Combinations of Chloroquine with Tigecycline and Telithromycin Induce Early Onset of Apoptosis in *Plasmodium falciparum* in vitro

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ABSTRACT

Objective. This study aimed to demonstrate that apoptosis in *Plasmodium falciparum* can be measured using kits originally designed for mammalian cells. The antimalarial chloroquine and antibiotics tigecycline and telithromycin were used to show the performance of the assays.

Methods. Nuclear stain DAPI fluorescence was used to estimate cytotoxicity. Apoptotic assays used were: CaspaTag™ for caspase activation, acridine orange for nuclear condensation, and TUNEL for DNA fragmentation.

Results. The IC50 values (95% confidence interval) for telithromycin (TL), tigecycline (TG) and chloroquine (CQ) were found to be 1.00 (0.47–1.53) μM, 4.56 (2.32–6.80) μM, and 0.019 (0.0089–0.029) μM, respectively. Activated caspase-like molecules seemed to be present in all erythrocytic stages, appearing to rise and fall with cell cycle progression with drug exposure appearing to dysregulate this pattern. Nuclear condensation and DNA fragmentation occurred late in the untreated erythrocytic life cycle of the parasite but were advanced by drug exposure.

Conclusion. The study shows that drug-induced apoptosis can be measured in *Plasmodium falciparum* using the methods. These assays could be used for drug discovery, in particular, using high throughput flow cytometry.

Key Words: antimalarial, antibiotic, cytotoxicity, apoptosis, P. falciparum

Introduction

The development of new drugs is encouraged in anticipation of the spread of drug-resistant *Plasmodium* strains.[1,2] There is, however, little incentive to develop new drugs because of the high costs and low profit margins.[3] Some antibiotics already in the market also exhibit antimalarial effects by targeting typical prokaryotic structures present in *Plasmodium* such as the mitochondria and the apicoplast.[4-9] In recent years, artemisinin-based combination therapies (ACT) have become the backbone of treatment for uncomplicated *falciparum* malaria, with antibiotics increasingly being used with classic malaria medications to treat bacterial co-infections in patients suffering from malaria.[1,6,10,11-15] A prudently selected antibiotics-based combination (ABC) for severe malaria could enhance treatment efficacy while delaying the selection of multiple drug resistance in endemic countries.[6,16]

The World Health Organization (WHO) has recommended that the mode of drug activity, specifically apoptosis, be assessed in the evaluation of new combinations.[17] It is known that *Plasmodium* and other protists undergo apoptosis, a process once thought to be confined to metazoan organisms.[18-20] Apoptosis is thought to be involved in the regulation of parasite growth.[21] Many of the drugs used for malaria treatment have the ability to induce irreversible damage on mature forms of the parasite but are not cytotoxic to human cell lines.[18,22-24] Specific interference of apoptosis in parasites might help explain this phenomenon. Ways of measuring cell death in parasites could help in discovering and designing drugs that exploit features of the death pathways.[25]

The study assessed the potential of combining a standard antimalarial drug, chloroquine with antibiotics telithromycin and tigecycline against *in vitro* cultures of *P. falciparum* and determined if cell death initiated by exposure of *P. falciparum* in vitro to these drug combinations resulted in hallmarks of apoptosis as measured by kits originally designed for mammalian cells.

Methods

Continuous culture of erythrocytic asexual stages

The chloroquine-sensitive strain Honduras-1/CDC was obtained from the American Type Culture Collection

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(ATCC) and maintained in continuous culture in Albumax-supplemented RPMI, as previously described.[25] Culture flasks were sparged with a gas mixture of 5% CO_2, 1% O_2, and 94% N_2 using a sterile, cotton-plugged Pasteur pipet and maintained at 37°C. Medium was replaced daily. Cultures were passaged once parasitemia reached 1%.

Prior to cytotoxicity and apoptosis assays, cultures were synchronized by enriching for young ring stage trophozoites using 5% D-sorbitol. Synchronized parasitized erythrocytes with a parasitemia of less than or equal to 0.5% were kept at a hematocrit of 2.5% for in vitro testing.

Determination of IC50 and synergy

For IC50 determination, serial two-fold dilutions of the drug were made across the plate in each row of a 96-well microtiter plate. Drugs were dosed singly in the following ranges: chloroquine (CQ), 2.27–581.55 nM; telithromycin (TL), 0.14–36.95 μM; and tigecycline (TG), 0.20–51.96 μM. Each of the drugs was present in duplicate rows at nine concentrations over a 256-fold range. For combinations, wells that contained a final concentration of 581.55 nM CQ and 36.95 μM TL or 51.26 μM TG were prepared and serially diluted two-fold down to 2.27 nM CQ and 0.14 μM TL or 0.20 μM TG. Solvent, parasite and erythrocyte controls were also included. Plates were maintained at 37°C for 72 hours.

Parasite numbers were estimated using the DAPI fluorescence assay adapted from Baniecki et al. with 30 μL of the DAPI fluorochrome mixture (20 mM Tris-HCl (pH 7.5), 5 mM EDTA (disodium salt), 0.0008% Saponin, 0.001% Trition X-100 and 1:10,000 final dilution of 5 mg/mL DAPI) being dispensed into each well.[26] The number of parasites in three fields of view per well was counted directly using the Olympus IX51 microscope (40X objective) with the use of DAPI filter (408 nm excitation; 460 nm emission).

