Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and digestion

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Supplementary Figures

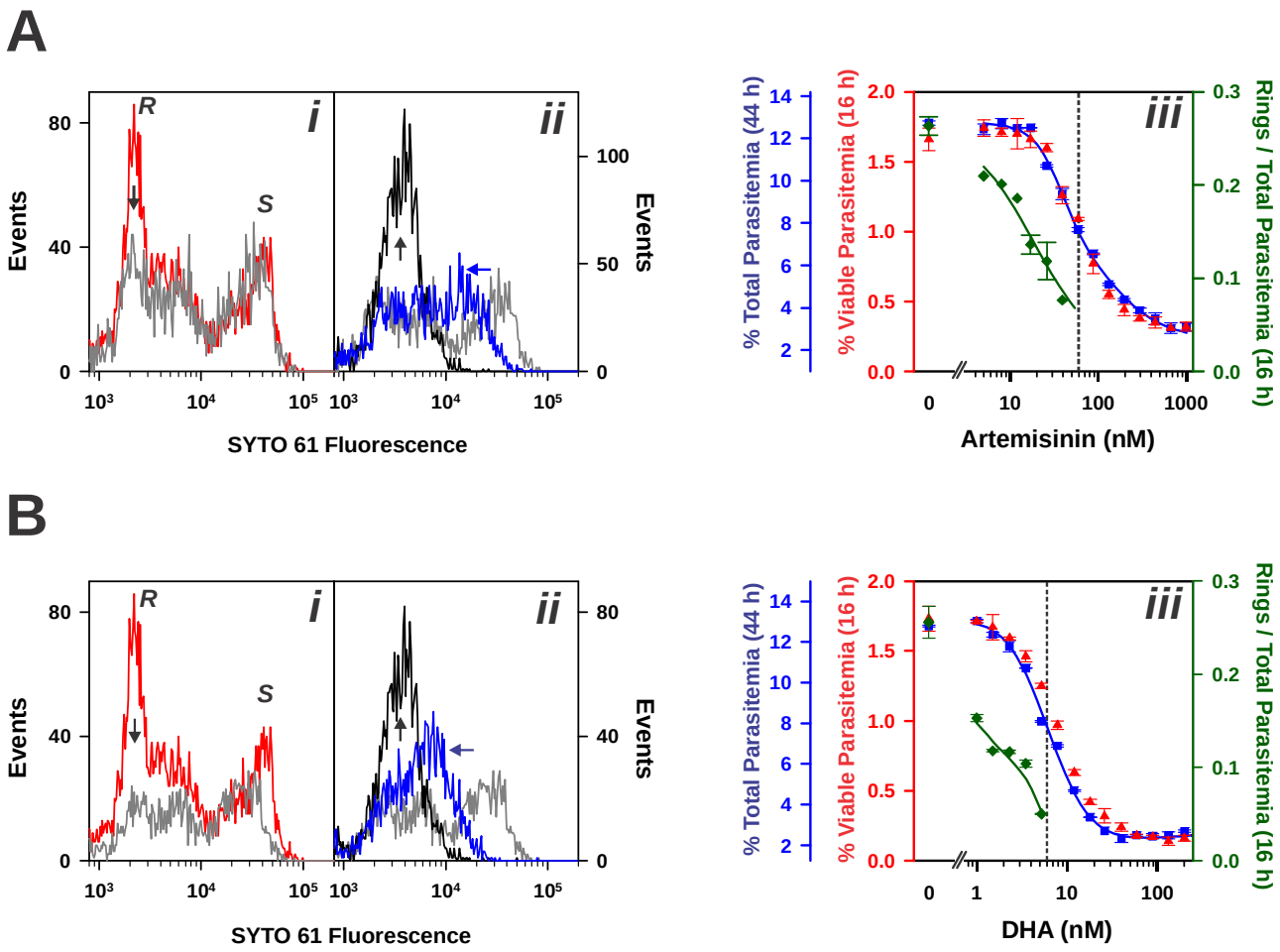

Figure S1. Artemisinin and DHA slow parasite growth in intact infected RBCs. Synchronized 3D7 parasites (2.5% parasitemia, 1% hematocrit) at the late ring/early trophozoite stage were treated for 4 h with artemisinin (A) or DHA (B) and analyzed by flow cytometry 16 and 44 h following treatment. (i, ii) SYTO 61 staining profiles of the parasite population measured 16 h after treatment with no drug (red curves) or with 12, 26 and 1000 nM artemisinin or 1.0, 5.2 and 200 nM DHA (corresponding to gray, blue and black curves, respectively). (iii) Correlation of total parasitemia measured 44 h after treatment (blue squares) with parameters measured at 16 h (see below). The dashed vertical lines correspond to the IC_{50} values of the respective drug. Note: The $IC_{50\text{-}4h}$ value is lower for these late ring/early trophozoite stage than for the mid-ring stage parasites in Fig. 1 (main text) because artemisinins are more active against the trophozoite stage (1-2) when hemoglobin digestion is most active (3). Error bars represent S.D. of triplicate measurements.

The SYTO 61 profiles of the controls measured 16 h after treatment (red profiles) show a mixture of low staining rings (R) and high staining schizonts (S) indicating that part of the parasite population (~10-20%) has progressed to the next cycle. Exposure of the parasites to sub-lethal concentrations of drugs (gray profiles) leads to a decreased population of rings (arrow in i) demonstrating that drug treatment delays the entry of the parasite population into the next cycle. The higher concentrations of drugs shown in (ii) produce the effects documented in Figure 1 of the main text: the highest drug concentrations produce non-viable cells that have very low SYTO staining (black profiles) whereas exposure to concentrations in the region of the drug IC_{50} value results in the progressive appearance of a non-viable parasite population (black arrows) and decreased staining of the viable population (horizontal arrows) with increasing drug concentration.

The viable parasitemia was quantitated as described in the main text by excluding the low staining population. Although this introduces some error as a fraction of the parasites in the controls that

have proceeded to the next cycle is also excluded, the majority of parasites (80-90%) are still in the same cycle as the drug treatment. For both artemisinin and DHA, a good correlation is observed between the viable parasitemia measured at 16 h with the total parasitemia at 44 h (blue squares, (iii)).

We used the ratio of rings to total parasites as a measure of the delay of parasite progression into the next cycle. In order to minimize inclusion of the unviable population which has a similar SYTO 61 profile as the rings, we employed a narrow gate to improve selectivity for viable rings and corrected for the presence of the unviable population using the 44 h parasitemia data. The resultant ratios (iii, green diamonds) show that retardation of growth is drug concentration dependent, beginning at sub-lethal concentrations of drug, and that the viable cells in the region of the IC_{50} value show increased growth retardation. These results demonstrate that the decreased SYTO 61 signals of the viable populations in experiments such as those shown in Figure 1 and 2 of the main text, reflect the presence of earlier stage parasites.

