Interactions between tafenoquine and artemisinin-combination therapy partner drug in asexual and sexual stage *Plasmodium falciparum*

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**Abstract**  
The 8-aminoquinoline tafenoquine (TFQ), a primaquine derivative, is currently in late-stage clinical development for the radical cure of *P. vivax*. Here drug interactions between TFQ and chloroquine and six artemisinin-combination therapy (ACT) partner drugs in *P. falciparum* asexual stages and gametocytes were investigated. TFQ was mostly synergistic with the ACT-partner drugs in asexual parasites regardless of genetic backgrounds. However, at fixed ratios of 1:3, 1:1 and 3:1, TFQ only interacted synergistically with naphthoquine, pyronaridine and piperaquine in gametocytes. This study indicated that TFQ and ACT-partner drugs will likely have increased potency against asexual stages of the malaria parasites, whereas some drugs may interfere with each other against the *P. falciparum* gametocytes.

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**1. Introduction**  
Artemisinin combination therapy (ACT) is currently the first-line treatment for falciparum malaria. It is administered as a co-formulated fixed-dose tablet with a fast-killing and rapidly eliminated artemisinin (ART) or its derivative combined with a slower-acting partner drug that has a longer elimination half-life (Eastman and Fidock, 2009). The majority of ACTs are highly effective against the asexual blood stages that cause clinical symptoms. However, they are mostly ineffective against *Plasmodium falciparum* mature gametocytes and the dormant liver stage of *P. vivax*. To suppress human to mosquito transmission in areas of emerging ART resistance in *P. falciparum*, a single dose of 0.25 mg base/kg of primaquine (PMQ), the only licensed gametocytocidal and liver schizonticidal 8-aminoquinoline, is recommended following ACT (White, 2013). For radical cure of vivax infections, the Walter Reed Army Institute of Research in collaboration with GlaxoSmithKline began testing a 5-phenoxyl PMQ derivative WR238605/SB-252263 or tafenoquine (TFQ) (Crockett and Kain, 2007). Compared to the 6–8 h half-life of PMQ, TFQ has a longer elimination half-life (t½) of two weeks and appears to have better bioavailability and enhanced hypnozoite suppression activity (Shanks et al., 2001; Li et al., 2014). One completed phase Ib clinical trial has already shown better protection against *P. vivax* relapses with a 3 day chloroquine (CQ) dosage + 300 mg TFQ on day 1 or 2 treatment versus CQ alone (Llanos-Cuentas et al., 2014). Nevertheless, these initial results need to be validated by the confirmatory ongoing phase III trial.

TFQ's mechanism of action is still currently unknown; nonetheless studies in *Leishmania* spp. and *Trypanosoma* spp. hypothesize on its ability to induce mitochondrial dysfunction (Carvalho et al., 2010, 2015). Curiously, studies in *Plasmodium* have yet to reveal the link between mitochondrial function and TFQ. What is clearly known is that both TFQ and PMQ have the disadvantage of potentially causing severe hemolytic anemia in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency (Llanos-Cuentas et al., 2014), therefore, screening for G6PD enzyme deficiency before treatment is recommended. To improve efficacy against the *P. vivax* hypnozoites, the Walter Reed Army Institute of Research in collaboration with GlaxoSmithKline began testing a 5-phenoxyl PMQ derivative WR238605/SB-252263 or tafenoquine (TFQ) (Crockett and Kain, 2007). Compared to the 6–8 h half-life of PMQ, TFQ has a longer elimination half-life (t½) of two weeks and appears to have better bioavailability and enhanced hypnozoite suppression activity (Shanks et al., 2001; Li et al., 2014). One completed phase Ib clinical trial has already shown better protection against *P. vivax* relapses with a 3 day chloroquine (CQ) dosage + 300 mg TFQ on day 1 or 2 treatment versus CQ alone (Llanos-Cuentas et al., 2014). Nevertheless, these initial results need to be validated by the confirmatory ongoing phase III trial.