Parasite counts obtained from the DAPI assay were plotted against the logarithm of the drug concentration, and the curve fitted by nonlinear regression using the median-effect equation ($\log (F_a/F_u) = m\log(D) - m\log(Dm)$) using the software CompuSyn (www.combosyn.com) to yield the dose–response curve and resultant inhibitory concentrations at 50% (IC50). Fu (fraction unaffected) was taken from the values obtained in the negative control while Fa (fraction affected) per well was obtained after counting the number of parasites left after drug treatment then dividing by Fu. For a system, since Fa + Fu = 1, when Fa = Fu, it follows that Fa / Fu = 1 and log (Fa/Fu) = 0, making the dose, D, at that point equal to Dm, the median dose or the IC50.[27-28]

Combination indices (CI) were evaluated for each combination using CI = D1/Dx1 + D2/Dx2 where D1 and D2 are doses of drugs 1 and 2 in "combination" that inhibits a system at x%, and Dx1 and Dx2 are doses of drugs "alone" that inhibit the system at x%. In drug combinations, for an additive effect, CI = 1, synergism is indicated when CI < 1, and antagonism is observed when CI > 1.[27]

Time course studies of apoptosis-like activity in parasite cultures exposed to drug combinations

Apoptosis was evaluated in parasites by exposing them to different drugs at doses representing concentrations at or close to the IC50s of the individual drugs indicated in Table 1. The tests were for caspase-like activity using the CaspaTag assay, nuclear condensation using acridine orange, and DNA fragmentation using TUNEL.

Table 1. Drug doses used for apoptosis testing. Tests were done in triplicate per 96-well plate

1	2	3	4	5	6
TL 1.23 μM	TG 6.83 μM	CQ 19.39 nM	TL : CQ 1.23 μM : 19.39 nM	TG : CQ 6.83 μM : 19.39 nM	Negative control

The assays were performed on parasitized cultures after exposure to individual and drug combinations for 8, 12, 24, 48, and 72 hours to determine the time in which apoptosis sets in upon drug exposure. Two hundred (200) parasitized erythrocytes per treatment per time point were scored for the presence or absence of the apoptosis markers. The assays were repeated thrice.

Cysteine protease activity assay using CaspaTag™

Caspase-like activity was observed using the CaspaTag™ Pan-caspase in situ assay kit, Fluorescein (Chemicon International, USA). This kit incorporates a membrane-permeable fluoromethyl ketone general inhibitor of caspases conjugated to carboxyfluorescein-Val-Ala-Asp (FAM.VAD.fmk), which binds irreversibly to activated caspases in 1:1 stoichiometry, to detect apoptotic cells via their green fluorescence.[25,29] Assay was done per manufacturer's instructions. Briefly, five μL of the freshly prepared CaspaTag FLICA working solution was added to each slide followed by incubation at 37°C in the dark for 1 hour. After washing with 1X PBS, pH 7.4, 10 μL of a 1:10,000 dilution of 200 μg/mL Hoechst stain was added and the slides were incubated for 30 minutes at room temperature. Putative caspases were visualized using the Olympus BX51 microscope (100X OIO) with Spectral Imaging attachment (Applied Spectral Imaging). Cells with active caspase-like molecules were viewed using the FITC filter (490 nm excitation; 520 nm emission) while Hoechst-stained cell nuclei were seen using the DAPI filter (408 nm excitation; 480 nm emission).[29]

Acridine orange (AO) staining

To detect nuclear chromatin condensation, parasitized erythrocytes were thin- smeared then fixed with 4% paraformaldehyde for 15 minutes at room temperature. An acridine orange (AO) mixture (12 μg/mL acridine orange, 0.13 M Na2HPO4, 0.35 M citric acid, 0.001 M Na2EDTA) was prepared. Ten (10) microliters of this AO mixture was added to the slide and incubated for 3 minutes at room

temperature in the dark.30-31 Samples were immediately observed using an Olympus BX51 microscope (100X OIO) using the FITC filter. Acridine orange-stained cells appeared uniformly green under a narrow-band FITC filter while cells with condensed chromatin fluoresced more strongly.[30-31]

TUNEL assay

DNA fragmentation was studied using the Click-iT® TUNEL Alexa Fluor® Imaging Assay (Invitrogen) as per manufacturer's instructions. Briefly, cultures were thin-smeared on slides and fixed with 4% w/v paraformaldehyde and permeabilization reagent (0.25% Triton X-100 in 1X PBS) and incubated for 20 minutes at room temperature. Cells were washed once with distilled deionized water. Next, TdT reaction buffer was added followed by the TdT reaction cocktail containing 5'-triphosphate and deoxynucleotidyl terminal transferase and the Click-iT® reaction cocktail after incubation for 60 minutes at 37°C. After washing, cells were counterstained with Hoechst 33342 and observed using the Olympus BX51 microscope (100X OIO). Fragmented DNA was detected by Alexa Fluor® 488 dye, fluorescing green as seen through a FITC filter, and the nuclei indicated by the counterstain seen through a DAPI filter.

Statistical analysis

DAPI *P. falciparum* viability assay data were analyzed using the student's t-test. Combination indices computed from three or four experiments were averaged and the 95% confidence intervals were determined from the standard deviations. Apoptosis assay data were analyzed using the Chi Square goodness of fit. Non-normally distributed data were first arcsin converted and analyzed using 2-way ANOVA. Means of several treatment groups were analyzed using Tukey's multiple comparisons test.

Results

In vitro susceptibility of P. falciparum to TL, TG and CQ

Parasites treated with different concentrations of telithromycin, tigecycline, and chloroquine showed a dose response in the fraction affected (Fa) values after drug treatment (Figures 1 and 2). Solvent controls were not toxic at the range of drug concentrations tested. It can be deduced from the dose–response curves of the two antibiotics that more of tigecycline is required to inhibit parasite growth by the same extent as compared to telithromycin and that chloroquine inhibits parasite survival at concentrations a hundred times less than that of the antibiotics.

The concentration of the drug that results in 50% inhibition of *P. falciparum* growth in vitro, a measure of its sensitivity, as specified by the drug's IC50 value, was determined by CompuSyn and tabulated in Table 2. All values are consistent with the literature.[11,32-36]

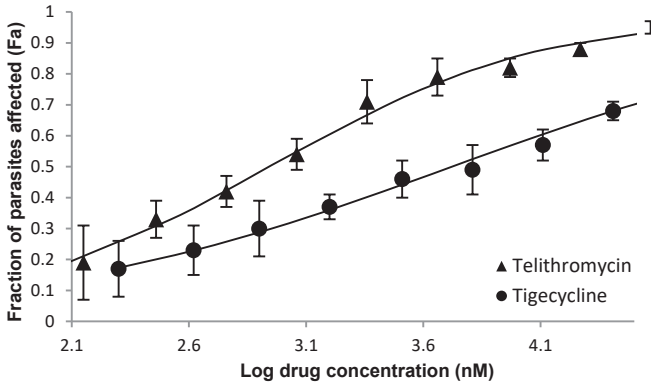


Figure 1. Dose response curves for telithromycin and tigecycline against P. falciparum Honduras-1/CDC after 72 hours of incubation. Error bars are 95% confidence intervals. Trendline indicates expected dose response from the median effect equation. N=6.

