also had a significant effect on the growth of the late trophozoite and late schizont stage parasites. It is possible that such a broad stage sensitivity of the drug might disrupt the biochemical processes of the parasite,

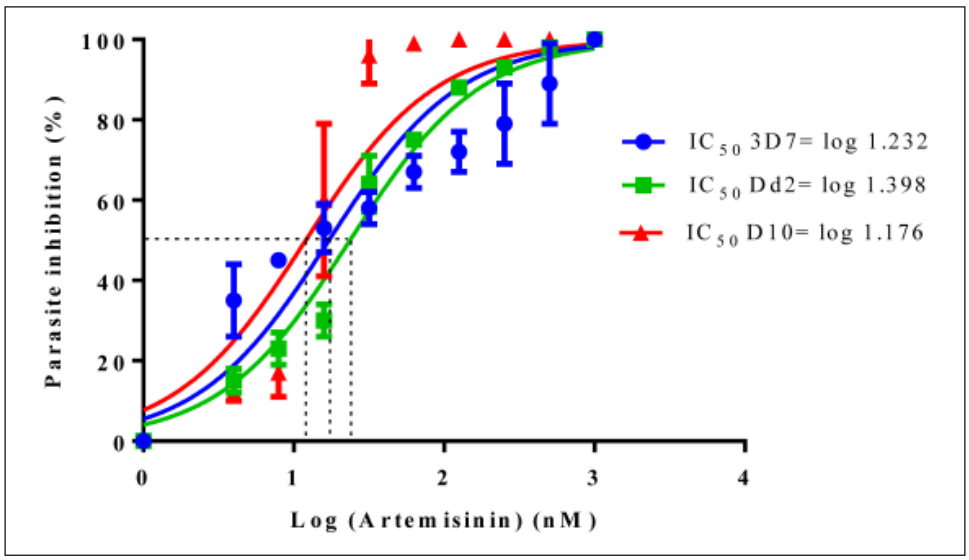

Figure 1. *In vitro* growth inhibition assay of 3D7, D10 and Dd2 parasite strains with artemisinin.

Table 1. Mean relative fluorescence intensity of SYBR Green I after 4-hour artemisinin treatments against different parasite stages

Stages	Mean (SD)					
	Control	IC_{20}*	IC_{50}*	IC_{70}*	IC_{80}*	IC_{100}*
Early ring	100.00 (0.00)	80.24 (17.24)	64.53[a] (3.33)	53.01[c] (14.57)	44.33[c] (4.79)	27.21[d] (14.62)
Mid ring	100.00 (0.00)	58.27[c] (15.60)	35.78[c] (15.06)	32.31[d] (18.82)	35.23[c] (16.82)	17.39[d] (10.30)
Late ring	100.00 (0.00)	66.67[b] (8.94)	74.36 (11.88)	73.92 (2.93)	62.97[a] (7.16)	53.72[c] (11.43)
Early trophozoite	100.00 (0.00)	73.84[a] (6.78)	61.54[a] (12.16)	61.06[b] (6.71)	40.73[c] (16.70)	23.10[d] (1.40)
Mid trophozoite	100.00 (0.00)	71.42[a] (11.09)	51.70[b] (7.71)	51.75[b] (17.90)	26.57[d] (17.94)	17.15[d] (10.03)
Late trophozoite	100.00 (0.00)	65.39[a] (7.58)	47.16[c] (7.59)	48.00[c] (4.85)	35.19[c] (8.64)	32.65[d] (8.08)
Early schizont	100.00 (0.00)	91.62 (5.53)	59.38[a] (17.03)	58.65[b] (9.21)	48.88[c] (13.44)	33.38[d] (2.25)
Late schizont	100.00 (0.00)	90.89 (6.01)	73.88 (25.51)	39.97[b] (2.02)	20.06[d] (3.75)	9.84[d] (1.73)

*One-way ANOVA test (p value < 0.05).
Dunnett's C post hoc: The mean of relative fluorescence intensity of SYBR Green I between untreated and treated parasites with artemisinin at IC_{20}, IC_{50}, IC_{70}, IC_{80} and IC_{100} for all parasite stages were statistically significance by ($p < 0.05$). The significance differences are indicated as follow: [a]($p \leq 0.05$), [b]($p \leq 0.01$), [c]($p \leq 0.001$), [d]($p \leq 0.0001$).

thereby causing its death. Moreover, a recent proteomics study revealed 120 protein targets of artemisinin, many of which were involved in crucial biological processes in the parasite (Wang *et al.*, 2015). Since hemoglobin metabolism is a vast process occurring in the parasite, heme or ferrous iron derived from the heme biosynthesis at ring (Abu Bakar *et al.*, 2010; Xie *et al.*, 2016) and trophozoite stages (Klonis *et al.*, 2011; Wang *et al.*, 2015) is thought to be responsible for the drug activation.