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activity must be carried out prior to dosing with PMQ or TFQ. Should TFQ be deployed in *P. falciparum* and *P. vivax* co-endemic zones that currently undergo *P. vivax* radical treatment with ACT or CQ, awareness of drug interactions between TFQ and ACT or between TFQ and CQ will be essential since drug interactions within the human host could enhance or reduce the efficacy of all the drugs involved. Unfortunately, *P. vivax* in vitro culture entails conditions which are challenging, requires a constant supply of reticulocytes, and yields very low parasitemias (Udomsangpet et al., 2007; Roosooong et al., 2015). Consequently, this study focused on TFQ – ACT-partner drug interactions using in vitro cultures of *P. falciparum* to elucidate potential off-target benefits/limitations of these drug combinations.

Limited in vitro studies pertaining to TFQ drug interactions have been performed. One study reported either antagonism or additivity between TFQ and CQ or amodiaquine (AMQ) (Gorka et al., 2013), whereas another study produced conflicting results of synergism when TFQ was combined with CQ (Bray et al., 2005). One other study reported synergism when TFQ is combined with ART at the 1:1 ratio (Rambhart et al., 2002). Neither study tested drug interactions against the full panel of ACT-partner drugs nor tested interactions in sexual-stage parasites. Drug interactions in gametocytes are expected to differ since asexual parasites have been shown to be 2000-fold more susceptible to most antimalarials, especially the first-line ACTs (Cabrera and Cui, 2015). Therefore, in this study we investigated the in vitro interactions between TFQ and the six ACT-partner schizonticides as well as CQ in intraerythrocytic asexual and sexual stages of *P. falciparum*. Using a SYBR Green I method for asexual stage parasites and a flow cytometry-based method for GFP-expressing gametocytes, fractional inhibitory concentrations (FICs) and isobolograms derived from pharmacologically relevant, fixed concentration ratios are used to determine decreased (antagonistic) or enhanced (synergistic) drug efficacy of ACT-partner drugs in the presence of TFQ. Because TFQ and ART derivatives reach maximum plasma concentrations (Tmax) at 15 h and <1.8 h respectively, and therefore are less likely to interact in vivo (Ali et al., 2010; Morris et al., 2011; Green et al., 2014), we only tested the long-lasting ACT-partner drugs, namely the 4-aminoquinolines AMQ and naphthoquine (NQ), the bisquinoline piperaquine (PPQ), the ary1 amino-alcohols lumefantrine (LMF) and mefloquine (MFQ), and the Mannich base pyronaridine (PND). These correspond to current ACTs used in different malaria endemic regions (Table S1), namely, artesunate-AMQ, ART-NQ, dihydroartemisinin (DHA)-PPQ, artemether-LMF, artesunate-MFQ and artesunate-PND (Eastman and Fidock, 2009; Benjamin et al., 2012; Pelfrene et al., 2015). CQ was included because CQ/PMQ is still the first-line treatment for vivax malaria in most *P. vivax* endemic areas (Baird, 2009). Parasites with different genetic backgrounds and differential drug susceptibilities to both CQ and the ART metabolite DHA were used to assess whether parasite genetic backgrounds affect asexual stage drug-drug interactions.