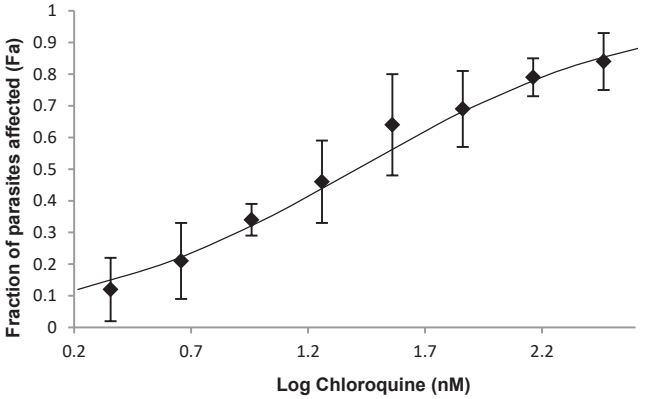
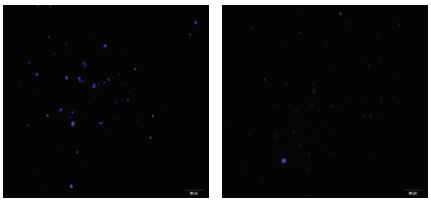


Figure 2. Dose response curve for chloroquine against P. falciparum Honduras-1/CDC after 72 hours of incubation. Error bars are 95% confidence interval. Trendline indicates expected dose response from the median effect equation. N=6.

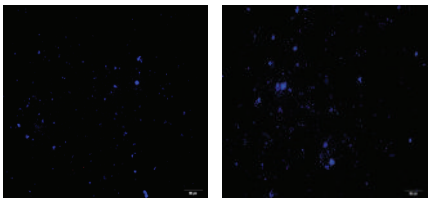
Table 2. *In vitro* susceptibility of P. falciparum Honduras-1/CDC to telithromycin, tigecycline and chloroquine after 72 hours of incubation. Values in parentheses represent the 95% confidence interval. N=6)

Drug (μM)	IC_{50}	IC_{75}	IC_{90}
Telithromycin	1.00 (0.47-1.53)	3.52 (1.59-5.46)	13.67 (3.52-23.82)
Tigecycline	4.56 (2.32-6.80)	39.55 (25.29-53.80)	309.76 (191.92-427.59)
Chloroquine	0.019 (0.0089-0.029)	0.095 (0.031-0.16)	0.47 (0-1.29)

The Combination Index (CI) indicating synergistic concentrations between chloroquine and telithromycin or tigecycline was also calculated by CompuSyn.[28] Of the nine concentrations tested over a 256-fold range for each combination, only TL at 1.16 μM and CQ at 18.18 nM (CI=0.58±0.19) (Figure 3), TG at 6.40 μM and CQ at 72.69 nM (CI=0.62±0.34), and TG at 3.20 μM and CQ at 36.35 nM (CI=0.66±0.34), appear to act synergistically against *P. falciparum.*



Telithromycin (nM)	1,116	1,116
Chloroquine (nM)	0	18.18
Number of Spots	120	57
% Inhibition	57.60	79.86



Telithromycin (nM)	0	0
Chloroquine (nM)	18.18	0
Number of Spots	157	283
% Inhibition	44.53	0

Figure 3. Representative images of wells containing the Honduras-1/CDC parasite and varying concentrations of telithromycin and chloroquine after 72 hours of incubation. Scale bar, 40 μm.

Detection of apoptotic markers in Plasmodium falciparum

Apoptosis in *P. falciparum* was induced using TL, TG, CQ or the combination of TL-CQ and TG-CQ. Three cell-based assays were conducted to detect caspase-like activation, chromatin condensation, and DNA fragmentation at specific time points after drug treatment as specified in Table 1. Considerable variation in the timing and proportion of parasites positive for apoptosis markers were observed pointing to a temporal profile consistent with what is known about programmed cell death (PCD).

Caspase-like activity

Caspase-like activity in all groups was not independent of time. The trend observed in the untreated culture (negative control) suggests a correlation with life cycle stage (Figure 4). Caspase-like activity at 48 hours was elevated in all treated cultures and in cultures at 72 hours treated with chloroquine alone or in combination with antibiotics. From the proportion of cells exhibiting caspase-like activity as shown in Figure 4, in general, it was observed that caspase-like activity increased for all treatments until it reached a specific time point where the highest proportion of caspase-like molecules in cultures was present. This was followed by a significant decrease in proportion of caspase-like molecules during the next time point. This observation is indicative of the action of caspase-like molecules and their probable role in initiating apoptosis in *P. falciparum* cultures.

It can be deduced that the general effect of drug administration is to increase the proportion of cells with activated caspase-like molecules. This can be seen at later time points, at 48 and 72 hours, during the end of the parasite life cycle (Figure 4). However, there is high background activity, particularly at 24 hours, masking this effect. Further molecular characterization is needed to accurately describe these findings.

