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The effects of anti-bacterials on the malaria parasite *Plasmodium falciparum*

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Abstract

Many anti-bacterial drugs inhibit growth of malaria parasites by targeting their bacterium-derived endosymbiotic organelles, the mitochondrion and plastid. Several of these drugs are either in use or being developed as therapeutics or prophylactics, so it is paramount to understand more about their target of action and modality. To this end, we measured *in vitro* growth and visualized nuclear division and the development of the mitochondrion and apicoplast in *Plasmodium falciparum* treated with five drugs targeting bacterial housekeeping pathways. This revealed two distinct classes of drug effect. Ciprofloxacin, rifampicin, and thiostrepton had an immediate effect: slowing parasite growth, retarding organellar development and preventing nuclear division. Classic delayed-death, in which the drug has no apparent effect until division and reinvasion of a new host by the daughter merozoites, was only observed for two drugs: clindamycin and tetracycline. These cells had apparently normal division and segregation of organelles in the first cycle but severe defects in apicoplast growth, subtle changes in the mitochondrion and a failure to complete cytokinesis during the second cycle. In two cases, the drug response in *P. falciparum* directly conflicted with reported responses for the related parasite *Toxoplasma gondii*, suggesting significant differences in apicoplast biology between the two parasites.

Keywords: Apicomplexa; *Plasmodium falciparum*; Apicoplast; Mitochondrion; Anti-bacterial; Delayed-death

1. Introduction

Malaria remains a major global health problem and resistance to existing drugs is lending increasing urgency to the search for new anti-malarials. Numerous anti-bacterials kill malaria parasites [1–4], and some antibiotics such as clindamycin and the tetracyclines are now commonly used in malaria prophylaxis or as components of multiple drug therapies [5–8]. However, despite their growing importance in clinical settings, we understand relatively little about the targets and cellular effects of these parasiticidal anti-bacterials. Endosymbiotic organelles derived from bacteria are the likely target of these drugs and malaria parasites contain both mitochondria and plastids that originate from bacterial symbionts, but whether the anti-bacterials target either or both of these organelles and at what stage of the malaria parasite life cycle remains largely unknown.

The recent identification and characterization of a vestigial chloroplast (apicoplast) in malaria parasites revealed putative targets for many of these anti-bacterial drugs and potentially rationalises their previously mysterious activities [9–11]. The apicoplast is essentially a reduced cyanobacterium living inside malaria parasites, and it has typical bacterial housekeeping machinery such as DNA replication, transcription, translation and post-translational modification as well as anabolic pathways for synthesis of fatty acids, isoprenoid precursors, haem and iron sulfur complexes.

A selection of anti-bacterials targeting these pathways have already been shown to kill malaria parasites [2,12], but rigorous target validation in malaria parasites has only been done for three of these drugs: ciprofloxacin, rifampicin and, thiostrepton. Ciprofloxacin targets the *Plasmodium falciparum* apicoplast genome, inhibiting DNA replication [13]. Rifampicin blocks bacterial type transcription and abrogates mRNA production by the apicoplast-encoded RpoB gene [14,15]. Thiostrepton inhibits translation of apicoplast-encoded TufA [16] and interacts specifically with the apicoplast ribosome [17,18]. Another bacterial translation blocker, tetracycline, is reported to interfere

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with mitochondrial function [19,20] but it is not known whether or not it also impacts apicoplast translation. However, there is a significant body of indirect evidence suggesting that the tetracycline derivative doxycycline impacts the apicoplast [29]. The target of clindamycin (another bacterial translation blocker) has not been examined in malaria parasites but in the related parasite *Toxoplasma gondii*, which is frequently used as a model for *Plasmodium*, clindamycin targets the large subunit of the apicoplast rRNA [21]. The marked similarity between the *Plasmodium* and *Toxoplasma* apicoplast supports the assumption that clindamycin also targets the apicoplast in malaria parasites.

An intriguing finding of early trials of anti-bacterials on malaria parasites was a dramatic increase in drug potency with prolonged exposure [22–24]. For example, extending clindamycin treatment from 48 to 96 h lowers the inhibitory concentration (IC_{50}) by three to four orders of magnitude [22]. In other words, it takes 1000–10,000 times as much drug to kill the parasites in 2 days as it does to kill them in 4 days [22]. This dramatic increase in potency with time is known as the delayed-death phenomenon and has been most thoroughly described in *T. gondii* [25–28]. Delayed-death refers to the fact that *T. gondii* parasites treated with an anti-apicoplast drug initially show no ill effects, growing and dividing at normal pace. Remarkably, the drugged parasites produce normal numbers of daughter parasites within the initial host cell, and these tachyzoite progeny emerge and each invades new host cells. However, at this stage, having entered a new host, the parasites fail to grow and eventually die [25,26]. The biological basis for this unusual drug effect remains elusive, but it has substantial implications for drug therapy strategies.

The delayed-death model from *T. gondii* has been applied somewhat indiscriminately in discussions of *Plasmodium*, and it has been assumed that delayed-death will also occur in malaria parasites treated with anti-bacterials targeting the equivalent apicoplast housekeeping processes. However, thus far only drugs targeting prokaryote-like translation have been shown to have increased potency with prolonged exposure in *P. falciparum* [22], and for only two of these, clindamyacin and doxycycline, is there evidence showing a delayed-death response in *Plasmodium* [29,30]. It remains an open question as to what effect other putative apicoplast targeting drugs have on the malaria parasite.

Drugs targeting anabolic or non-housekeeping apicoplast-localized processes, such as the synthesis of isoprenoids and fatty acids, cause immediate parasite death within the first intra-erythrocytic cycle [1,4,31]. As a further complication, some inhibitors are most active at certain stages of the parasite life cycle [31,32] while other drugs can cause the parasite to arrest at particular life stages [33,34]. Therefore, what constitutes a delayed-death response and which drugs show this type of effect remains largely unexplored. Despite this lack of data, delayed-death remains an important consideration when the development of new apicoplast-targeted anti-malarials is discussed, reflecting the clinical importance of the time frame of drug activity.