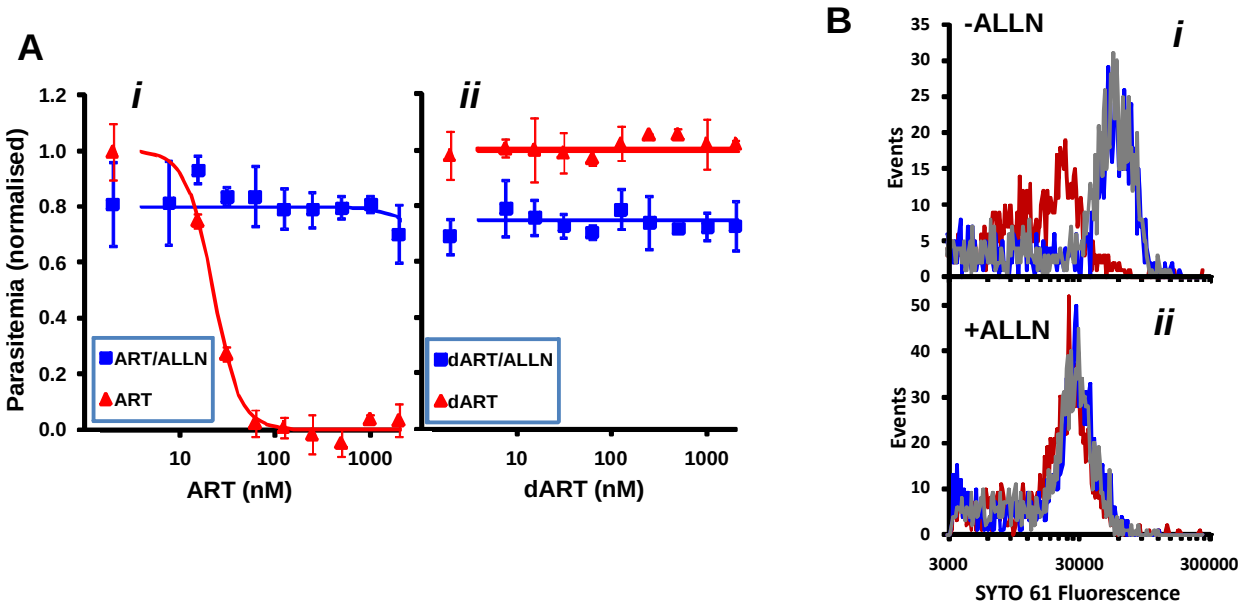

Figure S2. Deoxyartemisinin does not affect parasite viability or growth and shows no interaction with ALLN. Early-mid trophozoites (1% parasitemia, 0.2% hematocrit) were pre-incubated in the absence (red triangles) and presence (blue squares) of 5 µM ALLN for 45 min before the addition of either artemisinin (ART) or deoxyartemisinin (dART). Following a 4 h incubation, the drugs and ALLN were washed off and the cultures were analyzed for growth effects 9 h later and for parasite viability 36 h later. (A) Parasitemia 36 h after drug treatment. As described in the main text, artemisinin activity at concentrations up to 2 µM is almost completely inhibited by ALLN (i). In contrast, dART on its own shows no activity against the parasites at concentrations up to 2 µM and there is no additional effect of dART in the presence of ALLN (ii). Error bars represent S.D of triplicate measurements. (B) Growth analysis performed 9 h after drug treatment during the same cycle as the treatment. (i) Treatment with 60 nM ART (red curve) produces two SYTO 61 staining populations that show decreased staining compared to untreated samples (grey curve, corresponding to no drugs and no ALLN) as described in the main manuscript. In contrast, the staining profile in the presence of 2 µM dART (blue curve) is superimposable on the control showing that dART does not affect the growth of parasites. (ii) SYTO 61 staining profiles in the presence of ALLN. Parasites treated with either 2 µM ART (red curve) or dART (grey curve) show no difference in development stage to parasites treated with ALLN alone (grey curve).