### 2. Materials and Methods

#### 2.1. Chemical reagents

RPMI 1640 and Albumax II were purchased from Gibco Life Technologies (Grand Island, NY, USA). CQ diphosphate, AMQ dihydrochloride dihydrate, MFQ hydrochloride and TFQ succinate were purchased from Sigma-Aldrich (St. Louis, MO). PPQ tetraphosphate tetrahydrate was obtained from Chongqing Kangle Pharmaceuticals (Chongqing, China). NQ phosphate, PND tetraphosphate and LMF were obtained from Kunming Pharmaceuticals (Yunnan, China). PND, AMQ, NQ and CQ were dissolved in distilled water to make 20 mM stock solutions. PPQ was dissolved in 90% methanol + 10% 1 M HCl to make a 10 mM stock solution (Muangnoicharoen et al., 2009). TFQ, MFQ, and LMF were dissolved in dimethyl sulfoxide (DMSO: Alfa-Aesar, Ward Hill, MA) for stock solutions of 40, 20, and 40 mM, respectively. Cellulose acetate or nylan 0.2 µm membrane filters (VWR International, Radnor, PA) were used to sterilize water and DMSO-dissolved drugs, respectively. All drug stocks were stored at –80 °C until ready for use. Working drug concentrations ranging from 20 mM to 100 nM were freshly prepared as described below in malaria complete medium (MCM) on the same day of drug inhibition assay setup. SYBR Green I PCR Master Mix for asexual parasite growth inhibition assays was purchased from Invitrogen (Eugene, OR). Giemsa for parasite staining was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). Percoll for density gradient centrifugation was purchased from Sigma.

#### 2.2. Parasite culture

*P. falciparum* laboratory strains of different genetic backgrounds 3D7 (Africa: CQ sensitive), HB3 (Honduras: CQ sensitive), 7G8 (Brazil: CQ resistant), Dd2 (Indo-China: CQ resistant) and IPC5202 (Cambodia: ART resistant with Pfkelch13 R539T mutation) were obtained from MR4 (Manassas, VA) and maintained in a humidified 5% CO2 incubator at 37 °C in MCM containing RPMI 1640, 25 mM NaHCO3, 25 mM HEPES (pH 7.4), 11 mM glucose, 0.367 mM hypoxanthine and 5 µg/ml gentamycin supplemented with 0.5% Albumax II (Cabrera and Cui, 2015). MCM was changed daily and percentage parasitemia maintained below 6.5% at 2.5% hematocrit in O+ human red blood cells (RBCs) (Biological Specialty, Colmar, PA).

A 3D7-tubGFP parasite strain expressing GFP under the gametocyte-specific α-tubulin II gene promoter was used for gametocyte drug interaction assays (Wang et al., 2014). Gametocyte induction was as previously described with heparin sodium salt (Sigma-Aldrich) included to inhibit asexual parasite proliferation in gametocyte cultures (Miao et al., 2013). Late stage II gametocytes were purified by a 75%/35% Percoll gradient on day 4 post gametocyte induction. Gametocytes were maintained in gametocyte MCM containing 0.25% Albumax II + 5% heat-inactivated AB human serum (Interstate Blood Bank, Memphis, TN).

#### 2.3. SYBR green I drug inhibition assay for asexual stage *P. falciparum*

Parasites were synchronized with sterile pre-warmed 5% D-sorbitol (wt/vol) (J.T. Baker, Center Valley, PA) treatment for 9 min to enrich for ring-stage parasites four days after reviving from stocks stored in liquid nitrogen (Lambros and Vanderberg, 1979). The parasitemias of the cell cultures were determined by 10% Giemsa staining of thin blood smears and microscopy. Parasite cultures were pelleted via centrifugation at 900 × g for 5 min. Parasitemia was calculated from 1000 cells and parasite cultures diluted to 0.5% parasitemia and 2% hemotocrit by adding appropriate volumes of 50% freshly washed RBCs in incomplete medium (MCM minus Albumax II or serum). One hundred µl of the parasite sample was aliquoted into pre-loaded black 96-well plates containing 2 × concentrations of 100 µl working solutions of antimalarial drugs to make a final volume of 200 µl 1% hemotocrit and 0.5% parasitemia per well. Negative control wells without drug and with MCM, DMSO, or 1% M HCl/90% methanol dissolved in MCM, corresponding to the total amounts in the working drug solutions, were set up in parallel. Where possible, DMSO concentrations were kept below 0.4%. The plates were incubated for 72 h at 37 °C in a 5% CO2 humidified incubator then stored at –20 °C for at least 16 h to facilitate cell lysis. Lysis buffer (100 µl) consisting of 20 mM Tris (pH 7.4), 5 mM EDTA, 0.008% wt/vol saponin, and 0.08% Triton X-100 (vol/vol) with 0.2 µl of SYBR Green I was added to each 96-well
plate sample and mixed gently (Smilkstein et al., 2004). Plates were then incubated at room temperature for at least 1 h and SYBR Green I fluorescence corresponding to parasite density was determined using a FluoStar Optima plate reader (BMG Labtech, Cary, NC). Median inhibitory concentration (IC50) for each drug alone or in combination with TFQ was determined by three-parametric non-linear regression analysis of the resultant drug response growth curve using the GraphPad Prism 5 software (La Jolla, CA).