To address the significant gaps in our understanding of the cell biological effects of anti-bacterial drugs in *P. falciparum*, we decided to assess the response of asexual stage parasites to a selection of anti-bacterials. We focused on drugs believed to target DNA replication, transcription and protein translation in the apicoplast (Fig. 1A); so-called "housekeeping" functions whose disruption has generally been assumed to result in delayed-death effects. Here we describe the impact of these drugs on growth of synchronous cultures of parasites across two intra-erythrocytic life cycles. We also depict the effect of each drug on nuclear division, apicoplast development and mitochondrial development at each stage of growth to establish where drug impact takes place and what the consequences for cell cycle are.

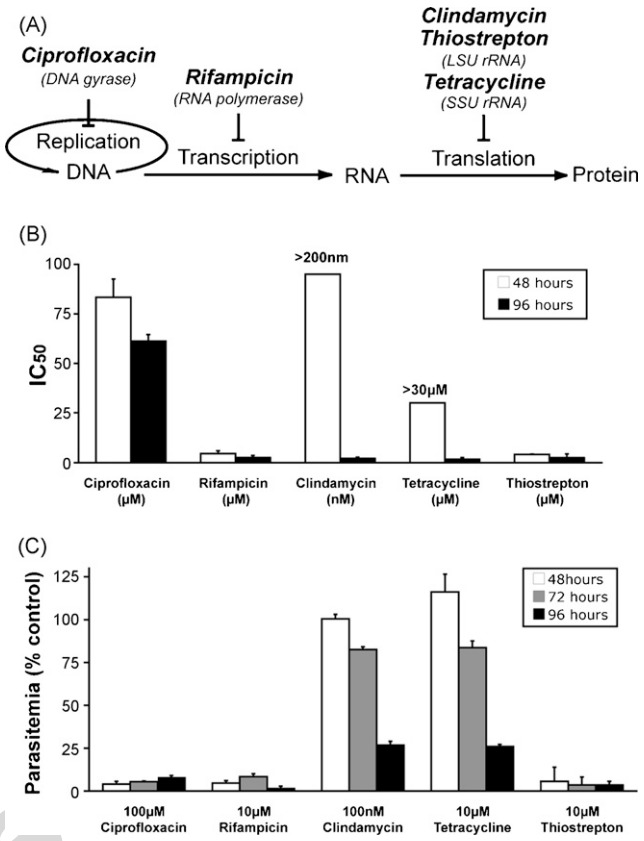


Fig. 1. Anti-bacterial drugs targeting the "housekeeping" functions of the apicoplast display two types of drug effect kinetics. (A) Schematic of the "housekeeping" functions of the *P. falciparum* apicoplast showing the targets of the anti-bacterial drugs used in this study. (B) IC_{50} values for *in vitro* drug trials over 96 h. No effect was detectable at 48 h for the highest concentrations of tetracycline (30 μM) and clindamycin (200 nM) tested. (C) Growth of *in vitro* cultures of *P. falciparum* over 96 h in the presence of "housekeeping" inhibitors at concentrations corresponding to the IC_{99}.

2. Materials and methods

2.1. Parasite strains and cell culture

P. falciparum strains D10, D10 ACP_L:GFP and D10 CS_L:YFP–ACP_L:RFP [35,36] were grown according to standard protocols [37] under appropriate drug selection. Parasites were synchronized with 5% sorbitol according to [38]. To obtain tightly synchronised cultures, two sorbitol treatments were carried out 12 h apart. Approximately 48 h after the initial synchronization, the cultures were synchronized again to eliminate any residual schizonts.

2.2. Drug trials

All chemicals were purchased from Sigma–Aldrich. Inhibitors were diluted to 2000× the maximum assay concentration in ethanol (ciprofloxacin, clindamycin), 70% ethanol, 30% water (tetracycline), methanol (rifampicin) or DMSO (thiostrepton). Drug trials were carried out in triplicate in 24 well plates, with each well containing 2% washed red blood cells (rbcs) and the desired drug concentration in 2 ml of media with 0.2% parasitemia. Seventy-five percent of the media was replaced prior to sample removal at 48, 72 and 96 h. Triplicate 100 μl samples of resuspended culture were transferred to 96 well plates for analysis. Parasitemia was assessed using a previously described fluorescence assay [39] modified by a reduction in the quantity of Sybr Green in the assay buffer to 0.1 μl/ml and normalization against an rbc only control. Fluorescence was assessed with an Ascent fluoroscan microplate reader (Labsystems). The solvents alone had no effect on parasite growth at the final concentrations used.

2.3. Microscopy

For parasite life stage counting, infected rbcs were fixed in methanol and stained in 5% Giemsa. Parasites were visualized on an Olympus microscope and a minimum of 10 fields and 1000 rbcs were counted for each time point. For live cell microscopy, parasite nuclei were labelled for 20 min with 20 μg/ml Hoechst 33258 and visualized with a Leica confocal microscope. To assess apicoplast segregation, 3 replicates of a minimum of 100 rbcs infected with ACP_LGFP parasites [36] were assessed for the presence of nuclear DNA (Hoechst 33258) and apicoplast fluorescence.

2.4. Western blot analysis

Serial samples for protein gel blot (Western) analysis were collected from a single culture at 12-h intervals and prepared as previously described [40]. Membranes were probed with mouse anti-GFP (1:2000; Roche) and rabbit anti-*P. falciparum* BiP antibody (1:1000) obtained through the Malaria Research and Reference Resource Center, NIH (MRA-20, contributed by John Adams; [41]) Primary antibody binding was detected using goat anti-mouse and goat anti-rabbit antibody conjugated to horseradish peroxidase (Pierce).

