Doxycycline has Distinct Apicoplast-Specific Mechanisms of Antimalarial Activity

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Abstract: Doxycycline (DOX) is a key antimalarial drug that is thought to kill *Plasmodium falciparum* parasites by blocking protein translation in the essential apicoplast organelle. Although parasite resistance to DOX has not emerged, clinical use is primarily limited to prophylaxis due to delayed second-cycle parasite death at 1-3 μM, the DOX concentration readily achieved in human plasma at current dosing. Slow antiparasitic activity is thought to be a fundamental limitation of DOX and other antibiotics that target apicoplast maintenance. We report that slightly higher 8-10 μM DOX concentrations kill parasites with first-cycle activity that blocks apicoplast biogenesis. This faster activity is rescued by exogenous isopentenyl pyrophosphate, an essential apicoplast product, confirming an apicoplast-specific target. Exogenous iron rescues first- but not second-cycle activity by 1 or 10 μM DOX, revealing that first-cycle activity involves a metal-dependent mechanism that is distinct from the canonical delayed-death mechanism. These results provide a new paradigm for understanding fundamental antiparasitic mechanisms of DOX and suggest the possibility of repurposing DOX as a faster-acting antimalarial at higher dosing, which remains well tolerated. Multiple mechanisms of DOX activity would be expected to limit parasite resistance.

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.

The tetracycline antibiotic, doxycycline (DOX), is a key antimalarial drug that is thought to kill eukaryotic *P. falciparum* parasites by inhibiting prokaryotic-like 70S ribosomal translation inside the essential apicoplast organelle (Fig. 1A) (2). Although stable *P. falciparum* resistance to DOX has not emerged, clinical use of DOX 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 plasma with current 100-200 mg dosage (5), continue to grow and replicate for 72-96 hours following treatment initiation and only die at the end of the second 48-hour intraerythrocytic growth cycle when they fail to form viable daughter parasites and expand into a third cycle (Fig. 1B) (2). Slow antiparasitic activity is believed to be a fundamental limitation of DOX and other antibiotics that block apicoplast maintenance (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 (7, 8). We set out to test and understand the mechanism of first-cycle DOX activity.

Prior ex vivo culture studies have shown that 200 μM isopentenyl pyrophosphate (IPP), an essential apicoplast product, rescues the delayed-death activity of 1-3 μM DOX, confirming an

apicoplast-specific target (Fig. 1B) (7). Using 48-hour growth inhibition and continuous growth assays, we confirmed that DOX concentrations >5 μM killed parasites with first-cycle activity (Fig. 1B & 1C). We observed that IPP shifted the 48-hour EC_{50} value from 4 ± 1 to 12 ± 3 μM (average ± SD, 4 biological replicates) (Fig. 1C), suggesting that first-cycle growth defects from 5-10 μM DOX involve an apicoplast-specific mechanism but that DOX concentrations >10 μM cause off-target defects outside this organelle. Using a continuous growth assay, we observed that IPP fully or nearly fully rescued first-cycle growth inhibition by 10 μM but not 20 or 40 μM Dox (Fig. 1B & 1D). We conclude that 10 μM DOX kills *P. falciparum* in the first growth cycle by an apicoplast-specific mechanism.

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_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 (Fig. 1E). Rescue by 200 μM IPP produced second-cycle parasite progeny with a dispersed GFP signal indicative of apicoplast loss (Fig. 1E). We conclude that 10 μM DOX blocks apicoplast biogenesis in the first treatment cycle.

What is the molecular mechanism of faster apicoplast-specific activity by 10 μM DOX? We first considered the simple 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 concentration. This model predicts that treating parasites with a combination of distinct apicoplast-translation inhibitors at delayed death-inducing concentrations 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/or 500 nM azithromycin and monitored growth over 3 intraerythrocytic cycles. Individual treatment with each antibiotic alone produced major growth defects in the third 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 (Fig. 2A). 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.

