Doxycycline has Distinct Apicoplast-Specific Mechanisms of Antimalarial Activity

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Abstract: Doxycycline (DOX) is a key antimalarial drug thought to kill Plasmodium parasites by blocking protein translation in the essential apicoplast organelle. Clinical use is primarily limited to prophylaxis due to delayed second-cycle parasite death at 1-3 μM serum concentrations. DOX concentrations >5 μM kill parasites with first-cycle activity but have been ascribed to off-target mechanisms outside the apicoplast. We report that 10 μM DOX blocks apicoplast biogenesis in the first cycle and is rescued by isopentenyl pyrophosphate, an essential apicoplast product, confirming an apicoplast-specific mechanism. Exogenous iron rescues parasites and apicoplast biogenesis from first- but not second-cycle effects of 10 μM DOX, revealing that first-cycle activity involves a metal-dependent mechanism distinct from the delayed-death mechanism. These results critically expand the paradigm for understanding the fundamental antiparasitic mechanisms of DOX and suggest repurposing DOX as a faster-acting antimalarial at higher dosing whose multiple mechanisms would be expected to limit parasite resistance.
INTRODUCTION

Malaria remains a serious global health problem, with hundreds of thousands of annual deaths due to *Plasmodium falciparum* parasites. The absence of a potent, long-lasting vaccine and parasite tolerance to frontline artemisinin combination therapies continue to challenge malaria elimination efforts. Furthermore, there are strong concerns that the current COVID-19 pandemic will disrupt malaria prevention and treatment efforts in Africa and cause a surge in malaria deaths that unravels decades of progress (1). Deeper understanding of basic parasite biology and the mechanisms of current drugs will guide their optimal use for malaria prevention and treatment and facilitate development of novel therapies to combat parasite drug resistance.

Tetracycline antibiotics like DOX are thought to kill eukaryotic *P. falciparum* parasites by inhibiting prokaryotic-like 70S ribosomal translation inside the essential apicoplast organelle (Figure 1) (2). Although stable *P. falciparum* resistance to DOX has not been reported, clinical use is largely limited to prophylaxis due to delayed activity against intraerythrocytic infection (3, 4). Parasites treated with 1-3 µM DOX, the drug concentration sustained in human serum with current 100-200 mg dosage (5), continue to grow for 72-96 hours and only die after the second 48-hour intraerythrocytic growth cycle when they fail to expand into a third cycle (2). Slow antiparasitic activity is believed to be a fundamental limitation of DOX and other antibiotics that block apicoplast-maintenance pathways (4, 6). First-cycle anti-*Plasmodium* activity has been reported for DOX and azithromycin concentrations >3 µM, but such activities have been ascribed to targets outside the apicoplast (2, 7, 8). A more incisive understanding of the mechanisms and parameters that govern first versus second-cycle DOX activity can inform and improve clinical use of this valuable antibiotic for antimalarial treatment. We therefore set out to test and unravel the mechanisms and apicoplast specificity of first-cycle DOX activity.
RESULTS AND DISCUSSION

First-cycle activity by 10 μM DOX has an apicoplast-specific mechanism. Prior studies have shown that 200 μM isopentenyl pyrophosphate (IPP), an essential apicoplast product, rescues parasites from the delayed-death activity of 1-3 μM DOX, confirming an apicoplast-specific target (7). To provide a baseline for comparison, we first used continuous-growth and 48-hour growth-inhibition assays to confirm that IPP rescued parasites from 1 μM DOX (Figure 2A) and that DOX concentrations >5 μM killed parasites with first-cycle activity (Figure 2A–C and Figure 2-figure supplement 1) as previously reported (2). To test the apicoplast specificity of first-cycle DOX activity, we next asked whether 200 μM IPP could rescue parasites from DOX concentrations >5 μM. We observed that IPP shifted the 48-hour EC$_{50}$ value of DOX from 5 ± 1 to 12 ± 2 μM (average ± SD of 5 independent assays, P = 0.001 by unpaired t-test) (Figure 2C and Figure 2-figure supplement 1), suggesting that first-cycle growth defects from 5-10 μM DOX reflect an apicoplast-specific mechanism but that DOX concentrations >10 μM cause off-target defects outside this organelle. We further tested this conclusion using continuous growth assays performed at constant DOX concentrations. We observed that IPP fully or nearly fully rescued parasites from first-cycle growth inhibition by 10 μM but not 20 or 40 μM Dox (Figure 2A & 2D and Figure 2-figure supplement 1). On the basis of IPP rescue, we conclude that 10 μM DOX kills *P. falciparum* with first-cycle activity by an apicoplast-specific mechanism.