3. Results

We set out to assess the nature of the effects of a number of anti-bacterial drugs that were predicted to target the "housekeeping" functions of the apicoplast (Fig. 1A). Given its clinical significance, we were particularly interested in the delayed-death response. To address the issue of variable drug effects at different life stages we used strict criteria to define delayed-death. Inhibitory compounds were applied to tightly synchronized cells within 4 h of invasion of new red blood cells, and parasite growth was assayed 48, 72 and 96 h later. A drug was only considered to cause delayed-death if there was no measurable growth inhibition after 48 h (first asexual cycle) of treatment with drug concentrations more than 10-fold higher than those needed to inhibit 50% of parasite growth following a further asexual cycle (96 h). Further, for the delayed effect to be considered delayed-death, parasite growth inhibition had to be insensitive to the presence or absence of drug in the second asexual cycle.

Under these stringent conditions, two distinct classes of drug effect are apparent (Fig. 1B and C). As previously reported [29,30] clindamycin and tetracycline showed stereotypical delayed-death. There is no observable effect on parasite growth in the first asexual cycle (48 h) even at drug concentrations greater than 10 times the IC_{50} measured after 96 h (200 nM versus 12.5 nm for clindamycin and 30 μM versus 2.5 μM for tetracycline). Drug efficacy increases during the second cycle (72 and 96 h), reflecting the progressive death of drug-treated daughter parasites during the course of their new infection in a second host cell (Fig. 1B and C). Parasite death occurred irrespective of whether clindamycin was present or absent from 48 to 96 h (Figs. 1C and 2B). It was somewhat surprising to note that tetracycline treatment also caused a delayed-death effect indistinguishable from that of clindamycin (Fig. 1B and C) as this is in stark contrast to the immediate effect of tetracycline reported in *T. gondii* [25,26].

The DNA gyrase inhibitor ciprofloxacin, which produces delayed-death in *T. gondii* [26], significantly inhibited malaria parasite growth within the first 48 h (Fig. 1B and C) again demonstrating a fundamental difference between parasite responses to drug inhibition. The kinetics of growth inhibition resulting from treatment with the RNA polymerase inhibitor rifampicin were similar to those seen in ciprofloxacin as were those of the translation inhibitor thiostrepton (Fig. 1B and C). The immediate effects of thiostrepton treatment contrasts with the effect of the other translation inhibitors tested (tetracycline and clindamycin).

To characterize the cellular effects of these drugs, we counted Giemsa-stained smears of drug-treated cultures to track progress through the stages of the *P. falciparum* life cycle. We also utilized transgenic parasites expressing nuclear encoded red fluorescent protein (mRFP) and enhanced yellow fluorescent protein (eYFP) constructs targeted to the apicoplast and mitochondria, respectively [35] to assess the impact these drugs have on the morphology and development of their two potential target organelles.

Under clindamycin and tetracycline treatment, progression through the first cell cycle was indistinguishable from that seen in untreated cells (Fig. 2A–C), as was the development of apicoplasts and mitochondria (Fig. 2D–F). The apicoplast divided normally and alignment of the organelles and nuclei occurred in the canonical manner (Fig. 2D–F). In the second cycle (48–96 h) the drug-treated parasites progressed normally to the trophozoite stage, but most failed to continue to schizogony (Fig. 2A–C). While some cells remained viable for a further 48 h, none of the drug-treated parasites were able to establish a successful third cycle infection, as evidenced by the total lack of ring stage parasites observed after 96 h (Fig. 2A–C).