Tetracycline antibiotics such as DOX tightly chelate a wide variety of di- and trivalent metal ions via their siderophore-like arrangement of exocyclic hydroxyl and carbonyl groups (Fig. 1A), with a reported affinity series of Fe^{3+}>Fe^{2+}>Zn^{2+}>Mg^{2+}>Ca^{2+} (11, 12). Indeed, tetracycline interactions with Ca^{2+} and Mg^{2+} 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 10 μM DOX. We observed no rescue by 10 μM $ZnCl_2$ (Zn^{2+} toxicity limit (14)) or 500 μM $CaCl_2$ (Fig. 2B). However, 500 μM $FeCl_3$ (and to a lesser extent 500 μM $MgCl_2$) fully or nearly fully rescued first-cycle growth inhibition by 10 μM DOX (Fig. 2C), but parasites still succumbed to second-cycle death in 1 or 10 μM DOX. We also observed that 500 μM $FeCl_3$ but not $CaCl_2$ rescued the first-cycle apicoplast-branching defect in 10 μM DOX (Fig. 2D). The

inability of 500 μM $FeCl_3$ to rescue second-cycle death by 1 or 10 μM DOX rules out an alternative model that iron prevents DOX uptake into the apicoplast. We also noted that $FeCl_3$ selectively rescued the apicoplast-specific first-cycle effects of 10 μM but not the off-target effects of 20-40 μM DOX (Fig. 2E). We conclude that 10 μM DOX kills parasites via a metal-dependent first-cycle mechanism that is distinct from the second-cycle delayed-death mechanism of 1 μM DOX.

What is the metal-dependent mechanism of 10 μM DOX, and why is first-cycle inhibition preferentially rescued by $FeCl_3$? Tetracyclines bind iron more tightly than other metal cations, with an equilibrium association constant of 10^{10} M^{-1} for 1:1 chelation of Fe^{3+} versus 10^4 M^{-1} for Mg^{2+} (11). There are at least two distinct models to explain the metal-dependent effects of 10 μM DOX, both of which could be operative. 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 (15). In this first model, rescue by exogenous $FeCl_3$ would be due to restoration of iron bioavailability.* Second, DOX could bind to additional macromolecular targets within the apicoplast (e.g., a metalloenzyme) via metal-dependent interactions that inhibit essential biomolecular functions required for organelle biogenesis. Exogenous 500 μM Fe^{3+} would then rescue parasites by disrupting these inhibitory interactions via competitive binding to and displacement of DOX. This second model would be mechanistically akin to diketo HIV integrase inhibitors like raltegravir that bind to active site Mg^{2+} ions to inhibit integrase activity but are displaced by exogenous metals (16, 17). We have on-going experiments to test and distinguish these possible mechanisms.

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*In this model, modest rescue by 500 μM $MgCl_2$ may reflect competitive displacement of DOX-bound iron to restore iron bioavailability. RPMI growth medium already contains ~400 μM Mg^{2+} prior to adding $MgCl_2$. Mg^{2+} availability is thus unlikely to be directly limited by 10 μM DOX.

These results provide a new 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 metal-dependent defect within the apicoplast that uniquely operates at 8-10 μM DOX, and a first-cycle metal-independent mechanism outside the apicoplast at ≥20 μM DOX. 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 it is clear that 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). The multiple mechanisms of DOX may explain why stable parasite resistance has not emerged (3, 4).

These results also clarify that DOX is not an intrinsically slow-acting antimalarial drug and that inhibition of apicoplast biogenesis can defy the delayed-death phenotype to kill parasites on a faster time-scale. The first-cycle, iron-dependent impact of 10 μM DOX on apicoplast biogenesis suggests that this organelle may be especially susceptible to therapeutic strategies that interfere with acquisition and utilization of iron and/or other metals. Finally, this work suggests the possibility of repurposing DOX as a faster-acting antiparasitic treatment at higher dosing. 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 (18, 19).