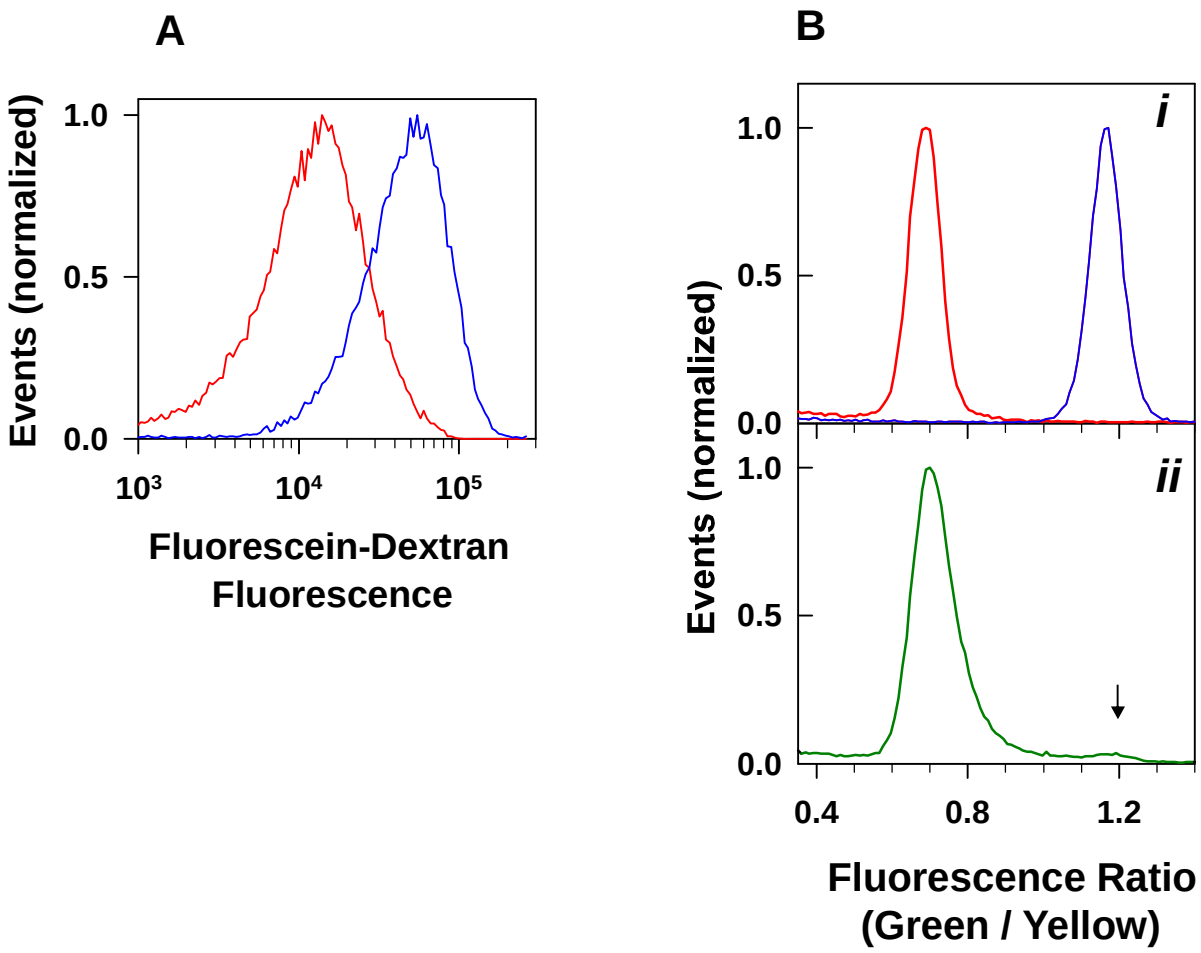

Figure S3. Fluorescein-dextran as a pH ratiometric probe in flow cytometry. Fluorescein is commonly employed as a pH ratiometric probe. Typically, pH dependent changes in the emission spectrum are monitored using two excitation wavelengths and a single emission wavelength(4). Although the emission spectrum of fluorescein does not exhibit a major spectral shift in the pH range 4-7, the spectrum does exhibit a change in shape over this pH range(5) that can be quantitated by measuring the ratio of green (505-530 nm) and yellow (565-610 nm) fluorescence at a single excitation wavelength (488 nm) with the optical set up of a commercial flow cytometer. We prepared saponin-released parasites from cultures grown in fluorescein-dextran containing resealed RBCs (prepared in the presence of 110 µM fluorescein-dextran) as described in the main text. (A) Green fluorescein-dextran fluorescence of saponin-released parasites in acidic (red) and alkaline (blue) buffers. The parasites were resuspended in isotonic buffers (150 mM NaCl) containing 20 mM 2-(N-morpholino)ethanesulfonic acid, pH 5.0, or 20 mM sodium phosphate, pH 7.5, in the presence of the ionophore, carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 10 µM), to dissipate the pH gradient in the intracellular compartments where the fluorescein-dextran is located. The fluorescence increases at the higher pH due to the larger extinction coefficient and quantum yield of the basic form of fluorescein(5). (B) Green/yellow fluorescence ratio of saponin-released parasites in (i) acidic and alkaline buffers (red and blue curves, respectively) and (ii) glucose-containing medium (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM glucose, 25 mM HEPES, pH 7). The ratio measurements comfortably distinguish between fluorescein-dextran located in the acidic digestive vacuole (pH 5.0-5.5) and that present in a neutral/alkaline environment. In the predominant population of parasites measured in glucose-containing medium the fluorescein is located in an acidic compartment, although in a small parasite population (indicated by the arrow in (ii)) the fluorescein is located in an alkanized environment. This minority population probably represents parasites that had been stressed or damaged by the saponin treatment. Measurements of parasite fluorescein-dextran fluorescence (as in Figure 2C) are potentially compromised by the

presence of non-viable cells which can significantly skew the average (cell-weighted) intensity towards high values if the acidic compartments become alkalinized. We avoided such effects in the experiments presented in Figure 2 by using a fluorescence ratio indicative of an acidic environment as an additional gate and by using the median green signal of the resultant population as a measure of uptake. In the experiment depicted in Figure 3, we used the median green fluorescence in the presence of CCCP because the lower intensities prevented accurate measurement of the signal under acidic conditions.