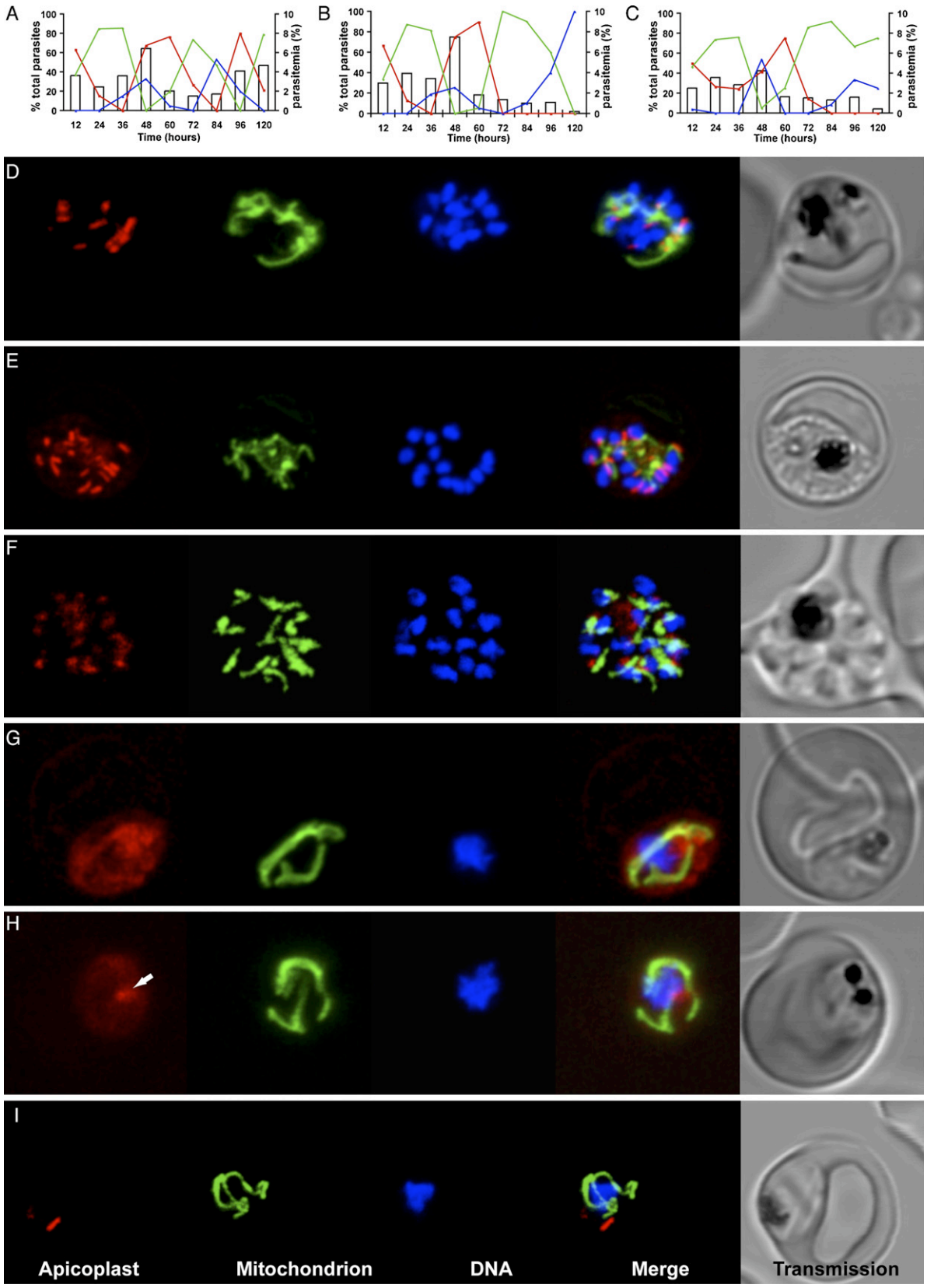

A
B
C
% total parasites
parasitemia (%)
Time (hours)
12 24 36 48 60 72 84 96 120
0 20 40 60 80 100
0 2 4 6 8 10
D
E
F
G
H
I
Apicoplast
Mitochondrion
DNA
Merge
Transmission

Table 1
Segregation of apicoplasts into daughter parasites after treatment for 48 h with clindamycin and tetracycline

Treatment	Cells with apicoplast (%)	Standard error
Control	98.87853692	0.753358023
100 nM clindamycin	96.60130719	2.239456431
10 μM tetracycline	96.75925926	2.080759727

Effects of delayed-death drugs clindamycin and tetracycline on apicoplast morphology are stark in the latter stages of the second cycle. Healthy looking spherical apicoplasts are clearly visible in the early stages of the second cycle, but as parasite development progresses the apicoplast becomes less distinct. In some cases a small spherical apicoplast persists in the presence of diffuse fluorescent background while in others the apicoplast is no longer recognizable as a distinct organelle (Fig. 2G and H). Counts of parasites shortly after the start of the second asexual cycle show no difference in terms of apicoplast content between the treated parasites and untreated controls (Table 1). This confirms that the absence of a recognizable apicoplast in later stages is not the result of missegregation of the apicoplast during merozoite formation during the first cycle.

The morphology of the mitochondrion is also affected by treatment with clindamycin and tetracycline, although these changes are more subtle. Seventy-two hours after the start of drug treatment, this organelle appears thicker with more limited branching in the drug-treated cells (Fig. 2G–I) and retains this unusual morphology through 96 h (data not shown). It is interesting to note that nuclear division continues in the drug-treated cells, albeit at a slower pace (data not shown). Despite the presence of multinucleate cells among the clindamycin and tetracycline treated parasites, neither the apicoplast nor the mitochondrion was observed to divide, merozoites never formed and no third-generation rings were seen.

A possible explanation for the lack of a detectable apicoplast in the second asexual cycle, and indeed for the ultimate death of the parasite, is a disruption of protein transport into the organelle. To test this we collected total protein from parasites (ACP_LGFP) expressing GFP targeted to the apicoplast via a fusion with the leader sequence of acyl carrier protein (ACP) [36]. In healthy parasites with a fully functional import process we normally observe two species of the reporter protein: an abundant processed GFP from which the targeting signal has been cleaved, and a much less abundant precursor with the transit peptide still attached that is en route to the apicoplast. This pattern can be clearly seen in untreated cells and in the first cycle of the tetracycline and clindamycin treated cells (Fig. 3A–C). However,