10 μM DOX blocks apicoplast biogenesis in the first cycle: Inhibition of apicoplast biogenesis in the second intraerythrocytic cycle is a hallmark of 1-3 μM DOX-treated *P. falciparum*, resulting in unviable parasite progeny that fail to inherit the organelle (2). IPP rescues parasite viability after the second cycle without rescuing apicoplast inheritance, such that third-cycle daughter parasites...
lack the organelle and accumulate apicoplast-targeted proteins in cytoplasmic vesicles (7). We treated synchronized ring-stage D10 (9) or NF54 (10) parasites expressing the acyl carrier protein leader sequence fused to GFP (ACP\textsubscript{L}-GFP) with 10 µM DOX and assessed apicoplast morphology 30-36 hours later in first-cycle schizonts. In contrast to the second-cycle effects of 1-3 µM DOX, the apicoplast in 10 µM DOX-treated parasites failed to elongate in the first cycle. Rescue by 200 µM IPP produced second-cycle parasite progeny with a dispersed GFP signal indicative of apicoplast loss (Figure 2E and Figure 2- figure supplement 2). We conclude that 10 µM DOX blocks apicoplast biogenesis in the first cycle.

First- and second-cycle effects of DOX on the apicoplast are due to distinct mechanisms. What is the molecular mechanism of faster apicoplast-specific activity by 10 µM DOX? We first considered the model that both 1 and 10 µM DOX inhibit apicoplast translation but that 10 µM DOX kills parasites faster due to more stringent translation inhibition at higher drug concentrations. This model predicts that treating parasites simultaneously with multiple distinct apicoplast-translation inhibitors, each added at a delayed death-inducing concentration, will produce additive, accelerated activity that kills parasites in the first cycle. To test this model, we treated synchronized D10 parasites with combinatorial doses of 2 µM DOX, 2 µM clindamycin, and 500 nM azithromycin and monitored growth over 3 intraerythrocytic cycles. Treatment with each antibiotic alone produced major growth defects at the end of the second cycle, as expected for delayed-death activity at these concentrations (6). Two- and three-way drug combinations caused growth defects that were indistinguishable from individual treatments and provided no evidence for additive, first-cycle activity (Figure 3A and Figure 3- figure supplement 1). These
results contradict a simple model that 1 and 10 µM DOX act via a common translation-blocking mechanism and suggest that the first-cycle activity of 10 µM DOX is due to a distinct mechanism.

**Exogenous iron rescues parasites from first- but not second-cycle effects of 10 µM DOX.**

Tetracycline antibiotics like DOX tightly chelate a wide variety of di- and trivalent metal ions via their siderophore-like arrangement of exocyclic hydroxyl and carbonyl groups (Figure 1), with a reported affinity series of Fe³⁺>Fe²⁺>Zn²⁺>Mg²⁺>Ca²⁺ (11, 12). Indeed, tetracycline interactions with Ca²⁺ and Mg²⁺ ions mediate cellular uptake and binding to biomolecular targets such as the tetracycline repressor and 16S rRNA (12, 13). We next considered a model that first-cycle effects of 10 µM DOX reflect a metal-dependent mechanism distinct from ribosomal inhibition causing second-cycle death. To test this model, we investigated whether exogenous metals rescued parasites from 10 µM DOX. We failed to observe growth rescue by 10 µM ZnCl₂ (toxicity limit (14)) or 500 µM CaCl₂ in continuous-growth (Figure 3B and Figure 3-figure supplement 1) or 48-hour growth-inhibition assays (Figure 3C). In contrast, 500 µM FeCl₃ (and to a lesser extent 500 µM MgCl₂) fully or nearly fully rescued parasites from first-cycle growth inhibition by 10 µM DOX (Figure 3C & 3D), although partial rescue was observed at FeCl₃ concentrations as low as 50 µM (Figure 3-figure supplement 1). However, parasites treated with 10 µM DOX and 500 µM FeCl₃ still succumbed to second-cycle, delayed death (Figure 3D and Figure 3-figure supplement 1), as expected for distinct mechanisms of first- and second-cycle DOX activity.