2.4. Flow cytometry-based drug inhibition assay for P. falciparum gametocytes

Stages II-V gametocytes of 3D7tauGFP, corresponding to days 4–12 post-gametocyte induction, at 0.04% gametocytoma and 1% hematocrit, were incubated in 2 × concentrations of serially diluted pre-loaded drug plates for 48 h at 37 °C in a 5% CO2 humidified incubator to determine the stage-specific inhibitory effects of drugs and drug combinations. Starting drug concentrations for gametocytes ranged from 2 to 10 μM, depending on the drug. Following incubation, aliquots from each well were diluted in a pre-warmed buffer containing 10 mM D-glucose, 10 mM HEPES (pH 7.4), 270 mM KCl, 270 mM NaCl, and 1.5 mM Na2HPO4 to minimize auto-fluorescence of MCM and dilute hematocrit to 0.4%. Resulting green fluorescence from live parasites that survived antimalarial drug exposure was detected by flow cytometry wherein 25,000 events per sample were collected on a Guava EasyCyte HT flow cytometer (EMD Millipore Corp., Billerica, MA). Fluorescence intensity (FI) was calculated by FI = (normalized events × mean green fluorescence) using FlowJo v10 software and plotted against drug concentrations to generate drug dose-effect curves. Median IC50 values for TFQ and TFQ-partner drug combinations were calculated using Graph Pad Prism 5.

2.5. Drug combination assays

For asexual stage parasites, TFQ was combined with ACT-partner drugs (LMF, PPQ, MFQ, AMQ, NQ, and PND) and CQ at fixed molar ratios (Table 1). Drug combination ratios were chosen to reflect TFQ and ACT-partner drug peak blood plasma concentrations (Cmax) (Table S2), as well as the in vitro median IC50 of each individual drug to ensure that proper drug-response curves were obtained. IC50 of individual drugs, and drugs in combination with TFQ against 3D7, 7G8, Dd2, and IPC5202 were measured for gametocytes. TFQ was combined in fixed ratios of 3:1, 1:1 and 1:3 in hundreds of μM concentrations as described elsewhere (Cabrera and Cui, 2015). Three biological replicates, each in duplicate, at 0.04% gametocytemia and 0.5% asexual stage parasitemia were performed to minimize inoculum effects (Hawley et al., 1998). The apparent IC50, which is the IC50 of the ACT-partner drug when combined with TFQ, was used to determine the fractional inhibitory concentration index (FICindex) using the formula: FICTFQ (Apparent IC50 of TFQ when combined with ACT partner drug/TFQ IC50) + FICACT partner drug (Apparent IC50 of ACT partner drug when combined with TFQ/ACT partner drug IC50). FICindex was calculated per fixed ratio and sum = 1 represents an additive drug interaction, < 1 a synergistic interaction, and > 1 an antagonistic interaction, which are represented by a diagonal line, concave curve, or convex curve isobologram when FICTFQ is plotted vs. FICACT partner drug, respectively (Gorka et al., 2013).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey HSD tests were used to determine the differences in IC50S for the different strains to the eight drugs under investigation using SAS software, version 14.1, University Edition Software (Cary, NC). A p-value of <0.05 was considered significant.