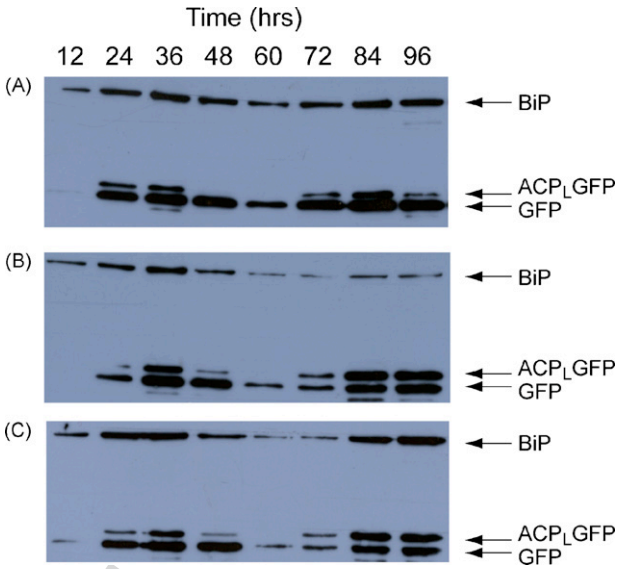


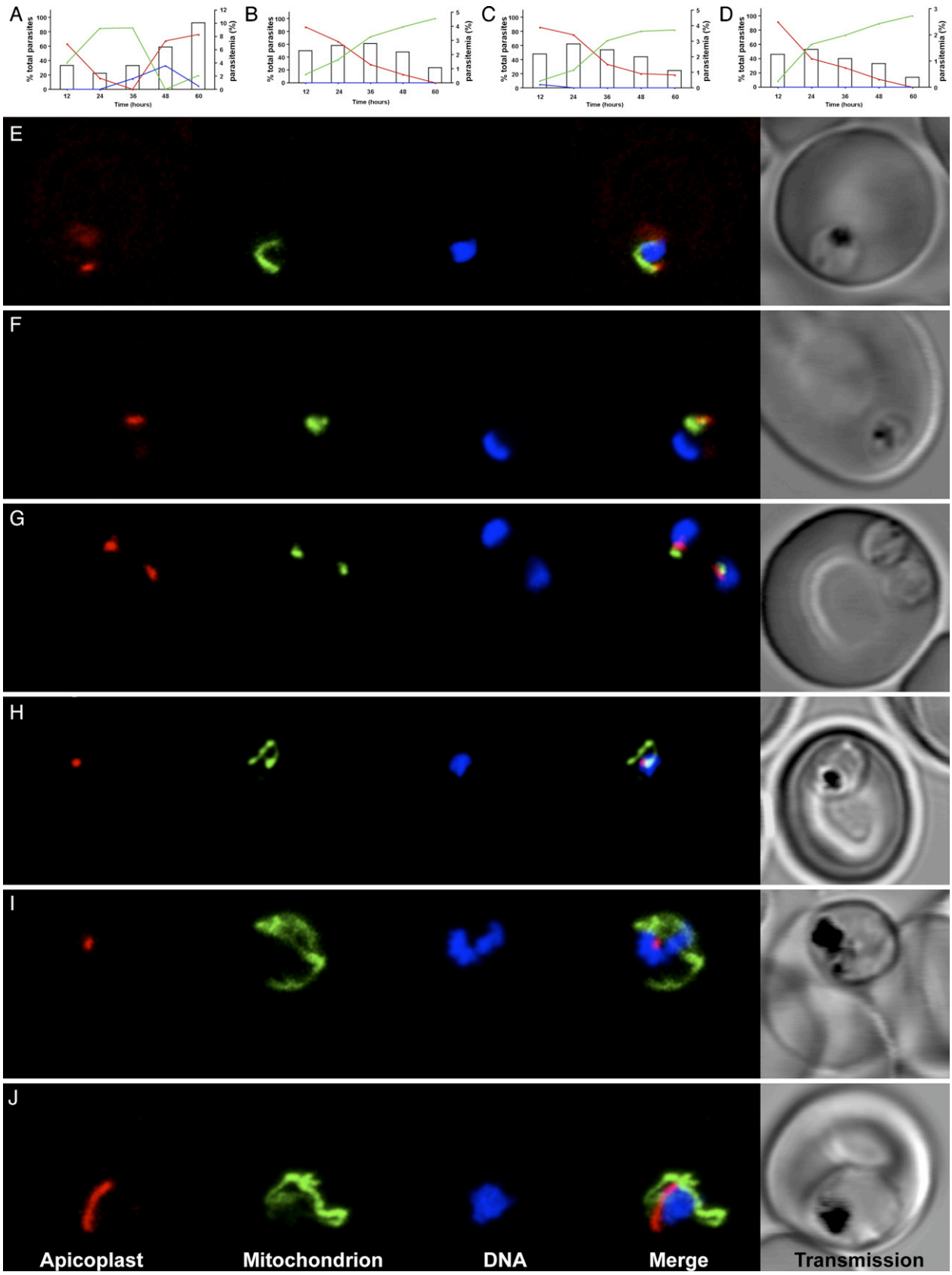
Fig. 3. The transport of proteins into the apicoplast is inhibited by clindamycin and tetracycline treatment but only in the second intra-erythrocytic cycle. Protein gel blots of *P. falciparum* expressing ACP_LGFP probed with monoclonal anti-GFP antibody and polyclonal anti-BiP antibody (loading control). (A) Untreated parasites. (B) Parasites treated from 0 to 48 h with 100 nM clindamycin and grown from 48 to 96 h without inhibitor. (C) Parasites treated from 0 to 48 h with 10 μM tetracycline and grown from 48 to 96 h without inhibitor.

during the second asexual cycle in the drug-treated cells, protein import into the apicoplast is severely disrupted, with the unprocessed protein (upper band) being of equal intensity to the processed protein (lower band) during the period of active translation (Fig. 3B and C).

In contrast to the delayed-death observed in response to clindamycin and tetracycline, treatment with a diverse array of other drugs targeting apicoplast genome replication, transcription and translation inhibits growth in the first asexual cycle (Fig. 1B and C). The cellular response to this diverse collection of drugs is remarkably consistent, showing a delay in the transition from ring stage to trophozoite stage and the subsequent failure to progress to schizogony (Fig. 4A–D).

Microscopic examination of the parasites shows significant defects in parasite growth. At 48 h post-invasion, untreated parasites are in the late stages of schizogony. These cells have multiple nuclei, a divided apicoplast and a highly branched mitochondrion (Fig. 2D). In contrast, parasites treated with ciprofloxacin, rifampicin or thiostrepton have a single spherical apicoplast and only minimally elongated mitochondria (Fig. 4E–G). Overall, the size and morphology of parasites treated with these drugs for 48 h is similar to untreated parasites in the early trophozoite stage seen ~20–24 h post-invasion

Fig. 2. Clindamycin and tetracycline treatment results in "delayed-death" and significant defects in the apicoplast and mitochondrial morphology. (A) Counts of Giemsa-stained blood smears of untreated *P. falciparum* cultures. (B) Counts of Giemsa-stained blood smears of cultures containing 100 nM clindamycin from 0 to 48 h and without inhibitor from 48 to 120 h. (C) Counts of Giemsa-stained blood smears of cultures containing 10 μM tetracycline from 0 to 48 h and without inhibitor from 48 to 120 h. White bars: total parasitemia; red lines: percent of parasites at ring stage; green lines: trophozoite stage; blue line: schizont stage. All parasites were subcultured 5:1 at 48 h and the untreated cultures were subcultured 5:1 at 96 h. (D) Scanning confocal microscopy images of live *P. falciparum* expressing ACP_LRFP (apicoplast—red) and CS_LYFP (mitochondria—green) and stained with Hoescht 33342 (DNA—blue) after 48 h without drug treatment. (E) Images taken after 48 h treatment with 100 nM clindamycin. (F) Images taken after 48 h treatment with 10 μM tetracycline. (G) Images of clindamycin treated parasite after 72 h. (H) Images of tetracycline treated parasite after 72 h. Arrow indicates spherical body which may be the apicoplast. (I) Images of untreated parasite after 72 h.