We also observed that 500 µM FeCl₃ but not CaCl₂ rescued first-cycle apicoplast-branching in 10 µM DOX (Figure 3E and Figure 3-figure supplement 2). These observations contrast with IPP, which rescued parasite viability in 10 µM DOX but did not restore apicoplast branching (Figure 2E). We further noted that FeCl₃ selectively rescued parasites from the
apicoplast-specific, first-cycle growth effects of 10 µM DOX but did not rescue parasites from the second-cycle effects of 1 µM DOX (Figures 3F) or the off-target effects of 20-40 µM DOX (Figure 3G and Figure 3-figure supplement 1). We conclude that 10 µM DOX kills parasites via a metal-dependent, first-cycle mechanism that blocks apicoplast biogenesis and is distinct from the second-cycle, delayed-death mechanism of 1 µM DOX.

**Metal-dependent mechanisms of first-cycle activity by 10 µM DOX.** What is the metal-dependent mechanism of 10 µM DOX, and why is there preferential rescue by FeCl₃?

Tetracyclines bind iron more tightly than other metals, with an equilibrium association constant of $10^{10}$ M⁻¹ for 1:1 chelation of Fe³⁺ versus $10^4$ M⁻¹ for Mg²⁺ (11). Although the 500 µM concentration of exogenous FeCl₃ required for maximal rescue of parasite growth in 10 µM DOX is large relative to the ~1 µM labile iron concentration estimated for the parasite cytoplasm (15), the intracellular iron concentration achieved by exogenous addition of 500 µM FeCl₃ remains unclear. Indeed, mechanisms of iron uptake and trafficking by blood-stage *P. falciparum* remain sparsely understood (15, 16), especially uptake across the four membranes that surround the apicoplast.

We first considered whether exogenous FeCl₃ might selectively rescue 10 µM DOX activity by blocking or reducing its uptake into the parasite apicoplast, since metal chelation has been reported to influence the cellular uptake of tetracycline antibiotics in other organisms (12). However, 500 µM FeCl₃ or MgCl₂ did not rescue second-cycle parasite death in continuous growth assays with 10 µM (Figures 3D) or 1 µM DOX (Figure 3F). Furthermore, exogenous iron resulted in only a small, 1.5-µM shift in EC₅₀ value from 0.5 to 2 µM in a 96-hour growth inhibition assay, in contrast to the 10.5-µM shift provided by IPP (Figure 3-figure supplement 1). These results strongly suggest that DOX uptake into the apicoplast is not substantially perturbed by exogenous...
iron. The inability of 500 µM FeCl₃ to rescue first-cycle activity by ≥20-µM DOX (Figure 3G) further suggests that general uptake of DOX into parasites is not substantially affected by exogenous iron.