3. Results

3.1. ACT-partner drugs differentially inhibit asexual and sexual stages of P. falciparum

Prior to drug interaction studies, we measured the IC50 of each drug in the asexual stages of HB3, 3D7, 7G8, Dd2, and IPC5202 (Table 2). The drug susceptibilities of the five different strains to each of the eight antimalarial drugs were in the nanomolar range and not significantly different from each other, with a notable exception of the significantly higher CQ IC50 values of 7G8, Dd2, and IPC5202. The 7G8 and IPC5202 strains also exhibited significantly lower and higher IC50, respectively, to MFQ (ANOVA p < 0.0001) (Table 2). The growth inhibition of the asexual stages of the same five strains to TFQ are in the micromolar range, and likewise not significantly different from each other. The median drug susceptibility of stage 3D7tauGFP gametocytes to the same eight antimalarial drugs ranged from 5.9 ± 1.3 to 723.6 ± 79.2 μM (Table 2). LMF had the least potent activity against stage 3D7tauGFP while AMQ was the most effective. The rest of the drugs have moderate gametocytocidal activity in the micromolar range. In our experimental conditions, TFQ IC50's were in the lower micromolar range against stage II-IV gametocytes and not significantly different from each other (Table 3).

3.2. TFQ synergizes ACT-partner drugs in asexual parasites

To determine the drug combination interactions between TFQ and ACT-partner drugs in asexual parasites, FICs were used to construct isobolograms (Fig. 1) to elucidate whether the combinations are antagonistic (FICindex > 1), synergistic (FICindex < 1) or additive (FICindex = 1). TFQ mostly synergized the inhibition of almost all ACT-partners in all strains, except in IPC5202 where it showed an antagonistic relationship with NQ (Table 4, Fig. 1). In HB3 and 3D7, LMF had FICindex > 1 at high [LMF] and low [TFQ] (Fig. S1). 3D7 also had an FICindex > 1 at high [AMQ] and low [TFQ] (Fig. S1). An additive trend in HB3 and IPC5202 was observed at high [TFQ]/low-[CQ] and [TFQ]/low-[AMQ] respectively (Fig. S1). Low [TFQ] with high [NQ] was additive in 3D7 and antagonistic in Dd2, while [TFQ]/high-[NQ] was synergistic in IPC5202 (Fig. S1).

3.3. TFQ and ACT-partner drug combinations differentially affect sexual-stage parasites

To determine the interactions of TFQ with ACT-partner drugs, drug combinations in fixed ratios of 1:3, 1:1 and 3:1 were tested.

| Table 1 |
| Tafenoquine-ACT partner drug molar ratios. |
| Tafenoquine (μM) | 0.5: | 1.0: | 2.0: | 4.0: |
|------------------|-----|-----|-----|-----|
| AMQ              | 0.4 | 0.2 | 0.1 | 0.05 |
| NQ               | 0.4 | 0.2 | 0.1 | 0.05 |
| LMF              | 3.5 | 1.75 | 0.88 | 0.44 |
| MFQ              | 4.0 | 2.0 | 1.0 | 0.5 |
| PND              | 0.64 | 0.32 | 0.16 | 0.08 |
| PPQ              | 0.8 | 0.4 | 0.2 | 0.1 |
| CQ               | 2.4 | 1.2 | 0.6 | 0.3 |

Note: TFQ (0.5, 1.0, 2.0, 4.0 μM) was combined with ACT-partner drugs in fixed molar ratios.

* Five-fold less LMF concentration than the Cmax was used based on *in vitro IC50.*
Only NQ, PND and PPQ appeared to interact with TFQ synergistically, whereas the rest of the drug combinations showed antagonistic interactions at the molar ratios used (Table 4, Fig. 1). Of note, the antagonistic interaction due to MFQ was shifting towards additivity at high [TFQ] and low [MFQ] (Fig. S1).