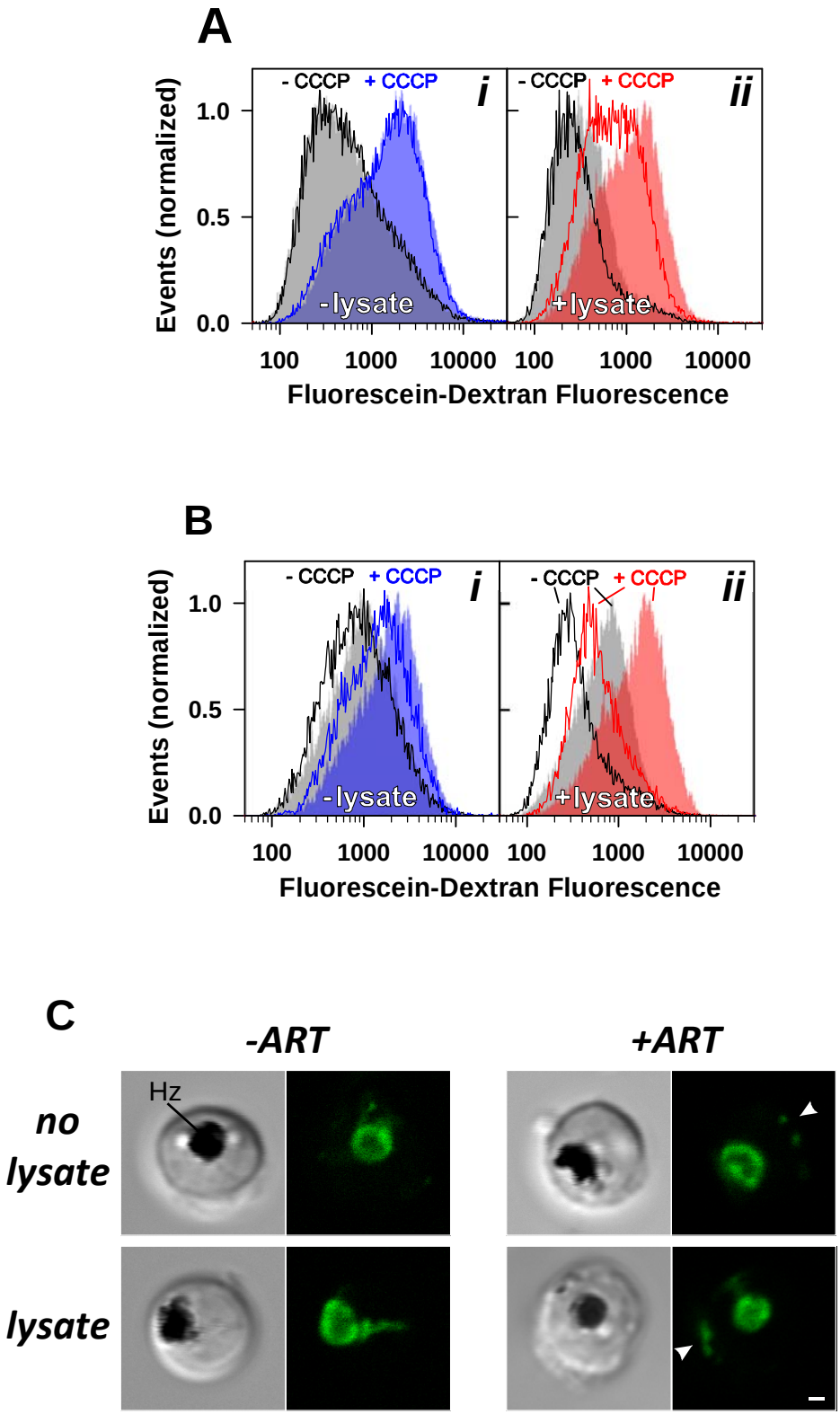

Figure S4. RBC lysate is needed for artemisinin- and DHA-mediated inhibition of endocytosis. The host cell membranes of RBCs infected with mid-trophozoite stage parasites were selectively permeabilized with 0.02% saponin, and free parasites were incubated for 4 h in medium containing fluorescein-dextran before being washed and analyzed by flow cytometry. Incubations were performed in the absence (i) and presence (ii) of RBC lysate containing 3.7 mM hemoglobin.

Shown are fluorescence histograms recorded in the presence (blue or red) and absence (grayscale) of the ionophore CCCP, which dissipates pH gradients. CCCP treatment causes an increase in fluorescein fluorescence when the environment changes from acidic to neutral or alkaline (Figure S2). Filled profiles correspond to measurements in the absence of drug. Open curves correspond to measurements in the presence of 220 nM artemisinin (A) and 250 nM DHA (B). These results show that free parasites take up the fluorescein-dextran into acidic compartments in both the absence and presence of RBC lysate. Uptake is reduced in the presence of the drugs, but the fluorescein-dextran that has been taken up remains in an acidic environment. (C) Fluorescein-dextran uptake by saponin-released parasites. Shown are differential interference contrast (grayscale) and fluorescence (green) images of fluorescein uptake by parasites in the absence and presence of RBC lysate and in the absence and presence of 1 μM artemisinin. In all cases, the majority of the fluorescence colocalized with the dark, hemozoin (Hz)-containing feature in the grayscale image, which corresponds to the parasite digestive vacuole. The small fluorescent puncta (examples indicated with arrowheads) correspond to fluorescein-dextran uptake into cytostomal vesicles. The brightness and contrast in each image has been adjusted to optimize the display of each panel. Bar = 1 μm.