METHODS. Culture of *P. falciparum* Dd2, D10, and NF54 strains was performed as previously described (20). Growth assay data points are the average ±SD of technical replicates. All growth assays were independently repeated 2-4 times. All reagents were purchased from Sigma.

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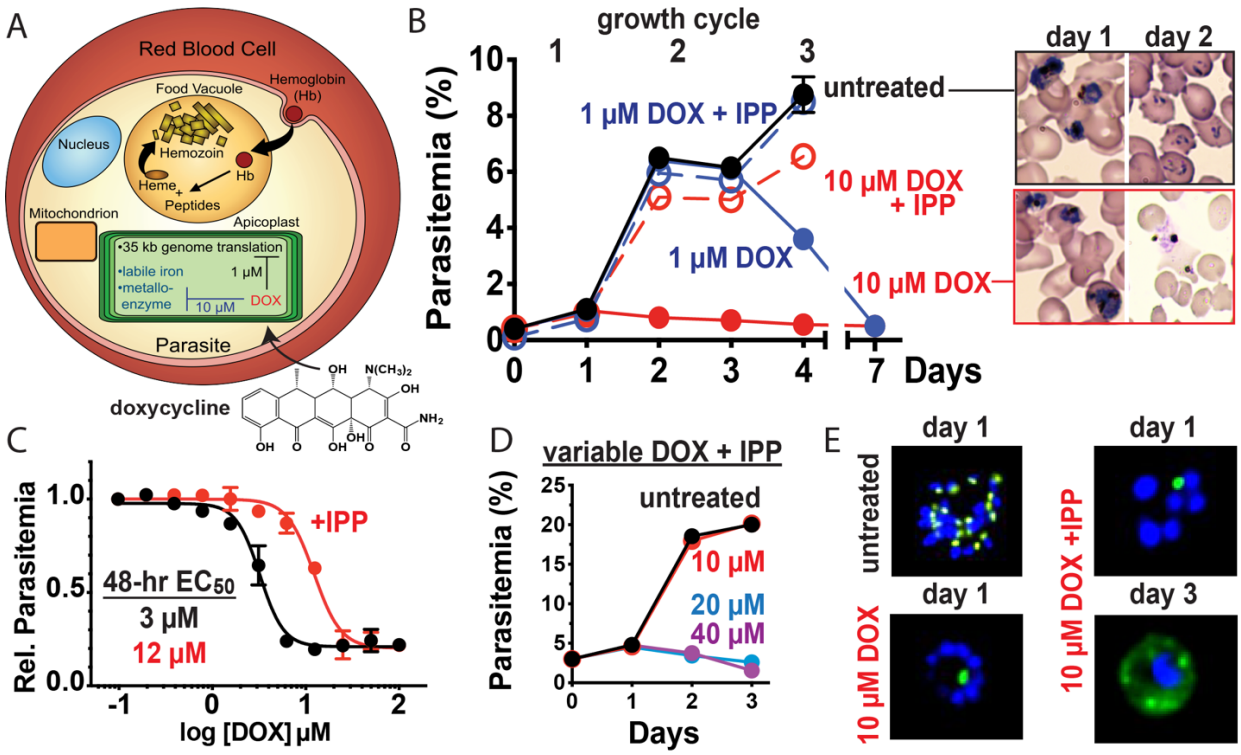

Figure 1. 10 μM doxycycline kills *P. falciparum* with first-cycle, apicoplast-specific activity. (A) Scheme of intraerythrocytic parasite. (B) Continuous growth assay of synchronized Dd2 parasites treated with 1 or 10 μM DOX ±200 μM IPP with Giemsa-stained blood smears. (C) 48-hour growth inhibition curve for DOX-treated Dd2 parasites. (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 = ACP_L-GFP, blue = nuclear Hoechst stain).