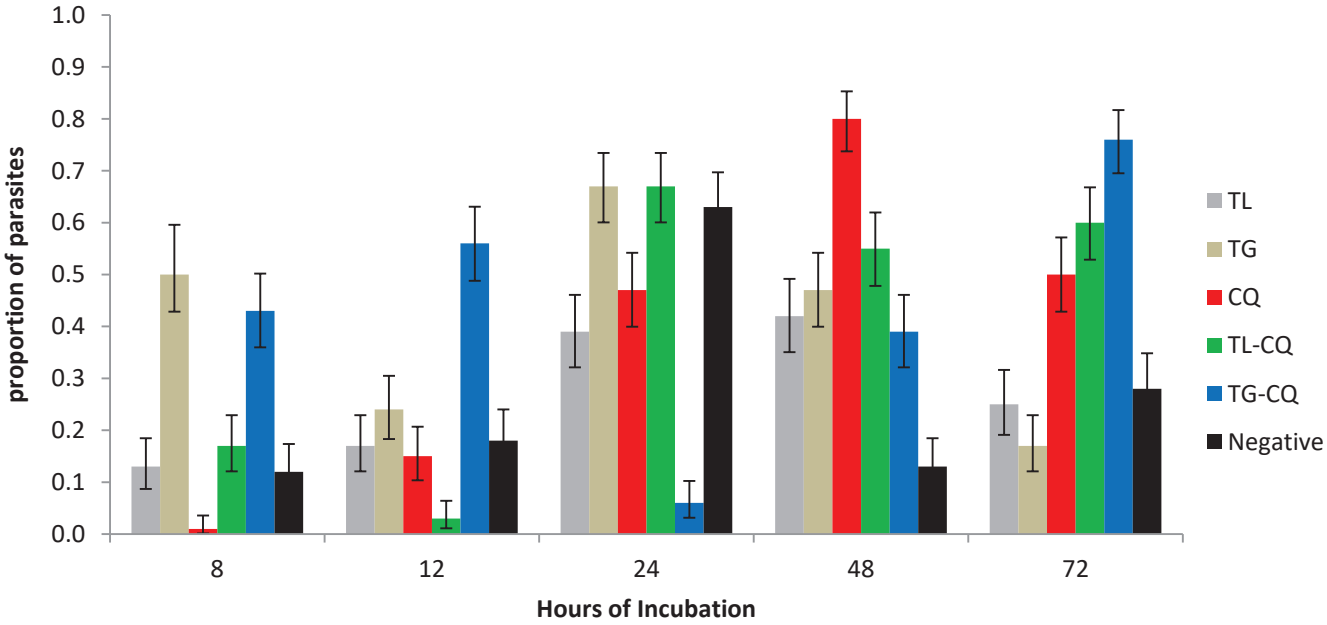


Figure 4. Proportion of caspase-like molecules in *Plasmodium falciparum* parasites exposed to different drugs compared to negative control. Error bars show 95% confidence interval of the proportion of positives.

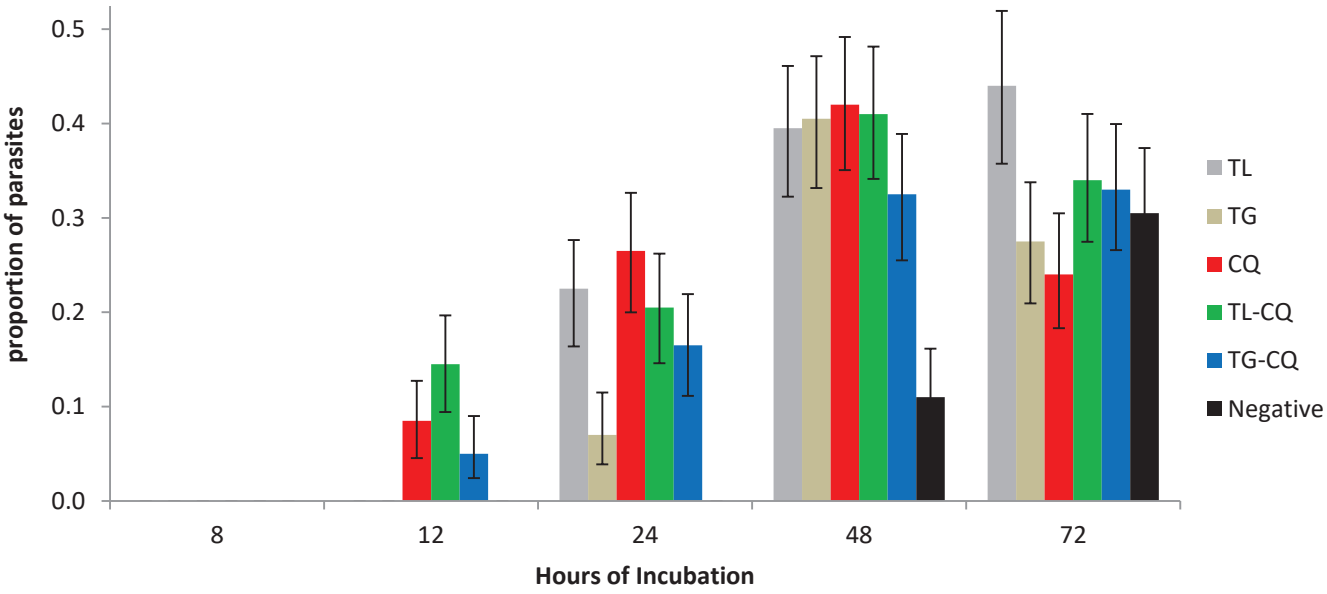


Figure 5. Proportion of cells with nuclear condensation in *Plasmodium falciparum* parasites exposed to different drugs compared to negative control. Error bars show the 95% confidence interval of the proportion of positives.