Morphology of the 3D7 parasites at different stages was observed upon artemisinin treatments for 4 hours (Figure 2). Control parasites appeared to be morphologically normal (right panels) (n = 30; three experiments). No morphological changes were observed when early ring stage parasites (n = 30; three experiments) were treated with artemisinin. This is expected as the actively heme metabolizing stages have been implicated in activating artemisinin (Abu Bakar *et al.*, 2010, Klonis *et al.*,

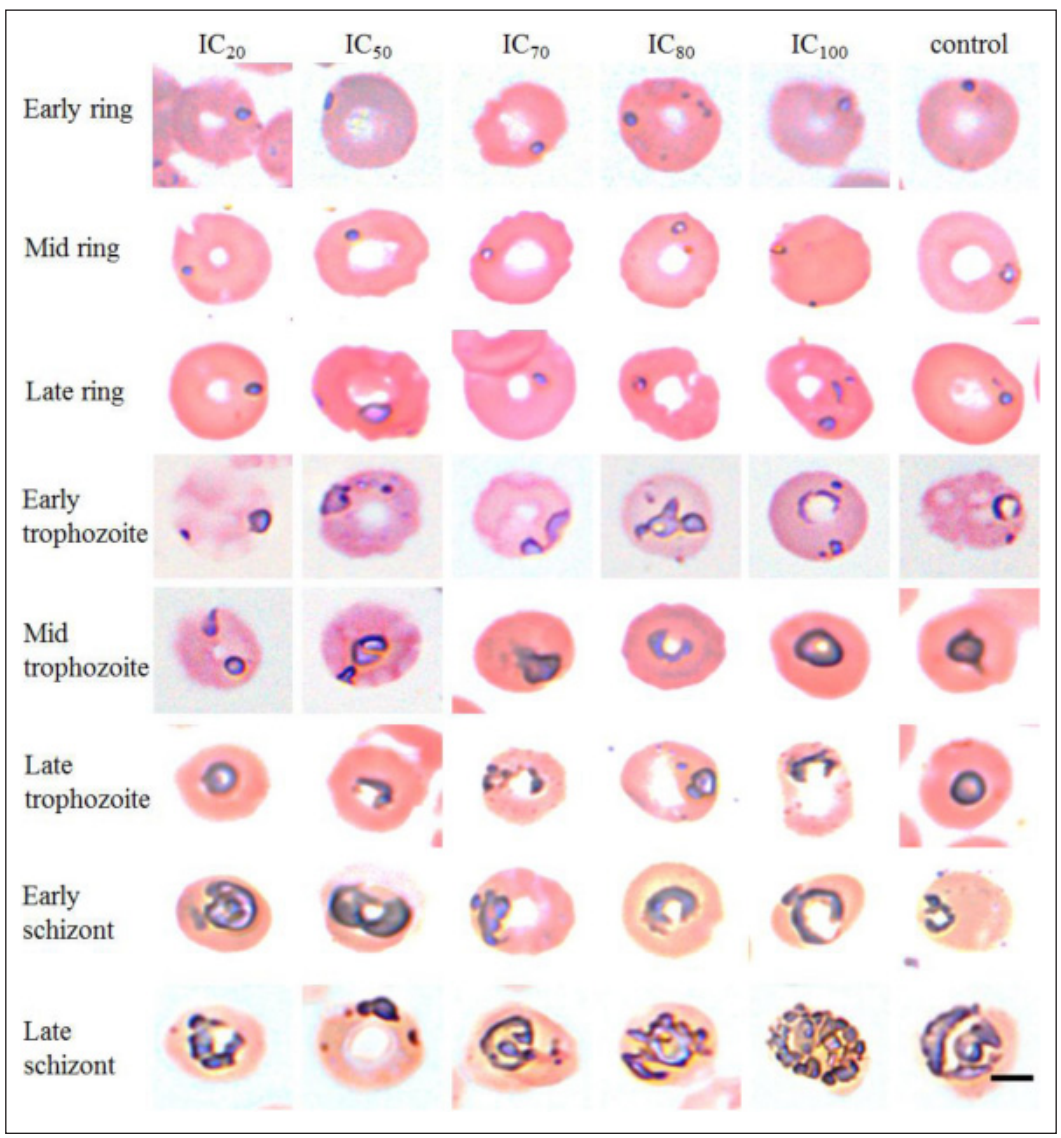

Figure 2. Morphology of 3D7 parasites treated with artemisinin at 0.2-60x its IC_{50} value for 4 hours. Scale bar: 5 µm

2011; Wang *et al.*, 2015). The majority of artemisinin-treated mid and late ring stage parasites (n = 30; three experiments) stopped their progression to late rings and early trophozoites, respectively. This was evidenced by the decrease in size and the contracted, pyknotic appearance of the rings, showing inhibition of the progression to mature stages. The effect of artemisinin on the trophozoites was much more pronounced than the ring stage parasites, consistent with an enhanced hemoglobin degradation flux. The treated trophozoites (n = 30; three experiments) were observed smaller and denser while untreated trophozoites underwent nuclear division to become schizonts as evidenced by a marked punctate appearance in the parasite cytoplasm. It is evident that artemisinin had a rapid action and its antimalarial activity was against all parasite stages, mainly directed to trophozoites. These findings agreed with the findings published in literature (Björkman & Bhattarai, 2005; Krishna *et al.*, 2004, Yang *et al.*, 2016).

The effect of artemisinin on DV pH

Resealed cells were proved to be able to support parasite growth and maturation (Mohd-Zamri *et al.*, 2017; Abu Bakar *et al.*, 2010). In this study, resealed erythrocytes containing TMR-dextran, an endocytic marker, were inoculated with mature stage parasites (3D7) and treated with 60 times the IC_{50} value of artemisinin (1 µM) for 4 hours. At the mid ring stage, the fluorescence intensity of TMR-dextran in the endocytic structures of control and treated parasites was intense and intact (Figure 3A-B, green arrow). No significant alteration in the distribution of the LysoSensor Blue labeling of the DV (Figure 3B, yellow arrow) was observed following artemisinin treatment, indicating no damage to the integrity of the DV membrane. To