A
B
C
D
% total parasites
parasitemia (%)
Time (hours)
12
24
36
48
60
E
F
G
H
I
J
Apicoplast
Mitochondrion
DNA
Merge
Transmission

(Fig. 4H). At this stage, untreated parasites have spherical apicoplasts and elongated mitochondria (Fig. 4H). While it is difficult to determine whether the arrested development of the organelles is a cause or effect of the slower growth of the parasite, examination of the few cells managing to survive to 72 h under drug treatment suggests that parasites continue to develop. At this stage, treated cells appear similar to untreated cells after 30 h, complete with nuclear division and mitochondrial elongation, but without the expected elaboration of the apicoplast (Fig. 4I and J). This suggests a direct effect on the apicoplast in addition to the overall growth retardation. Infected erythrocytes were not detectable in blood smears after 96 h of treatment with these drugs.

4. Discussion

The effects of drugs proposed to target the "housekeeping" functions of the apicoplast fall into two broad categories. The more common response to such drugs, seen in parasites treated with DNA replication, transcription and *some* translation inhibitors, is a disruption of parasite development during the first asexual cycle. This manifests itself as a retarded transition from rings to trophozoites or a reduced rate of development during the trophozoite stage (Fig. 4A–I). The development of the mitochondria is inhibited, with normal elongation and branching only occurring in the few parasites that survive beyond 48 h of drug treatment. The apicoplast does not, however, appear to elongate or branch whatsoever—even in cells with an overall morphology similar to late-stage trophozoites, when such apicoplast development generally begins (Fig. 4I and J). These parasites never complete schizogony or produce merozoites (Fig. 4A–D).

In contrast to the immediate effects of ciprofloxacin, rifampicin and thiostrepton, treatment with the translation inhibitors clindamycin and tetracycline results in the delayed-death drug response (Fig. 1B and C). Parasite growth and merozoite formation are normal during the first asexual cycle under drug treatment (Fig. 2A–F) and red blood cells can be successfully invaded by the daughter merozoites (Table 1). It is only during the subsequent infection that the response to the drug occurs. Although some nuclear DNA replication occurs in this second cycle, the parasites fail to progress through schizogony, merozoite formation is not observed and no third-generation ring stage parasites are produced (Fig. 2A–C). There are severe defects in the morphology of the apicoplast (Fig. 2G–H), and a disruption of protein transport into the apicoplast (Fig. 3A–C). It is not clear whether the inhibition of transport is the cause of the observed apicoplast effects, or results from them, or both. The mitochondrion is also affected. In the asexual cycle following drug treatment, the branching pattern of this organelle is less elaborate and mitochondria develop a thickened appearance (Fig. 2G–I).

Which of these drug effects – immediate death or delayed-death – result directly from disruption of the apicoplast? Two factors are important in determining the efficacy of drugs targeting parasite organelles: the susceptibility of the target enzyme to inhibition, and the ability of the drug to reach the target. Genomic data and *in vitro* enzymatics can address target susceptibility but target accessibility depends on the physical characteristics of the cells and organelles. Even when such physical characteristics are well understood, their impact is difficult to predict. Therefore, identifying specific drug targets requires *in vivo* assessment of drug effects on the putative target pathways.

This type of *in vivo* data is available for the effects of the DNA gyrase inhibitor ciprofloxacin. In both *Toxoplasma* and *Plasmodium* ciprofloxacin specifically disrupts apicoplast DNA replication and has no effect on the nuclear or mitochondrial genomes [13,26]. Ciprofloxacin is thus assumed to specifically target the apicoplast. In *Toxoplasma*, ciprofloxacin treatment clearly results in delayed-death [26] whereas we show here that ciprofloxacin has an immediate effect in *P. falciparum* (Fig. 4). This apparent contradiction may reflect differences in the structure and replication process of the apicoplast genome between the two parasites. The *P. falciparum* apicoplast genome exists as closed circles whereas *T. gondii* apicoplast DNA comprises principally linear tandem arrays [13,42]. Both apicoplasts share the rolling circle type of DNA replication but *P. falciparum* also has a twin D-loop replication mechanism absent from *T. gondii*, and the latter mechanisms appears to be most sensitive to ciprofloxacin [13]. Whether these differences in apicoplast genome organization and replication explain the differences seen in parasite response to ciprofloxacin remains an open question.

Treatment with rifampicin specifically inhibits apicoplast transcription [14,15] and we show immediate death in *P. falciparum*. Mitochondrial transcription in *P. falciparum* is likely accomplished by a rifampicin-insensitive phage type RNA polymerase, so this drug is assumed not to perturb mitochondria directly [43]. Rifampicin has also been shown to target ABC type transporters in the mammalian liver, and the antagonistic effect observed when rifampicin is used in combination with chloroquine or quinine has prompted speculation that rifampicin might impact parasite transporters [44,45]. It is difficult to interpret the importance of these *in vivo* studies, however, as rifampicin increases liver activity and may act by reducing the effective concentration of the partner drug [45]. Further, the similarity of drug effects seen for ciprofloxacin, thiostrepton and rifampicin

Fig. 4. Treatment with ciprofloxacin, rifampicin and thiostrepton slows parasite growths and prevents apicoplast elongation and branching. (A) Counts of Giemsa-stained blood smears of untreated *P. falciparum* cultures. (B) Counts of Giemsa-stained blood smears of cultures containing 100 μM ciprofloxacin from 0 to 60 h. (C) Counts of Giemsa-stained blood smears of cultures containing 10 μM rifampicin from 0 to 60 h. (D) Counts of Giemsa-stained blood smears of cultures containing 10 μM thiostrepton from 0 to 60 h. White bars: total parasitemia; red lines: percent of parasites at ring stage; green lines: trophozoite stage; blue line: schizont stage. (E) Scanning confocal microscopy images of live *P. falciparum* expressing ACP_LRFP (apicoplast—red) and CS_LYFP (mitochondria—green) and stained with Hoescht 33342 (DNA—blue) after 48 h treatment with 100 μM ciprofloxacin. (F) Images taken after 48 h treatment with 10 μM rifampicin. (G) Images taken after 48 h treatment with 10 μM thiostrepton. (H) Images of untreated controls after 24 h. (I) Images of ciprofloxacin treated parasites after 72 h. (J) Images of untreated parasites after 30 h.

treatment suggests that they all target the same organelle—the apicoplast.