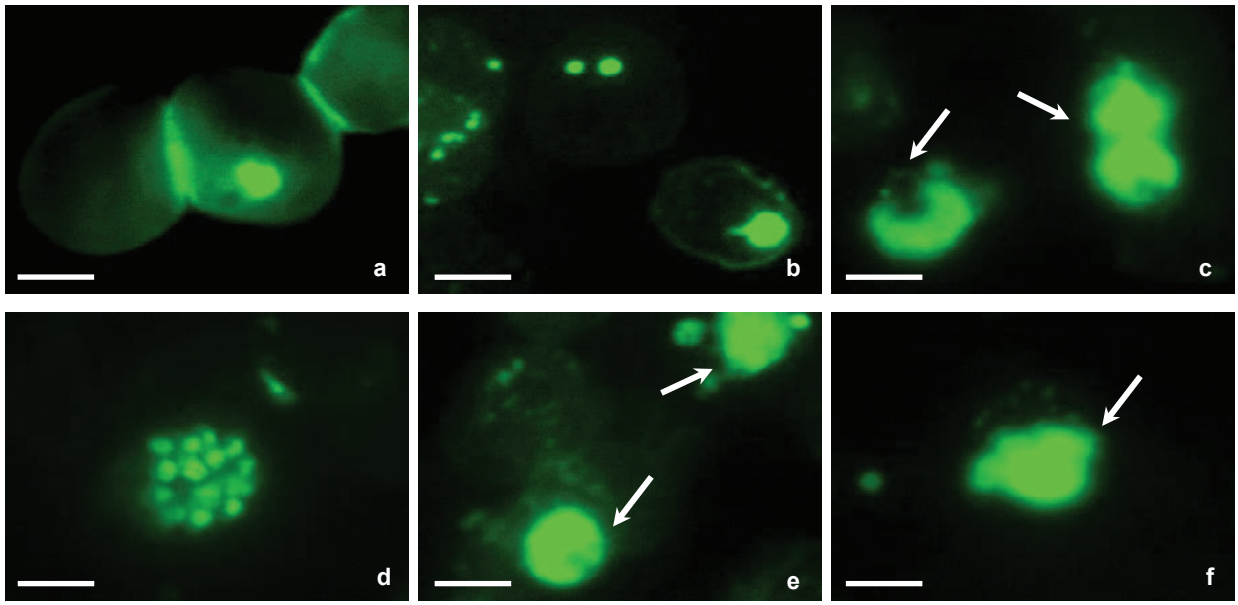


Figure 6. Detection of nuclear chromatin condensation in *P. falciparum* Honduras-1/CDC treated with chloroquine. *P. falciparum*-infected erythrocytes were stained with Acridine Orange (AO). Intense green fluorescence (indicated by white arrows) is displayed by nuclei with condensed chromatin. Incubation is as follows: a) 8 hours, b) 12 hours, c) 24 hours, d) and e) 48 hours and f) 72 hours. Scale bar, 5 μm.

Nuclear condensation

No chromatin condensation was seen in all cultures at 8 hours, but increased thereafter. The proportion of the population containing nuclei with condensed chromatin was highly variable with time post-incubation as can be seen in Figure 5. The number of cells with condensed chromatin was seen to peak after incubation for 48 hours while untreated cultures had a proportion of their population (around 0.3) displaying chromatin condensation after 72 hours, which was not apparent during the four earlier time points. Nuclear condensation is also expected in untreated cultures if parasites undergo apoptosis normally. It was found that nuclear condensation is not independent of time for all treatments ($p<0.0001$), with drug treatment advancing the onset of the marker.

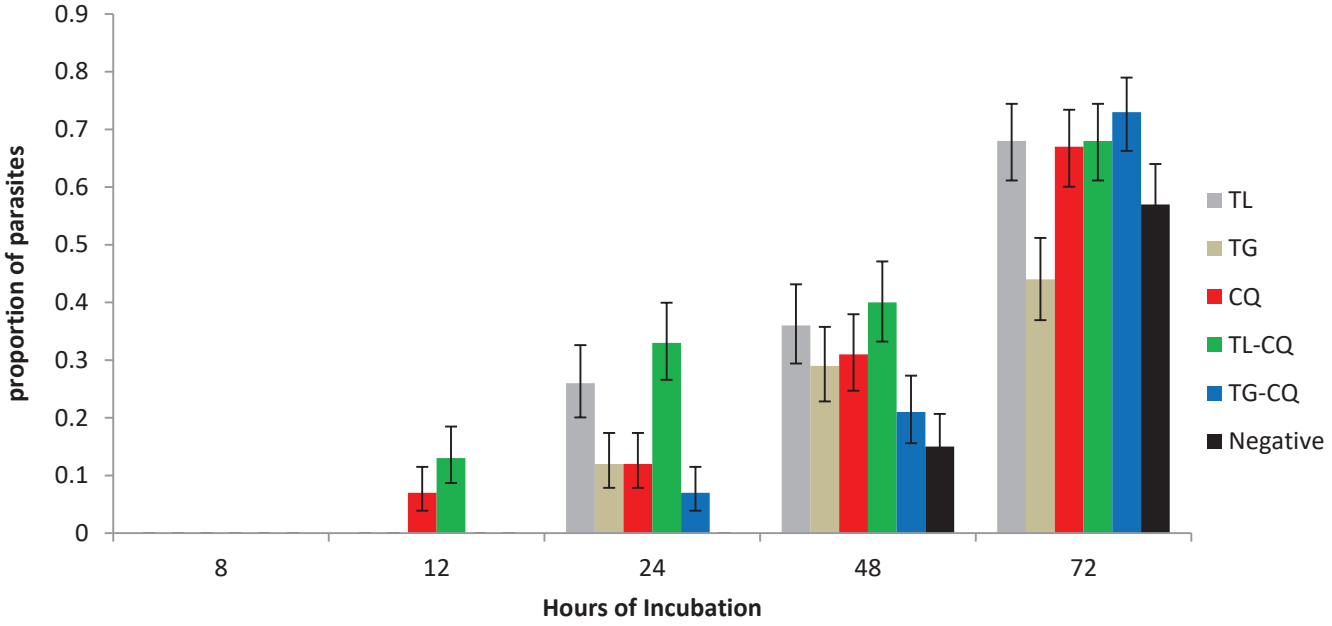


Figure 7. Proportion of cells with fragmented DNA in *Plasmodium falciparum* parasites exposed to different drugs compared to negative control. Error bars show the 95% confidence interval of the proportion of positives.