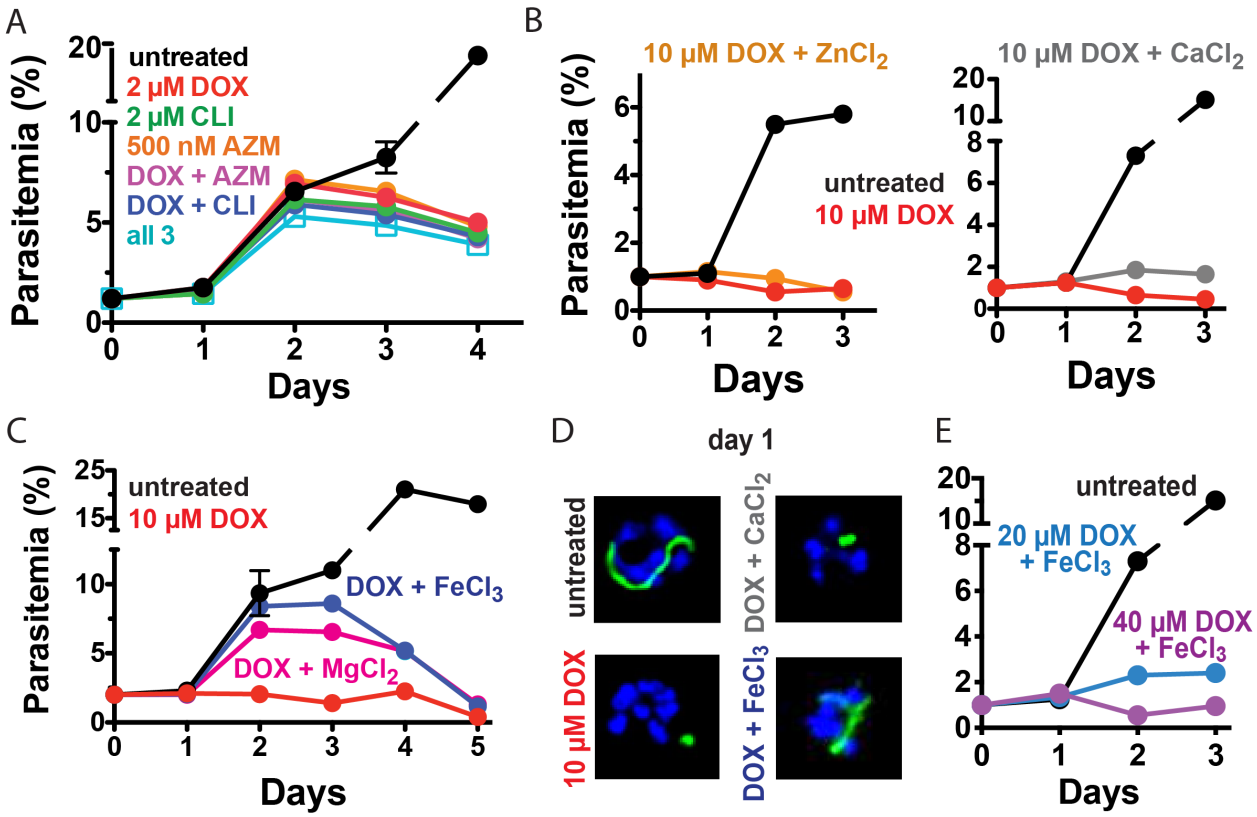

Figure 2. 10 μM DOX kills *P. falciparum* with a first-cycle, metal-dependent mechanism. Continuous growth assay of synchronized Dd2 parasites treated with (A) DOX, clindamycin (CLI), and/or azithromycin (AZM); (B) 10 μM DOX and 10 μM $ZnCl_2$ or 500 μM $CaCl_2$; or (C) 10 μM DOX and 500 μM $FeCl_3$ or $MgCl_2$. (D) Epifluorescence images of synchronized parasites treated as rings with 10 μM DOX ±500 μM $FeCl_3$ or $CaCl_2$ and imaged 36 hours later (green = ACP_L-GFP, blue = nuclear Hoechst stain). (E) Continuous growth assay of synchronized Dd2 parasites treated with 20 or 40 μM DOX and 500 μM $FeCl_3$.