4. Discussion

Resistance of P. vivax to CQ is spreading and a shift from CQ to ACT followed by PMQ is recommended for radical cure in areas of emerging P. vivax CQ resistance (Price et al., 2014). Currently, PMQ is the only drug licensed for this purpose. Its derivative, TFQ was co-administered with CQ and found to improve relapse-free efficacy in a phase Iib clinical trial and has been shown to be adequate for a first-line treatment for P. falciparum infections common (Snounou and White, 2004; Smithuis et al., 2010). If deployed for P. falciparum, knowledge of the TFQ-ACT partner drug interactions in P. falciparum would benefit areas co-endemic for P. falciparum and P. vivax where mixed infections are common (Snounou and White, 2004; Smithuis et al., 2010). If deployed for P. vivax, TFQ will inevitably be in the human circulation at the same time as CQ or ACT-partner drugs, therefore, assessing whether its presence is beneficial for drug potency is necessary for both P. falciparum and P. vivax parasites.

In P. falciparum strains with different sensitivities to CQ and ART, TFQ showed mostly synergistic interactions in asexual-stage parasites when combined with ACT-partner drugs (Table 4). This is desirable because an augmentation of ACT drug inhibition in the presence of TFQ will likely result in faster cure rates and fewer asexual parasites surviving to later differentiate into infectious gametocytes. This synergism could be due to TFQ and the ACT-partner drugs having different mechanisms of action thereby evading competitive inhibition of the same drug targets. TFQ is reported to exhibit low levels of hematin polymerization inhibition which is likely to supplement the action of the quinolines by further inhibiting hemoglobin digestion and sequestration in the asexual stage parasites, but its action in the gametocyte stages remains speculative (Vennerstrom et al., 1999; Duffy and Avery, 2013). In Leishmania and Trypanosoma, TFQ has been reported to interfere with mitochondrial function leading to apoptosis (Carvalho et al., 2010, 2015), an activity reminiscent of that of PMQ on Plasmodium erythrocytic stages (Beaudoin and Aikawa, 1968) and gametocytes (Lanners, 1991). However, in accordance with other findings, TFQ by itself was ineffective in inhibiting asexual-stage parasites as compared to the ACT-partner drugs, suggesting that its effects relating to hemoglobin digestion may be insignificant (Bray et al., 2005; Gorka et al., 2013; Cabrera and Cui, 2015).

The few studies that have investigated TFQ interactions in vitro in asexual stage P. falciparum reported either synergistic (Bray et al., 2005) or antagonistic interactions with CQ (Gorka et al., 2013). The differences may be due to the drug concentrations used to test the drug interactions. Whereas in the earlier study Cmax ratios were used (Bray et al., 2005), the latter used ratios of in vitro IC50 values which were pharmacologically relevant for CQ but not for TFQ (Gorka et al., 2013). In contrast, a 0.5–4 μM concentration range was used in the present study. Taken together and with the exception of NQ, TFQ appeared to have a positive inhibitory effect on asexual P. falciparum when combined with ACT-partner drugs, regardless of genetic background and CQ or ART susceptibility at fixed ratios taking the Cmax and t1/2 into consideration. The molar ratios where the ACT-partner drug interactions shift towards additivity or antagonism are all at low [TFQ] and high concentrations of the ACT-partner drug which is not easy to attain in vivo because of the long t1/2 of all the drugs tested in this study and subsequently favors the synergism observed in the middle range of the molar ratios tested. In the case of NQ, the molar ratios tested are probably not optimal for synergism to occur and testing for different concentrations and ratios might yield better results (Berenbaum, 1978; Tallarida, 2011). It should be noted that while ART and TFQ have been shown to be synergistic in an ex vivo study (Ramharter et al., 2002), ART derivatives were not tested here since they should have long been eliminated from circulation before the peak TFQ concentration is reached.