The effect of drug treatment on nuclear condensation was also apparent from Figure 6. Life stages of the parasite can also be deduced from nuclear shape and size in the pictures presented. Ring stage parasites are seen after 8 and 12 hours of incubation in (a) and (b), a schizont with intact nuclei can be seen in (d) after 48 hours. Normal nuclei and parasites with condensed chromatin are both present as separate populations in culture during the specified time points.

DNA fragmentation

Fragmentation of DNA is a well-defined end point in programmed cell death. It was found that DNA fragmentation is not independent of time for all treatments ($p<0.0001$). No DNA fragmentation was observed in all test cultures after 8 hours of incubation. DNA fragmentation was identified only in CQ and TL-CQ-treated cultures after 12 hours. At 24 hours, DNA fragmentation was initially detected in cultures exposed to TL, TG, and the combination TG-CQ. DNA fragmentation was only observed in untreated cultures after 48 hours of culture while those that were subjected to drug pressure tested positive for TUNEL at much earlier time points (Figure 7). The number of TUNEL-positive cells increased for each culture regardless of treatment until the 72-hour period, as shown in Figure 7, indicating that all of these drugs induced in situ DNA fragmentation in *Plasmodium falciparum*.

Discussion

In this study, it was found that the antibiotics telithromycin and tigecycline are active against *P. falciparum* and that they are synergistic with chloroquine at specific doses in vitro. The drugs, singly or in combination, inhibited parasite growth and were associated with markers typical of apoptosis. Caspase-like activity, nuclear chromatin condensation, and DNA fragmentation were observed to follow a temporal sequence consistent with what is known about the apoptosis program. Since these kits may be used in flow cytometry, our results suggest that a high throughput platform for drug discovery may be developed.

The employment of DAPI in a high-throughput (HTS) assay was based on the premise that since mature erythrocytes lack RNA and DNA, binding of the dye is specific for malarial DNA in any erythrocytic stage of *P. falciparum* development. This ensured that detection of stained parasite DNA by the DAPI reporter is dependent on parasite replication. Viability assays were done after incubation times of 48, 72, and 96 hours. Counts after 48 and 96 hours did not seem to exhibit any dose-response for any drug (data not shown). There are two probable reasons for this observation. The first is that the expected effect of the drugs on the parasites is not apparent before the first schizogonic cycle (~48 hours) as is the case with antibiotics. On the other hand, after 96 hours, parasite growth was overwhelming, making it difficult to establish any dose response.

Antimalarial compounds in the market and new molecules under investigation act in various ways that may be combined to improve drug treatment. Tigecycline and telithromycin bind to ribosomal subunits, blocking the entry of transfer RNA and preventing protein synthesis by halting the incorporation of amino acids into peptide chains and thus limiting bacterial growth.[37-38]

A number of antibiotics have been characterized and approved for human use; they have been incorporated into antimalarial treatment regimens. Because of their relatively