further investigate the effect of artemisinin on the pH of the DV as well as endocytic compartments, a ratiometric pH indicator, SNARF-1-dextran, was incorporated into resealed erythrocytes. The ratio of the fluorescence intensities at two emission wavelengths can be used to estimate the pH of the cellular compartments where the SNARF-1-dextran is located. Late ring and mid trophozoite stage parasites (D10) developing in these cells were treated with 1-4 times the IC_{50} value of artemisinin for 4 hours and observed by live cell confocal microscopy (Abu Bakar *et al.*, 2010). The look-up table generated (Figure 4) shows acidic compartments with pH values <6 (R_{ry} values <0.8) appear green, whereas neutral to basic compartments (R_{ry} values >1.2) appear blue-purple.

SNARF-1-dextran accumulated within the endocytic compartments of control late ring stage parasites and appeared green in the R_{ry} image, indicating that these compartments are acidic (Figure 4A). These endocytic compartments represent the early endocytic compartments of the ring stage parasites (Abu Bakar *et al.*, 2010; Hanssen *et al.*, 2010; Mohd-Zamri *et al.*, 2017). In treated late ring stage parasites, however, the compartments remained acidic with pH values <6. The similar results were observed in treated mid trophozoites with the extra-DV compartments (white arrowheads) adjacent to the prominent DV were both remained acidic (Figure 4B). In contrast, the redistribution of the LysoSensor Blue from the DV to punctate structures following artemisinin treatment was reported by del Pilar Crespo *et al.* (2008), suggesting a loss of the pH gradient across the DV membrane or restructuring of this compartment. The vacuolar-type proton-pumping ATPase (V-type H^+-ATPase) and pyrophosphatase (V-type H^+-pyrophosphate) are thought to be responsible for maintaining an acidic DV (Hayashi *et al.*, 2000; Saliba *et al.*, 2003; Spillman *et al.*, 2008). Pyrophosphatase proved to be able to compensate the DV pH sufficiently in case of impaired H^+-ATPase activity (Moriyama *et al.*, 2003; Saliba *et al.*, 2003). Therefore, changes of DV pH at the therapeutic artemisinin concentrations used in this study might be counteracted by the action of another H^+-linked secondary transporter. Another study by Elandalloussi *et al.* (2005) supported the unaffected ATPase activity in the parasite plasma membrane after drug treatment, which might explain the results of our findings.