Compared to asexual stage parasites, gametocyte IC50 values were in the micromolar range which is consistent with other studies (Peatey et al., 2012; Duffy and Avery, 2013; Cabrera and Cui, 2015). Since many antimalarial drugs are active against gametocytes up to stage III, presumably coinciding with a halt in hemoglobin digestion, the drug susceptibilities of gametocytes of developmental stages were investigated (Wang et al., 2014). Yet, this study did not find significant changes in gametocyte susceptibility to TFQ over time (Table 3), which is similar to PMQ’s in vitro activity against different stages of gametocytes (Wang et al., 2014). With the exception of NQ, PND and PPQ, all TFQ-ACT partner drug interactions in stage IV gametocytes were antagonistic. One significant difference between the experimental design of the asexual and sexual stages was the use of molar ratios highly divergent from Cmax in the gametocytes, which were in the micromolar range. Pharmacologically relevant concentrations based on Cmax may not be enough to result in dose-effect curves necessary to reach the IC50 values for gametocytes (Cabrera and Cui, 2015). It is unclear why TFQ-ACT partner drug interactions in gametocytes are different...
from the asexual stage parasites. Optimistically, comparisons to different parasite strains with varying genetic backgrounds may yield clues on what systems or targets in the gametocytes are affected. Based on the purported PMQ target and observed organelle differences in the asexual and sexual stages which show significant structural, metabolomic, and transcriptional differences in the parasite mitochondrion of asexual vs. sexual stage parasites (Okamoto et al., 2009), a hypothesis based on the gametocyte mitochondria as a TFQ target is favorable. Clearly from our results, the 4-aminoquinolines (PMQ and TFQ) and the aryl amino-alcohols (LMF and MFQ) have antagonistic interactions with TFQ in the gametocyte stages and molar ratios tested. Interestingly, the definitions for synergy or antagonism, however, are arbitrary and it may very well be that had the threshold mean sum of FIC set at 2 or 4 instead of 1, synergistic or additive interactions with TFQ would have been observed for all the ACT-partner drugs tested with the single exception of AMQ (Bell, 2005). And again, different molar concentrations and combination ratios can yield varying results in
Table 4
Tafenoquine-ACT partner drug interactions in asexual and sexual stages of P. falciparum.

| ACT partner drug | HB3 | 3D7 | 7G8 | Dd2 | IPC5202 | 3D7-act rifGP | 3D7-fl GP |
|------------------|-----|-----|-----|-----|---------|-------------|---------|
| AMQ              | Syn | Syn | Syn | Syn | Syn     | Syn*        | Syn     |
| NQ               | Syn | Syn | Syn | Syn | Syn*    | Syn*        | Syn     |
| LMF              | Syn*| Syn | Syn | Syn | Syn*    | Syn         | Syn     |
| MQF              | Syn | Syn | Syn | Syn | Syn*    | Syn*        | Syn     |
| PND              | Syn | Syn | Syn | Syn | Syn     | Syn         | Syn     |
| PPQ              | Syn | Syn | Syn | Syn | Syn     | Syn         | Syn     |
| CQ               | Syn | Syn | Syn | Syn | Syn*    | Syn*        | Syn     |

Asexual stages of different strains and 3D7-act GFP gametocytes were tested with different fixed ratios of TFQ + ACT partner drugs shown in Table 1 for asexual parasites and 1:1, 1:3 and 3:1 for gametocytes. The mean sum of FICindices were used to interpret synergistic (Syn < 1), antagonistic (Ant > 1), and additive (Add = 1) interactions.

a At [TFQ]high:[partner drug]low.
b Only at [TFQ]low:[partner drug]high.