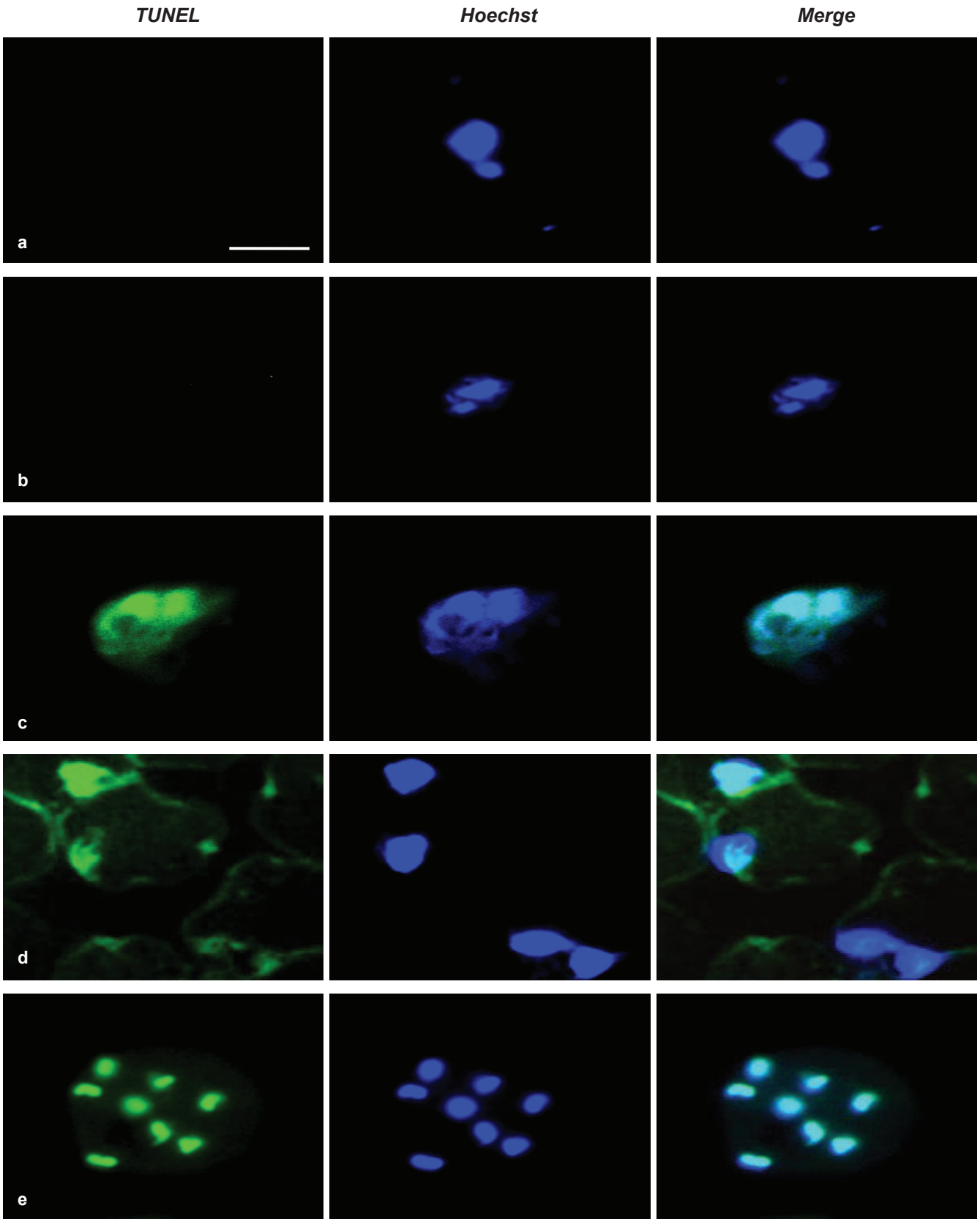


Figure 8. Staining of DNA double-strand nicks using TdT-mediated dUTP nick-end labeling (TUNEL) in P. falciparum Honduras-1/CDC treated with Telithromycin. Hoechst was used to stain nuclei (middle column). Incubation is as follows: a) 8 hours, b) 12 hours, c) 24 hours, d) 48 hours and e) 72 hours. Scale bar, 6 μm.

slow antimalarial action and enhanced activity after prolonged contact, antibiotics are best administered in conjunction with a rapidly acting antimalarial, such as chloroquine, quinine or artemisinin. The *in vitro* activity of telithromycin and tigecycline against intraerythrocytic stages of *P. falciparum* (Figure 1) and their known efficacy in vivo suggest the use of these antibiotics or their analogues as potential antimalarial drugs.[5,33-34] These two antibiotics were chosen as model compounds in this study for their individual effects and possible synergism when combined with another model compound, chloroquine.

Despite some controversy, it has been shown that protozoan parasites are capable of displaying morphological and cellular markers often associated with apoptosis. However, there is considerable variation in the timing and proportion of parasites positive for apoptosis, both across species and according to the markers used.[19] A significant proportion of parasites exposed to TL, TG, and CQ, alone or in synergistic doses, displayed several independent biochemical and morphological markers that are associated with typical mammalian apoptotic phenotypes. Although most apoptosis assays have been developed for mammalian cells, the protocols involved still share a molecular basis, making them applicable to parasites.

A proportion of *P. falciparum* in untreated cultures was also thought to undergo apoptosis as indicated by the same markers seen in treated cultures. However, these untreated cultures only tested positive for apoptotic markers after significantly longer incubation times relative to those exposed to drugs (Figures 4, 5 and 7) suggesting that programmed cell death is a normal part of the parasite life cycle, ensuring the elimination of unfit or defective parasites as previously reported.[20-21,39]

Part of the debate surrounding parasite apoptosis stems from the absence of canonical caspases in protozoans. Caspase-like activity during apoptosis has been described for several unicellular organisms that lack classical caspases, but possess one or more metacaspases, distant caspase homologues present in non-metazoan organisms, including plants, fungi, and protozoa.[20,25,40] Limited information is available concerning the role of metacaspase-like genes that have been annotated from the *P. falciparum* genome.[40] To date, no activity of a purified metacaspase has been reported, and from work on mammalian cells, an apoptosis-independent function of metacaspases is also a possibility.[20] Meslin et al. have suggested that a clan CD protease PfMCA1 (a metacaspase homologue) from chromosome 13 was a likely mediator of cell death because it had the required predicted catalytic cysteine and histidine residues in the correct context for an active enzyme; but a similar metacaspase-like sequence on chromosome 14 failed to present clear features associated with apoptosis.[40-41] In previous studies, the observation that *Plasmodium* apoptosis was inhibited by general caspase inhibitors and the more specific caspase-3 subfamily inhibitor, Z.DEVD.fmk, suggests that caspase-like activity and aspartate specificity may be as crucial for apoptosis in *Plasmodium*, as it is for mammalian cells.[29]

Given these conflicting results, the utility of metacaspase gene expression to detect apoptosis in *P. falciparum* remains to be seen. However, it is the presence of these putative catalytic cysteine residues and the desire to assay cells in the early stages of apoptosis that argued for the use of CaspaTag™ to study apoptosis in *Plasmodium*.[41-42] Still, it is not necessarily the case that the fluorescent caspase inhibitor in the kit would be as specific in *Plasmodium* as with other cells. Results must be approached with caution until relationships between mammalian caspases and *P. falciparum* metacaspases have been completely elucidated and clearly established. Nonetheless, assaying the activation of death executors is an attractive option when linking apoptosis to developmental schedule.

Cells that show both caspase-like activity and membrane integrity are said to be in the early apoptotic stages while those that show caspase-like activity and loss of membrane integrity at the same time are said to be in the late apoptotic or dying stages.[31] The appearance of caspase-like molecules was the only marker of apoptosis that was evident after 8 hours in culture, most especially for cells exposed to TG and TG-CQ. At this time point, since there were no cells that tested positive for both nuclear chromatin condensation and DNA fragmentation, it can be surmised that parasite nuclei are still intact going into the 12-hour period. Caspase-like activity continued to increase in the subsequent time points for all cultures. Relative to cells that tested positive for chromatin condensation and DNA fragmentation, caspase-like activity for all cultures was significantly lower at the 72-hour time point suggestive of imminent parasite death.

Subsequent markers are more straightforward. Morphological changes and final breakdown into apoptotic bodies result in a loss of volume of the nucleus leading to nuclear condensation and DNA degradation.[23,42] Chromatin condensation and DNA fragmentation were apparent after 48 hours of incubation for all cultures and drug treatment advanced both the onset and the proportion of cells exhibiting positive results (Figures 6 and 8).

After 12 hours of incubation, cultures treated with CQ and the combinations TL-CQ and TG-CQ were seen to have a small population, less than 10%, of nuclei with condensed chromatin (Figure 4). Cultures treated with the antibiotics TL and TG alone did not contain any cells with condensed chromatin. This may be because the action of these antibiotics, which act via "delayed death" effect.[2,4] Though effective in parasite inhibition, the action of antibiotics is still slow compared to the standard antimalarial CQ. Nuclear condensation detected in cultures treated with drug combinations may be attributed to the action alone of the fast-acting CQ.