We propose two distinct models to explain the metal-dependent effects of 10 µM DOX, both of which could contribute to apicoplast-specific activity. First, DOX could directly bind and sequester labile iron within the apicoplast, reducing its bioavailability for Fe-S cluster biogenesis and other essential iron-dependent processes in this organelle. Indeed, prior work has shown that apicoplast biogenesis requires Fe-S cluster synthesis apart from known essential roles in isoprenoid biosynthesis (17). In this first model, rescue by exogenous FeCl₃ would be due to restoration of iron bioavailability, while modest rescue by 500 µM MgCl₂ may reflect competitive displacement of DOX-bound iron to restore iron bioavailability. RPMI growth medium already contains ~400 µM Mg²⁺ prior to supplementation with an addition 500 µM MgCl₂, and thus Mg²⁺ availability is unlikely to be directly limited by 10 µM DOX. Consistent with a general mechanism that labile-iron chelation can block apicoplast biogenesis, we observed in preliminary studies that the anti-Plasmodium growth inhibition caused by the highly-specific iron chelator, deferoxamine (DFO) (16), could be partially rescued by IPP, fully rescued by exogenous FeCl₃, and involved a first-cycle defect in apicoplast branching/division (Figure 3- figure supplement 3). Development of targeted and incisive probes of labile iron within subcellular compartments remains an ongoing challenge in biology (18), especially in the Plasmodium apicoplast where iron uptake, concentration, and utilization remain sparsely understood. To evaluate the impact of DOX on iron availability in the apicoplast, we are developing protein-based probes of lipoic acid and isoprenoid biosynthesis, as these two apicoplast-dependent processes require Fe/S-cluster biosynthesis for activity (17).
In a second model, DOX could bind to additional macromolecular targets within the apicoplast (e.g., a metalloenzyme) via metal-dependent interactions that inhibit essential functions required for organelle biogenesis. Exogenous 500 µM Fe$^{3+}$ would then rescue parasites by disrupting these inhibitory interactions via competitive binding to DOX. This second model would be mechanistically akin to diketo acid inhibitors of HIV integrase like raltegravir that bind to active site Mg$^{2+}$ ions to inhibit integrase activity but are displaced by exogenous metals (19, 20). To test this model, we are developing a DOX-affinity reagent to identify apicoplast targets that interact with doxycycline and whose inhibition may contribute to first-cycle DOX activity.

**Conclusions and implications.** These results critically expand the paradigm for understanding the fundamental mechanisms of DOX activity against *P. falciparum* malaria parasites. These mechanisms include a delayed, second-cycle defect at 1-3 µM DOX that likely reflects inhibition of 70S apicoplast ribosomes, a first-cycle iron-dependent defect within the apicoplast that uniquely operates at 8-10 µM DOX, and a first-cycle iron-independent mechanism outside the apicoplast at ≥20 µM DOX (Figure 1). Pharmacokinetic studies indicate that current 100-200 mg doses of DOX achieve peak human serum concentrations of 6-8 µM over the first six hours which then decrease to 1-2 µM over 24 hours (5). Although current DOX treatment regimens result in delayed parasite clearance in vivo, both apicoplast-specific mechanisms of DOX likely operate over this concentration range and contribute to parasite death (2). These multiple mechanisms of DOX, together with limited antimalarial use of DOX in the field, may explain why parasites with stable DOX resistance have not emerged (3, 4).

There has been a prevailing view in the literature that delayed-death activity is a fundamental limitation of antibiotics like DOX that block apicoplast maintenance (21, 22). Our
results emphasize that DOX is not an intrinsically slow-acting antimalarial drug and support the emerging paradigm (23-25) that inhibition of apicoplast biogenesis can defy the delayed-death phenotype to kill parasites on a faster time-scale. The first-cycle, iron-dependent impacts of 10 µM DOX or 15 µM DFO on apicoplast biogenesis also suggest that this organelle may be especially susceptible to therapeutic strategies that interfere with acquisition and utilization of iron, perhaps due to limited uptake of exogenous iron and/or limited iron storage mechanisms in the apicoplast.

Finally, this work suggests the possibility of repurposing DOX as a faster-acting antiparasitic treatment at higher dosing, whose multiple mechanisms would be expected to limit parasite resistance. Prior studies indicate that 500-600 mg doses in humans achieve sustained serum DOX concentrations ≥5 µM for 24-48 hours with little or no increase in adverse effects (26, 27). DOX is currently contraindicated for long-term prophylaxis in pregnant women and young children, two of the major at-risk populations for malaria, due to concerns about impacts on fetal development and infant tooth discoloration, respectively, based on observed toxicities for other tetracyclines (28). Recent studies suggest that these effects are not associated with short-term DOX use (28, 29), but more work is needed to define the safety parameters that would govern short-term use of DOX for treatment in these populations. Recent development of tetracycline derivatives with improved activities may provide another option to deploy this important class of antibiotics for antimalarial treatment (30).
METHODS

Materials: All reagents were cell-culture grade and/or of the highest purity commercially available. The vendor and catalog number are given for individual compounds when first mentioned.