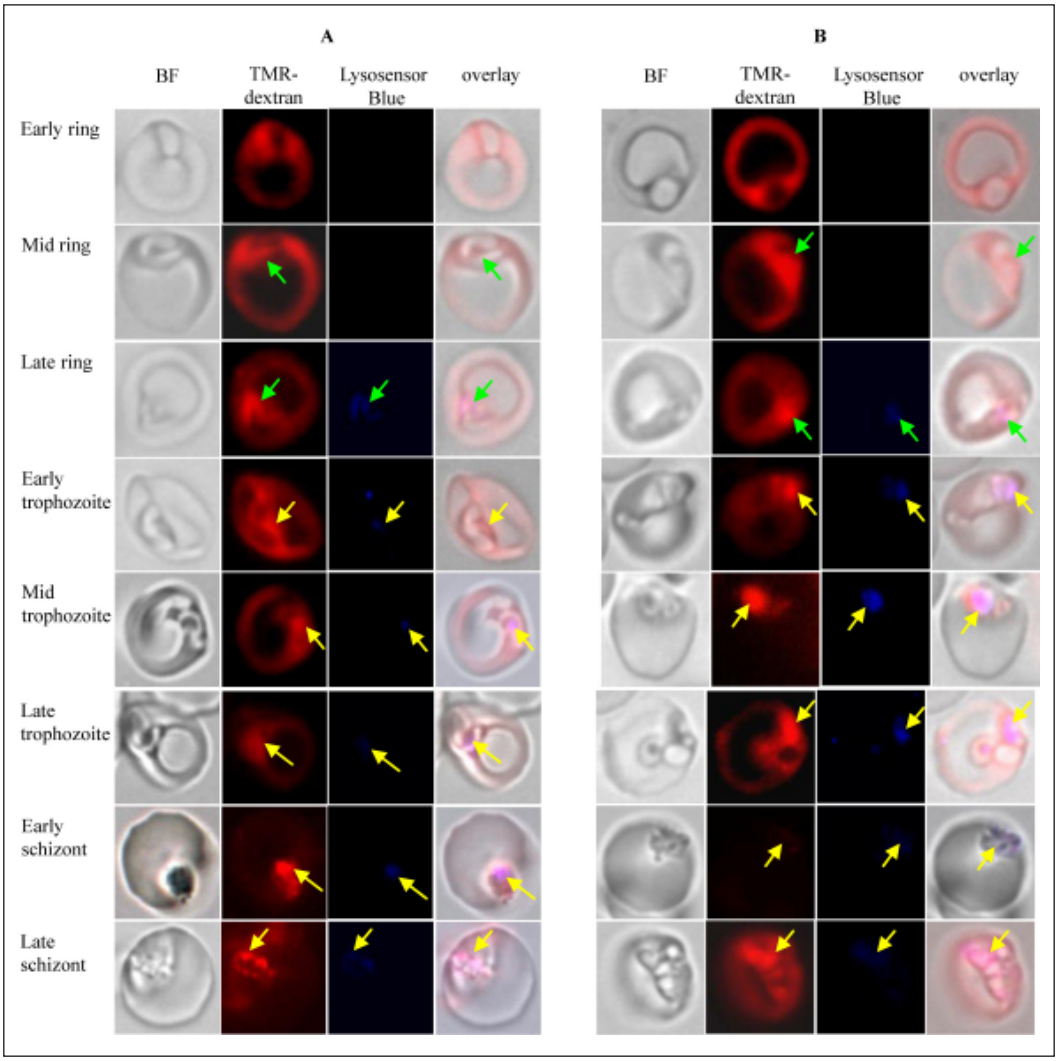

Figure 3. Analysis of DV pH after treatment with 1000 nM artemisinin for 4 hours
The parasites were labeled with LysoSensor Blue for 20 minutes before observed under fluorescence microscope at 100x magnification. (A) The acidic DV of the control and treated parasites appeared as early as at the late ring stage as shown by the LysosSensor Blue labelling (green arrow). (B) No alterations of LysoSensor Blue labelling of the DV following treatments with the highest concentration of artemisinin were observed in both untreated and treated cells. Scale bars: 5 µm.