Target validation for drugs inhibiting translation has been confounded by the difficulty in generating specific and sensitive antibodies to the proteins encoded on the parasite organellar genomes. Thiostrepton is the only translation inhibitor to have been shown to block *Plasmodium* apicoplast translation in whole parasites [16]. These *in vivo* experiments confirmed data from genomic and biochemical analysis showing that the apicoplast-encoded rRNA is targeted by thiostrepton while the nuclear and mitochondrial version are resistant to this drug [14,17,18]. Unfortunately, there are no reports on the kinetics of the response of *Toxoplasma* to thiostrepton.

Another approach used to directly assess inhibition of organellar translation is to block cytosolic translation with cycloheximide and observe changes in cycloheximide-resistant protein. The cycloheximide-resistant proteins should include some or all of the three proteins encoded on the mitochondrial genome [46] plus the ~30 proteins encoded on the apicoplast genome [11]. In *P. falciparum* culture, 1 μM tetracycline inhibits the synthesis of two cycloheximide-resistant proteins but has no observable effect on several others [19]. This sensitivity to tetracycline and insensitivity to cycloheximide is consistent with organellar translation inhibition, but unfortunately the inhibited proteins were not identified nor localized to a specific organelle.

Similar experiments in *T. gondii* recovered 19 cycloheximide-resistant proteins that were sensitive to tetracycline at concentrations that cause death in the first intra-cellular cycle [47]. Surprisingly, treatment with clindamycin at concentrations that induce delayed-death had no effect on these cycloheximide-resistant proteins [47]. There are no reports concerning the identity of the cycloheximide-resistant proteins, making it difficult to confirm their localization. However, the large number of recovered proteins eliminates the mitochondrion as the sole target, as this organelle encodes only three proteins [48].

To date, there is only indirect evidence for the target of drugs causing delayed-death in *P. falciparum*. Tetracycline appears to inhibit mitochondrial function. The mitochondrial enzyme dihydroorotate dehydrogenase (DHOD) is dependant on a functional electron transport chain, which in turn requires the synthesis of proteins encoded by the mitochondrial genome [46]. DHOD can, therefore, be used to assess inhibition of mitochondrial translation. Such assays show that tetracycline treatment inhibits the *in vivo* activity of DHOD, indicating that tetracycline interferes with the translation of mitochondrially encoded genes [20].

Other indirect approaches to assessing the effect of tetracyclines on apicoplast translation include measuring apicoplast transcription as a proxy for translation, and using genome replication and organellar morphology as an indicator of a specific organellar target [29,15]. The apicoplast encodes two subunits of RNA polymerase, so inhibiting apicoplast translation should lead to suppression of transcription. However, tetracyclines only have a weak and delayed effect on apicoplast transcription, even when applied at extremely high concentrations [15]. Conversely, thiostrepton – which definitely impacts apicoplast translation [16] – severely inhibits apicoplast transcription within a few hours of application [15], so the two translation inhibitors have fundamentally different effects on expression. Similarly, the effects of tetracycline and thiostrepton on apicoplast morphology and apicoplast genome copy number are markedly different (Figs. 2 and Figs. 2F and 4G [29]), which begs the question as to whether thiostrepton or tetracyclines have additional targets outside the apicoplast resulting in different kinetics of parasite death.

Interpreting these indirect assays is complicated by the extensive transfer of genes from the mitochondria to the nucleus that has occurred during the course of apicomplexan evolution. Only three mitochondrial genes (all involved in electron transport) have been retained on the mitochondrial genome with the rest being encoded in the nucleus and translated into proteins by the tetracycline resistant ribosomes in the cytosol [49]. This means that it is unlikely that mitochondrial transcription, genome replication or mitochondrial morphology would be affected by inhibiting mitochondrial translation and, therefore, a mitochondrial target for the tetracyclines cannot be ruled out. Clearly it will be necessary to make direct assays of the translation systems in each organelle to determine which is (are) the target of tetracyclines.

Our analysis of different bacterial housekeeping targeting drugs and their ability to inflict delayed-death on the malaria parasite leaves us with a paradox. Three drugs (ciprofloxacin, rifampicin and thiostrepton) have specific targets in the apicoplast and kill parasites immediately. Tetracycline, which could target mitochondrial translation or apicoplast translation, or both, produces delayed-death, as does another bacterial translation blocker, clindamycin. The gene sequences of *Plasmodium* organellar rRNAs show that the mitochondrial rRNA carries base changes that confer some clindamycin resistance in bacteria [50]. Moreover, clindamycin-resistant mutants in *T. gondii* have altered apicoplast rRNA sequences suggesting that this drug almost certainly targets the apicoplast [21]. Finally, we observe impacts of clindamycin on apicoplast morphology and function (Figs. 2G and 3A–C). How then can drugs with apparently similar organelle targets produce different cellular responses?