Parasite culture: All experiments were performed using Plasmodium falciparum Dd2, ACPI-GFP D10 (9), or ACPI-GFP NF54 (10) parasite strains. Parasite culturing was performed as previously described (31) in Roswell Park Memorial Institute medium (RPMI-1640, Thermo Fisher 23400021) supplemented with 2.5 g/L Albumax I Lipid-Rich BSA (Thermo Fisher 11020039), 15 mg/L hypoxanthine (Sigma H9636), 110 mg/L sodium pyruvate (Sigma P5280), 1.19 g/L HEPES (Sigma H4034), 2.52 g/L sodium bicarbonate (Sigma S5761), 2 g/L glucose (Sigma G7021), and 10 mg/L gentamicin (Invitrogen Life Technologies 15750060). Cultures were maintained at 2% hematocrit in human erythrocytes obtained from the University of Utah Hospital blood bank, at 37 °C, and at 5% O2, 5% CO2, 90% N2.

Parasite Growth Assays: Parasites were synchronized to the ring stage either by treatment with 5% D-sorbitol (Sigma S7900) or by first magnet-purifying schizonts and then incubating them with uninfected erythrocytes for five hours followed by treatment with 5% D-sorbitol. Results from growth assays using either of these synchronization methods were indistinguishable, and 5% sorbitol was used for synchronization unless stated otherwise. For continuous growth assays, parasite growth was monitored by diluting sorbitol-synchronized parasites to ~0.5% starting parasitemia, adding additional treatments (antibiotics, IPP, and/or metal salts) at assay initiation, and allowing culture expansion over several days with daily media changes. Growth assays with
doxycycline (Sigma D3447), clindamycin (Sigma C6427), and azithromycin (Sigma 75199) were conducted at 0.2% DMSO at the indicated final drug concentration. Growth assays with ZnCl₂ (Sigma 208086), CaCl₂ (Sigma C4901), MgCl₂ (M8266), FeCl₃ (Sigma 236489), deferoxamine (Sigma D9533), and/or IPP (NH₄⁺ salt, Isoprenoids IPP001) were conducted at the indicated final concentrations. Parasitemia was monitored daily by flow cytometry by diluting 10 µl of each parasite culture well from 2-3 biological replicates into 200 µl of 1.0 µg/ml acridine orange (Invitrogen Life Technologies A3568) in phosphate buffered saline (PBS) and analysis on a BD FACSCelesta system monitoring SSC-A, FSC-A, PE-A, FITC-A, and PerCP-Cy5-5-A channels. For EC₅₀ determinations via dose-response assay, synchronous ring-stage parasites were diluted to 1% parasitemia and incubated with variable (serially two-fold diluted) DOX concentrations ±200 µM IPP, ±50 µM mevalonate (Cayman 20348), ±500 µM FeCl₃, or ±500 µM CaCl₂ for 48-120 hours without media changes. Parasitemia was determined by flow cytometry for 2-4 biological replicates for each untreated or drug-treated condition, normalized to the parasitemia in the absence of drug, plotted as the average ±SD of biological replicates as a function of the log of the drug concentration (in µM), and fit to a 4-parameter dose-response model using GraphPad Prism 8.0. All growth assays were independently repeated 2-4 times on different weeks and in different batches of blood. The 48-hour EC₅₀ values determined from five independent assays for DOX ±IPP were averaged and analyzed by unpaired t-test using GraphPad Prism 8.0.