In conclusion, mid ring to schizont stage *P. falciparum* were sensitive and susceptible to artemisinin. However, the pH of the DV and small endocytic structures was shown not to be an initiator of inhibition of the parasite growth and changes in parasite morphology. Our findings suggest that the mechanism of action of artemisinin needs to be re-examined and that DV targets should be further re-evaluated. We suggest that the flow cytometry-based assay could be used for the rapid, simple and quantitative measurement of the DV pH on a large number of cells.

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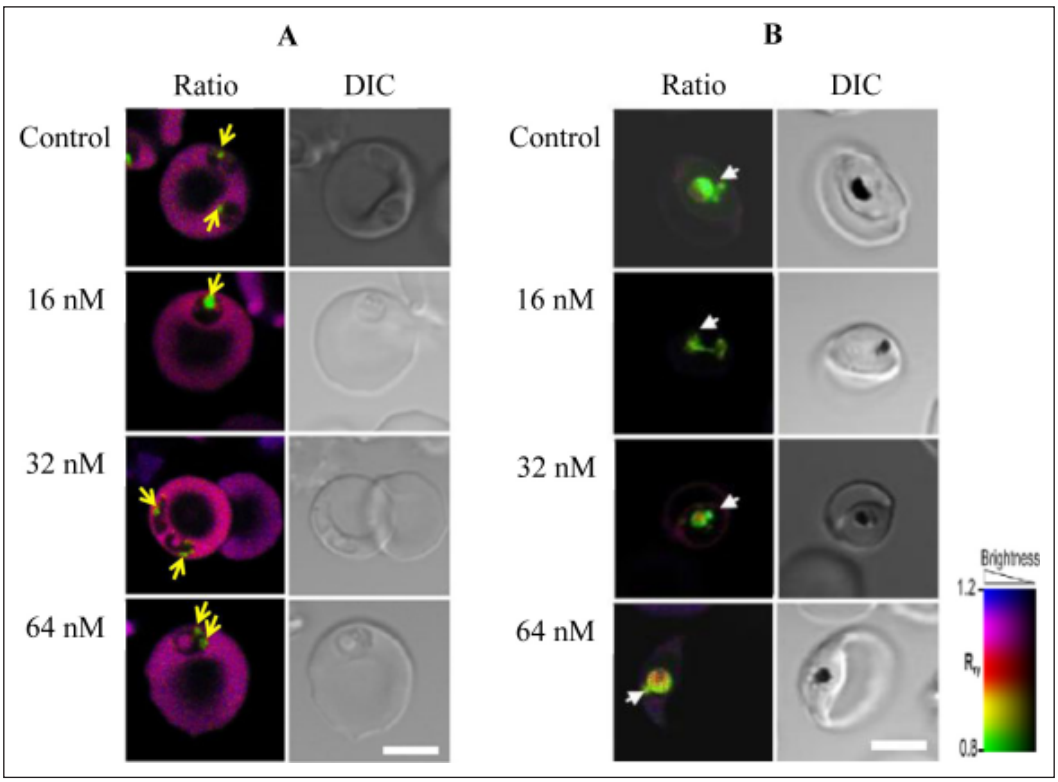

Figure 4. pH of endocytic structures and DV analysis after after 4-hour artemisinin treatment
The colour at ratio images reflects the R_{ry} value from the look up table. Neutral to basic compartments appear blue-purple (R_{ry} = >1.2), whereas compartments with pH values <6 appear green (R_{ry} = <0.8). (A) The endocytic compartments of late ring stage parasites (yellow arrowheads) and (B) the DV and extra-DV compartments of mid trophozoite stage parasites (white arrowheads) appeared green (yellow arrowheads), indicating that the compartments remained acidic upon artemisinin treatment. Scale bars: 5 µm.

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