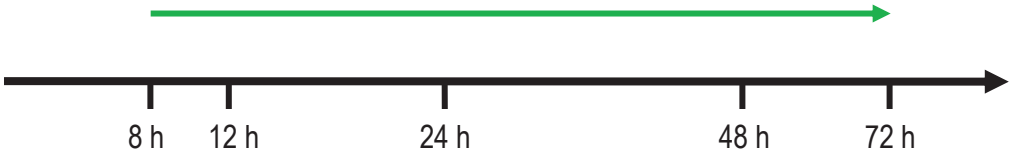


Figure 9. Onset of caspase-like activity (green arrow) in *Plasmodium falciparum* cultures for all drug treatments and no-drug control. For all cultures, caspase activity is seen as early as 8 hours in culture and continues until the last time point.

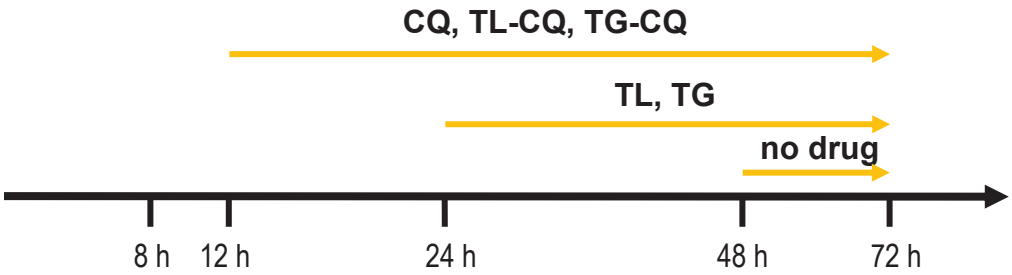


Figure 10. Onset of nuclear condensation (orange arrow) in *Plasmodium falciparum* cultures. For cultures treated with CQ, TL-CQ and TG-CQ, nuclear condensation is seen after 12 hours of incubation and continues until the last time point. In cultures treated with TL and TG, cells with condensed nuclei were observed after 24 hours in culture while the no-drug control was observed to have cells with condensed nuclei only after 48 hours in culture.

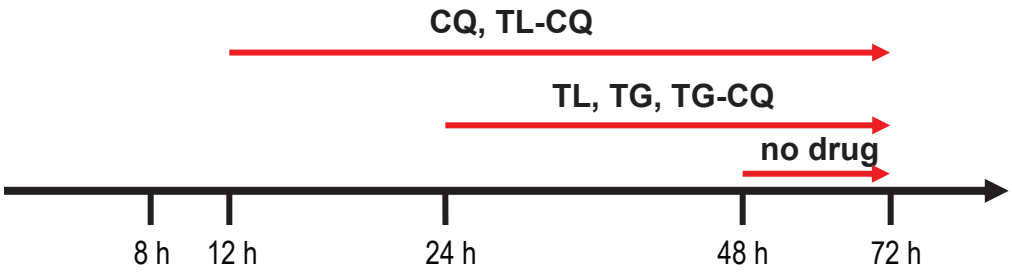


Figure 11. Onset of DNA fragmentation (red arrow) in *Plasmodium falciparum* cultures. For cultures treated with CQ and TL-CQ, DNA fragmentation is seen after 12 hours of incubation and continues until the last time point. In cultures treated with TL, TG and TG-CQ, DNA fragmentation was observed after 24 hours in culture while the no-drug control was observed to have cells with fragmented DNA only after 48 hours in culture.

Chromatin condensation, most especially in treated cultures, was apparent after 48 hours of incubation. Results for TUNEL also mirrored that of the AO assay's, suggesting that parasites that were in the early apoptotic stages had already transitioned to the late apoptotic stage after 48 hours and were dying or already dead after 72 hours. Overall, chromatin condensation and DNA fragmentation in treated and untreated cultures increased with time.

Interpreting acridine orange fluorescence intensity depends on the general appearance of cells on a slide. Parasites change size as they go through their erythrocytic stages. One must, therefore, carefully distinguish fluorescence due to stage effects. In contrast, the fluorescence observed with the TUNEL assay is highly specific to its target, nicked DNA strands. The assay is readily adaptable to any organism, is less perturbed by background fluorescence from non-specific signals, and is easy to score.

Other features of apoptosis-like cell death in *Plasmodium*, such as cell shrinkage, nuclear condensation, loss of mitochondrial membrane potential, and phosphatidyl-serine translocation have been shown in other studies.[19-20,41] The complexity of mechanisms in other taxa suggest that multiple pathways are also involved in protozoan parasites.[20,42]

In general, caspase activation is followed by nuclear condensation and DNA fragmentation.[29] At present, the involvement of caspase-like molecules leading to apoptosis in *P. falciparum* is speculative but the close agreement in the proportion of parasites displaying this marker and condensed chromatin and DNA fragmentation over time indicates some connection between their action and PCD. These events may be followed using assays that are generally used for metazoan cells but modified to account for the greater number of membranes probes may need to cross in order to reach their targets in the parasite. Figures 9–11

summarize the temporal patterns indicative of apoptosis-like cell death observed in *P. falciparum* treated and non-treated cultures. They also show that chloroquine is a good positive control.

This study has shown that markers for apoptosis-like cell death can be detected in *P. falciparum* and may be used to characterize drug activity. Flow cytometry, in particular, will allow more rapid and more accurate measurement of apoptosis in *P. falciparum*, allowing for a promising high-throughput model for drug discovery.

Recommendations

The most important recommendation from this study is to optimize the same apoptotic assays presented in a high throughput flow cytometry platform to test compound libraries including natural products. Flow cytometry is a versatile technique for studying a wide variety of mechanisms of drug action. For example, one lead (MMV-08138) from the Malaria Box collection of the Medicine for Malaria Venture was identified by a high throughput flow cytometry screen designed to reveal drugs targeting the parasite's apicoplast.[43] The compound has been targeted for modification to improve its drug-like properties.

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