This study for the first time investigated TFQ-ACT partner drug interactions on parasites with different genetic backgrounds, at pharmacologically relevant drug concentrations, and with the use of gametocytes. The major limitations to this study were the omission of tests in P. vivax because of the difficulty of maintaining it in continuous culture. The drug-drug interactions in gametocytes were performed only in the 3D7 genetic background. In addition, the fixed-ratios for gametocytes were higher than Cmax, which may not be physiologically relevant. Lastly, the use of AMQ instead of its active metabolite in vivo, mono desethyl-amodiaquine, may not result in the same interaction as the metabolite. Thus, in vivo studies will need to be carried out to validate these results as many host factors come into play. For example, drugs such as CQ and MFQ are known to increase the number of gametocytes, which might be problematic assuming antagonism is reproduced in vivo (Price et al., 1996; Buckling et al., 1999). Other drugs such as TFQ, MFQ, LMF, and PPQ have a higher bioavailability when taken with food (Crockett and Kain, 2007; Nguyen et al., 2008). With regard to mixed infections, P. falciparum and P. vivax mixed infections have been reported to show differences in severity, onset of one or the other, and the number of gametocytes etc. compared to single infections (Price et al., 1999; Snounou and White, 2004). In addition, TFQ Cmax may be variable depending on cytochrome P450 metabolism and gender, with women displaying higher plasma concentrations than men (Edstein et al., 2007; Vuong et al., 2015). It has been shown that unlike PMQ, the efficacy of TFQ is not diminished in CYP2D6 intermediate metabolizers nor is TFQ pharmacokinetics affected by CYP2D6 (St Jean et al., 2016). An ongoing study is currently evaluating the safety, tolerability and hemolytic potential of TFQ in G6PD-deficient healthy female volunteers (Clinical-Trials.gov NCT01205178).

Taken together, TFQ’s mostly synergistic effect on the asexual stage parasites is promising because although it is being targeted for P. vivax, it has some benefit to P. falciparum infection in the case of mixed infection. If the presence of TFQ with ACT enhances the inhibitory effect on asexual parasites, less asexual parasites will commit to gametocytophylaxis and transmission. Although being developed for P. vivax, it is important to note that earlier studies of TFQ against P. falciparum showed chemo-prophylactic activity (Buckling et al., 1999; Shanks et al., 2001). Care will need to be taken when choosing an ACT to take prior to TFQ dosage as the least desirable ones are those with a consensus of antagonism in similar in vitro and in vivo studies. A study testing the DHA-PPQ and artemether-LMF ACT interactions with TFQ has been completed elsewhere (ClinicalTrials.gov identifier: NCT02184637) and summarily reported no adverse pharmacokinetic interactions between said ACTs and TFQ (Green et al., 2016). TFQ does however cause severe hemolysis in individuals with reduced G6PD activity so in vivo studies in humanized mice and humans with both normal and reduced G6PD activity studies will also need to be undertaken (Rochford et al., 2013). In summary, the future of malaria chemotherapy lies in the identification of combination therapies to maximize inhibitory effects to parasites and to slow down the evolution of resistance; drug-drug interactions such as this, with varied parasite strains and drug tolerance will therefore be imperative to test the different candidates in pre-clinical trials. Furthermore, in vivo TFQ-ACT/CQ drug interaction work in humanized mice of varying G6PD activity as well as human clinical trials are a necessary follow-up to results reported here since host factors such as gender, immunity, drug metabolism as well as diet, among others, might influence the results obtained.

Abbreviations

ACT Artemisinin Combination Therapy
AMQ Amodiaquine
ART Artemisinin
CQ Chloroquine
DHA Dihydroartemisinin
LMF Lumefantrine
MFQ Mefloquine
NQ Naphthoquinone
PMQ Primaquine
PND Pyronaridine
PPQ Piperaquine
TFQ Tafenoquine
MCM Malaria Complete Medium
G6PD Glucose-6-Phosphate Dehydrogenase
GFP Green Fluorescent Protein
IC50 Median inhibitory drug concentration
Cmax Maximum plasma concentration following a single drug dose
FIC Fractional Inhibitory Concentration
atubIGFP Green fluorescent protein expression driven by α-tubulin II [PlasmoDB: PF3D7_042230] promoter
MW Molecular Weight
DMSO Dimethyl sulfoxide

Competing interests

The authors have no competing interests to declare.

Authors’ contributions

MC and LC conceived of the study. KK designed and performed all the experiments, analyzed data and drafted the manuscript. MC helped with experimental design, trouble-shooting, performed data analysis and figure preparation, and edited the manuscript. LC assisted with data interpretation and edited the manuscript. All authors have read and approved the final manuscript.

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Appendix A. Supplementary data

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