**Fluorescence Microscopy:** For live-cell experiments, parasites samples were collected at 30-36 or 65 hours after synchronization with magnet purification plus sorbitol treatment (see above). Imaging experiments were independently repeated twice. Parasite nuclei were visualized by incubating samples with 1-2 µg/ml Hoechst 33342 (Thermo Scientific Pierce 62249) for 10-20
minutes at room temperature. The parasite apicoplast was visualized in D10 (9) or NF54 mevalonate-bypass (10) cells using the ACPleader-GFP expressed by both lines. Images were taken on DIC/brightfield, DAPI, and GFP channels using either a Zeiss Axio Imager or an EVOS M5000 imaging system. Fiji/ImageJ was used to process and analyze images. All image adjustments, including contrast and brightness, were made on a linear scale. For indicated conditions, apicoplast morphologies in 20-40 parasites were scored as elongated, focal, or dispersed; counted; and plotted by histogram as the fractional population with the indicated morphology. Analysis of replicate samples indicated standard deviations that were <10% for all samples in the percentage of parasites displaying a given apicoplast morphology in a given condition. Unpaired t-test analysis using GraphPad Prism was used to evaluate the significance of observed population differences.

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**Figure 1.** Scheme of intraerythrocytic *P. falciparum* parasite depicting doxycycline, its canonical delayed-death mechanism at 1 µM inhibiting apicoplast genome translation, the novel metal-dependent mechanism(s) in the apicoplast explored herein at 10 µM, and off-target activity outside the apicoplast at >20 µM.
Figure 2. 10 µM doxycycline kills *P. falciparum* with first-cycle, apicoplast-specific activity. (A) Continuous growth assay of synchronized Dd2 parasites treated with 1 or 10 µM DOX ±200 µM IPP with (B) Giemsa-stained blood smears for days 1-3. (C) 48-hour growth-inhibition curve for DOX-treated Dd2 parasites ±200 µM IPP. (D) Continuous growth assay of synchronized Dd2 parasites treated with 10 – 40 µM DOX and 200 µM IPP. (E) Epifluorescence images of synchronized parasites treated as rings with 10 µM DOX ±200 µM IPP and imaged 36 or 65 hours later (green = ACP1-GFP, blue = nuclear Hoechst stain). Data points in growth assays are the average ±SD of biological replicates. All growth assays were independently repeated 2 – 4 times using different batches of blood (shown in Figure 2- figure supplement 1).

The following figure supplements are available for figure 2:

**Figure supplement 1.** Additional, independent growth assays with DOX and IPP.

**Figure supplement 2.** Additional epifluorescence images of DOX-treated parasites and analysis.
Figure 3. 10 μM DOX kills *P. falciparum* with a first-cycle, metal-dependent mechanism. Continuous growth assays of synchronized Dd2 parasites treated with (A) DOX, clindamycin (CLi), and/or azithromycin (AZM) and (B) 10 μM DOX and 10 μM ZnCl₂ or 500 μM CaCl₂. (C) 48-hour growth inhibition assay of D10 parasites treated with DOX without or with 500 μM FeCl₃ or CaCl₂. (D) Continuous growth assay of synchronized Dd2 parasites treated with 10 μM DOX and 500 μM FeCl₃ or MgCl₂. (E) Epifluorescence images of synchronized parasites treated as rings with 10 μM DOX ±500 μM FeCl₃ or CaCl₂ and imaged 36 hours later (green = ACPL-GFP, blue = nuclear Hoechst stain). (F) Continuous growth assay of synchronized Dd2 parasites treated with 1 μM DOX and 500 μM FeCl₃ or MgCl₂. (G) Continuous-growth assay of synchronized Dd2 parasites treated with 20 or 40 μM DOX and 500 μM FeCl₃. Data points in growth assays are the average ±SD of biological replicates. All growth assays were independently repeated using different batches of blood (shown in Figure 3-figure supplement 1).