The *Plasmodium* apicoplast and mitochondria are intimately linked. The parasite's haem biosynthesis pathway is a conjoined hybrid between two originally complete and separate pathways from the mitochondrion and apicoplast [10,51–53]. Moreover, apicoplasts and mitochondria appear to share a physical link during most of the asexual cycle, indicative of some degree of interdependence [35,54]. It has also been proposed that the apicoplast delivers "housekeeping" components (such as tRNAs) to the mitochondrion, which encodes no tRNAs itself but requires them for translation of the mitochondrial genome [55]. Thus, disrupting the activity of one organelle could have a significant impact on the development and morphology of the other. This could explain how targeting mitochondrial translation with tetracycline could result in delayed-death, but it does not adequately explain the distinct difference in cellular response between the two translation blockers, clindamycin and thiostrepton, that both appear to target the apicoplast. A more thorough understanding of the interactions between these organelles will be required to unravel this problem.

Table 2
Comparison of drug kinetics and putative targets in *P. falciparum* and *T. gondii*

Drug	Drug kinetics		Target—validation		References
	P. falciparum	*T. gondii*	*P. falciparum*	*T. gondii*	
Ciprofloxacin	Immediate	Delayed	Apicoplast—direct via apicoplast genome copy number	Apicoplast—direct via apicoplast genome copy number, microscopy	[13,26]
Clindamycin	Delayed	Delayed	Not determined	Apicoplast—direct via clindamycin resistance mutation	[21]
Rifampicin	Immediate	Immediate	Apicoplast transcription—direct via apicoplast transcript levels	Not determined	[14,15,60]
Tetracycline	Delayed	Immediate	Mitochondria—indirect via DHOD activity, apicoplast transcription	Apicoplast—suggested by cycloheximide-resistant translation	[15,20,47]
Thiostrepton	Immediate	Not determined	Apicoplast translation—direct via apicoplast translation, indirect via *in vitro* drug binding	Not determined	[16,18]

The drug responses in *P. falciparum* are not consistent with those seen in *T. gondii* (Table 2). Ciprofloxacin causes delayed-death in *T. gondii* and it clearly interrupts apicoplast genome replication [26]. Ciprofloxacin also specifically targets apicoplast genome replication in *P. falciparum* [13] but kills the parasites in the first asexual cycle of treatment—immediate death (Fig. 4). Tetracycline causes delayed-death in *P. falciparum* but does not appear to cause delayed-death effects in *T. gondii*, although very high concentrations of tetracycline are required to kill *T. gondi* so the effects may be non-specific [25]. Indeed, doxycycline – another member of the tetracycline class – is effective against *T. gondi* at much lower concentrations and has been reported to give rise to a delayed-death response [21], so the delayed-death effect is probably consistent between the two parasites but there remains conflicting evidence as to the exact target(s) of the tetracycline type drugs. Clindamycin is the only drug unequivocally shown to cause delayed-death in both parasites, and it very likely impacts apicoplast translation specifically. We thus have a spectrum of responses to anti-bacterials between the two parasites and no obvious way to discriminate whether the delayed-death phenomenon is due exclusively to perturbation of the apicoplast and/or the mitochondrion at this stage.

Although there are significant similarities between *Plasmodium* and *Toxoplasma*, it is not surprising that they respond so differently to the same drug. Such differences may simply reflect a differential ability of the drugs to penetrate the multiple membranes surrounding the various organelles. If drugs are unable to interact with specific organellar targets, they may result in vastly different responses; as demonstrated by the differing efficacy of tetracycline treatment in the two organisms.

Host cell and lifestyle differences may also be reflected in differing drug responses. A postulated function of the apicoplast in *P. falciparum* is to maintain the high levels of antioxidant enzymes necessary to deal with the oxidative byproducts of haemoglobin degradation [56]. *T. gondii* does not degrade haemoglobin so reactive oxygen species are presumably less of an issue in its asexual life cycle. Therefore, the role of the apicoplast in synthesizing antioxidants may be less important to cellular growth and survival in *T. gondii* than in *P. falciparum*, so disrupting apicoplast function may have less severe effects on *Toxoplasma*.

Differences in the developmental processes of the two parasites could also be reflected in drug response. To form daughter cells, *P. falciparum* undergoes an atypical cell cycle in which there are multiple nuclear divisions and extensive organellar elaboration prior to daughter cell formation [35]. Shortly before merozoite formation, the cell components undergo a process of alignment that ensures the correct partitioning of nuclei and organelles in the daughter cells [35,57]. The regulated nature of this process suggests the presence of a number of developmental checkpoints, and the inability of ciprofloxacin, rifampicin and thiostrepton treated cells to reach schizogony (Fig. 4A–D) could reflect an arrest at the trophozoite-to-schizont transition. The disruption of the apicoplast in the second cycle of clindamycin and tetracycline treatment (Fig. 2G and H) suggests that schizogony cannot be completed without a viable apicoplast. *T. gondii* asexual replication occurs via endodyogeny, a process in which two daughter cells are formed in a single mother cell and which does not appear to require extensive realignment of nuclei or organelles [58]. In *Toxoplasma* the disruption or loss of the apicoplast appears to have no effect on the *T. gondii* division process [26,59]. Therefore, disruption of the apicoplast may arrest development in *P. falciparum* while having no direct effect growth and division in *T. gondii*.

By examining the cellular responses to drugs targeting the "housekeeping" functions of the apicoplast, we have shown that inhibitors with confirmed targets in the apicoplast arrest parasite development in the first cycle whereas tetracycline, which appears to target the mitochondrion, shows delayed-death kinetics. Paradoxically, clindamycin also shows delayed-death and its target is most probably apicoplast translation. Moreover, it now seems likely that drugs targeting one organelle (mitochondrion or apicoplast) can impact on the other organelle due to the intimate structural and biochemical links between these two organelles. It is now paramount to identify the exact target(s) of these anti-bacterials in each parasite in order to unravel their effects at the cellular level. These findings will

have implications for future development of drugs targeting the apicoplast.

Clearly, concerns that all apicoplast targeting drugs – or even a subset that target organellar "housekeeping" functions – can only cause delayed-death are unwarranted. Inhibitors of apicoplast pathways can thus be explored as treatments for acute infections, as well as prophylactics and secondary components of combination therapies, with some degree of optimism.

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