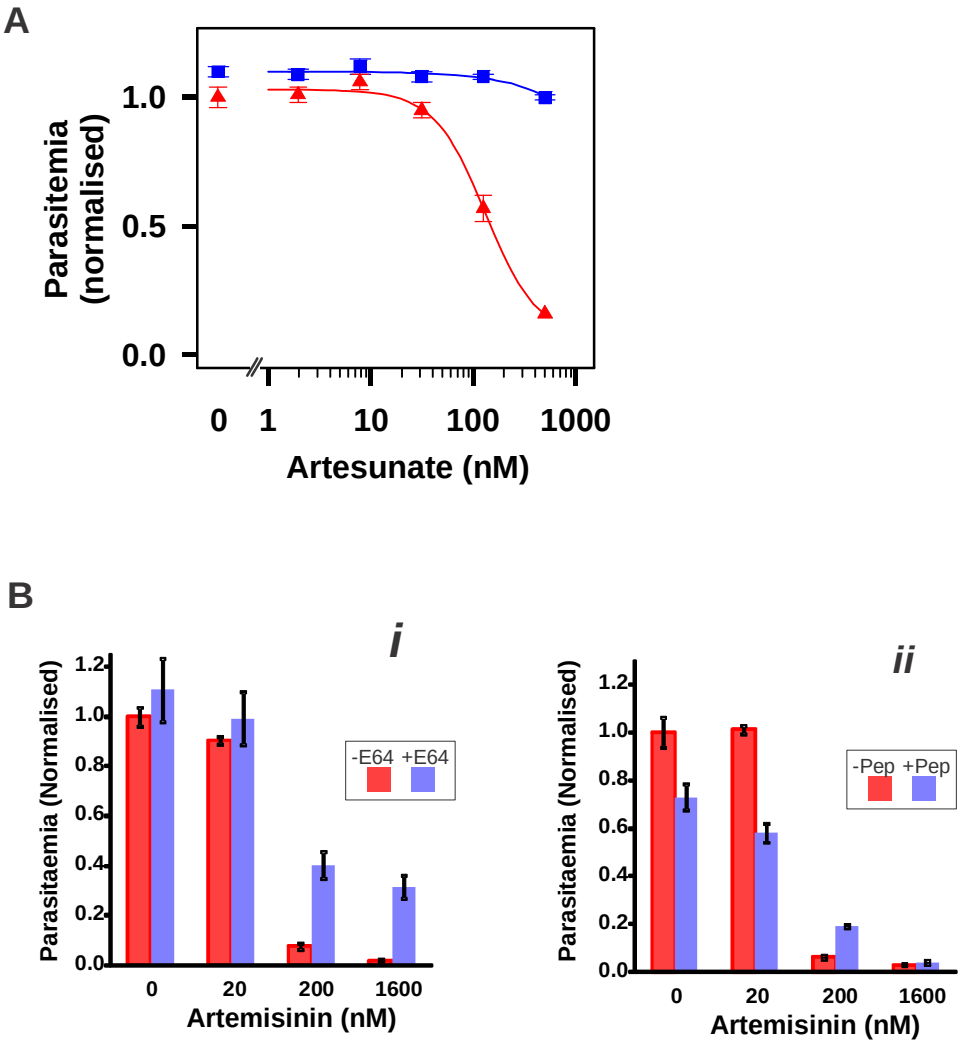

Figure S5. Endoperoxide activity is antagonized by cysteine but not aspartic protease inhibitors. (A) Inhibition of artesunate activity by ALLN. Trophozoite stage parasites (2% hematocrit, 0.2% parasitemia) were pre-incubated for 1 h in the absence (red triangles) and presence (blue squares) of 5 µM ALLN before the addition of artesunate. After 4 h, the cells were washed, cultured for 72 h and analyzed by flow cytometry. Parasitemias are normalized to untreated controls (no endoperoxide or inhibitor). (B) Interaction of artemisinin with E-64 (i) or pepstatin A (ii). Synchronized early ring stage parasites (1% parasitemia) were pretreated for 24 h in the absence (red) or presence (blue) of 10 µM E-64 or 120 µM pepstatin A before adding artemisinin for 2 h. The drugs were removed and samples cultured for 48 h before analysis of parasitemia by flow cytometry. All parasitemias are normalized to untreated controls (no endoperoxide or inhibitor). Error bars represent S.D. of triplicate measurements.

Supplementary References

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