The following figure supplements are available for figure 3:

**Figure supplement 1.** Additional independent growth assays with DOX, antibiotics, and metals.

**Figure supplement 2.** Additional images and analysis of parasites treated with DOX and metals.

**Figure supplement 3.** Effect of deferoxamine on parasite growth and apicoplast biogenesis.
**Supplemental Figures**

**Figure 2- figure supplement 1.** Additional, independent growth assays with DOX and IPP. (A) Additional continuous growth assays of synchronized Dd2 parasites treated with 1 or 10 µM DOX ±200 µM IPP. (B) Independent 48-hour growth-inhibition assays for DOX-treated Dd2, D10, NF54 parasites in the absence or presence of 200 µM IPP or 50 µM mevalonate. Dd2 and NF54 parasites were synchronized by single treatment with 5% D-sorbitol. D10 parasites were synchronized by magnet purification of schizonts followed by 5-hour incubation with uninfected erythrocytes and treatment with 5% D-sorbitol. Data points in individual plots are the ave ±sd of 2-4 biological replicates. Each plot reflects an independent assay performed with a different batch of blood. (C) Scatter plot of doxycycline EC₅₀ values from independent assays ±IPP with calculated ave ± sd and analysis by unpaired t-test. (D) Independent continuous growth assay of synchronized Dd2 parasites treated with 10 – 40 µM DOX and 200 µM IPP.
**Figure 2- figure supplement 2.** Additional epifluorescence images of DOX-treated D10 parasites. Magnet plus sorbitol-synchronized parasites were treated as rings with 10 µM DOX ±200 µM IPP and imaged 1 or 3 days later (green = ACP₅-GFP, blue = nuclear Hoechst stain). 20-40 parasites were examined for each treatment condition on each given day for duplicate experiments, and data were plotted as the average percentage of parasites in each population that displayed an elongated, punctate, or dispersed apicoplast GFP signal. For clarity, error bars are not displayed but standard deviations were <10% in all conditions. Cell-percentage differences were analyzed by unpaired t-test (P values in parentheses, ns = not significant).
**Figure 3- figure supplement 1.** Additional, independent growth assays with DOX, other antibiotics, and metals. Additional continuous growth assays of synchronized Dd2 parasites treated with (A) DOX, clindamycin (CLI), and/or azithromycin (AZM); (B) 500 µM of CaCl$_2$, MgCl$_2$, or FeCl$_3$ alone; (C) 10 µM DOX and 5 µM ZnCl$_2$ or 250 µM CaCl$_2$; (D) 10 µM DOX treated with 50 - 500 µM FeCl$_3$ or 500 µM MgCl$_2$. Individual data points are the ave ± sd from 2-4 biological replicates. (E) 96-hour growth-inhibition assays for DOX-treated D10 parasites in the absence or presence of 200 µM IPP, 500 µM FeCl$_3$, or 500 µM CaCl$_2$. Parasites were synchronized by magnet purification of schizonts followed by 5-hour incubation with uninfected erythrocytes and treatment with 5% D-sorbitol. Data points are the ave ±sd of 3 biological replicates.
Figure 3- figure supplement 2. Additional epifluorescence images of D10 parasites treated with DOX and metals. Magnet plus sorbitol-synchronized parasites were treated as rings with 10 µM DOX ±500 µM FeCl₃ or CaCl₂ and imaged 36 hours later (green = ACP₁-GFP, blue = nuclear Hoechst stain). 20-40 parasites were examined for each treatment condition on each given day for duplicate experiments, and data were plotted as the average percentage of parasites in each population that displayed an elongated, punctate, or dispersed apicoplast GFP signal. For clarity, error bars are not displayed but standard deviations were <10% in all conditions. Cell-percentage differences were analyzed by unpaired t-test (P values in parentheses, ns = not significant).
Figure 3- figure supplement 3. Effect of deferoxamine on parasite growth and apicoplast biogenesis. (Left) Continuous growth assay of synchronized Dd2 parasites cultured without or with 15 µM deferoxamine (DFO), 15 µM FeCl₃, or 200 µM IPP. (Right) Fluorescence microscopy of live, synchronized D10 parasites untreated or treated with 15 µM DFO and imaged 36 hours later (green = ACP₇-GFP, blue = nuclear Hoechst stain).