International Journal for Parasitology: Drugs and Drug Resistance 7 (2017) 131–137

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

Invited article

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

Karen Kemirembe [1], Mynthia Cabrera [1], Liwang Cui*

Department of Entomology, The Pennsylvania State University, University Park, PA 16802, USA

ARTICLE INFO

Article history:
Received 13 January 2017
Received in revised form
4 March 2017
Accepted 8 March 2017
Available online 11 March 2017

Keywords:
Plasmodium falciparum
Tafenoquine
Malaria
Gametocyte
Drug interaction
8-Aminoquinoline
Isobologram
ACT

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.
© 2017 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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, a 14-day course of PMQ (0.25 mg base/kg/day) is normally prescribed (Baird and Hoffman, 2004). 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 ($t_{1/2}$) 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 IIb clinical trial has already shown better protection against *P. vivax* relapses with a 3 day chloroquine (CQ) dosage + 300 mg TFQ on day 1or 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

* Corresponding author.
E-mail address: luc2@psu.edu (L. Cui).
[1] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.ijpddr.2017.03.002
2211-3207/© 2017 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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 (Udomsangpetch et al., 2007; Roobsoong 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 (Ramharter 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 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 (T_{max}) 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 aryl 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 nylon 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% CO_2 incubator at 37 °C in MCM containing RPMI 1640, 25 mM $NaHCO_3$, 25 mM HEPES (pH 7.4), 11 mM glucose, 0.367 mM hypoxanthine and 5 μg/L 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 $3D7^{\alpha\text{-}tubII GFP}$ 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% hematocrit 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% hematocrit and 0.5% parasitemia per well. Negative control wells without drug and with MCM, DMSO, or 10% 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% CO_2 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 (IC_{50}) 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 3D7$^{\alpha tubIIGFP}$, corresponding to days 4–12 post-gametocyte induction, at 0.04% gametocytemia and 1% hematocrit, were incubated in 2 × concentrations of serially diluted pre-loaded drug plates for 48 h at 37 °C in a 5% CO_2 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 Na_2HPO_4 to minimize autofluorescence 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 version 10 software and plotted against drug concentrations to generate drug dose-effect curves. Median IC_{50} 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 (C_{max}) (Table S2), as well as the *in vitro* median IC_{50} of each individual drug to ensure that proper drug-response curves were obtained. IC_{50} of individual drugs, and drugs in combination with TFQ against 3D7, 7G8, Dd2, IPC5202 and HB3 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 IC_{50}, which is the IC_{50} of the ACT-partner drug when combined with TFQ, was used to determine the fractional inhibitory concentration index (FIC_{index}) using the formula: FIC_{TFQ} (Apparent IC_{50} of TFQ when combined with ACT partner drug/TFQ IC_{50}) + $FIC_{ACT\ partner\ drug}$ (Apparent IC_{50} of ACT partner drug when combined with TFQ/ACT partner drug IC_{50}). FIC_{index} 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 FIC_{TFQ} is plotted vs. $FIC_{ACT\ partner\ drug}$, respectively (Gorka et al., 2013).

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[a]	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 and 4.0 μM) was combined with ACT-partner drugs in fixed molar ratios.
[a] Five-fold less LMF concentration than the C_{max} was used based on *in vitro* IC_{50}.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey HSD tests were used to determine the differences in IC_{50}s 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 IC_{50} 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 IC_{50} values of 7G8, Dd2, and IPC5202. The 7G8 and IPC5202 strains also exhibited significantly lower and higher IC_{50}, 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 IV 3D7$^{\alpha\text{-}tubIIGFP}$ 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 IV 3D7$^{\alpha\text{-}tubIIGFP}$ while AMQ was the most effective. The rest of the drugs have moderate gametocytocidal activity in the micromolar range. In our experimental conditions, TFQ IC_{50}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 ($FIC_{index} > 1$), synergistic ($FIC_{index} < 1$) or additive ($FIC_{index} = 1$). 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 $FIC_{index} > 1$ at high [LMF] and low [TFQ] (Fig. S1). 3D7 also had an FICindex >1 at high [AMQ] and low [TFQ] (Fig. S1). An additivity trend in HB3 and IPC5202 was observed at $[TFQ]_{low}$:$[CQ]_{high}$ and $[TFQ]_{low}$:$[AMQ]_{high}$, respectively (Fig. S1). Low [TFQ] with high [NQ] was additive in 3D7 and antagonistic in Dd2, while $[TFQ]_{high}$:$[NQ]_{low}$ 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 2
IC_{50}s of asexual and sexual stage *P. falciparum* parasites for different antimalarial drugs (mean ± SEM).

Drug	HB3 (nM)	3D7 (nM)	7G8 (nM)	Dd2 (nM)	IPC5202 (nM)	3D7$^{\alpha\text{-}tubIIGFP}$ (μM)
AMQ	9.9 ± 1.8	4.8 ± 0.7	19.0 ± 3.8	9.8 ± 1.0	10.3 ± 0.4	5.9 ± 1.3
NQ	12.1 ± 2.0	4.1 ± 0.9	3.9 ± 0.1	10.9 ± 1.3	5.9 ± 2.3	142.5 ± 15.8
LMF	8.9 ± 1.9	11.6 ± 0.7	6.9 ± 1.0	11.9 ± 1.2	38.7 ± 5.0	723.6 ± 79.2
MFQ	16.5 ± 1.6	13.0 ± 0.4	1.6 ± 0.6[a]	11.6 ± 1.3	28.8 ± 2.8[a]	16.5 ± 11.6
PND	3.3 ± 0.5	3.8 ± 0.8	4.8 ± 0.9	4.9 ± 0.6	2.7 ± 0.1	25.1 ± 7.9
PPQ	17.8 ± 1.8	23.6 ± 0.6	31.6 ± 2.6	15.3 ± 1.5	19.1 ± 0.7	197.7 ± 12.6
CQ	18.5 ± 1.5	24.5 ± 1.4	318.0 ± 14.4[a]	308.7 ± 24.5[a]	416.3 ± 27.7[a]	27.6 ± 0.3
TFQ	1875.2 ± 418.9	1719.5 ± 321.3	2400.7 ± 193.1	1129.8 ± 201.0	2045.3 ± 290.1	9.8 ± 1.4

Various strains of asexual stage parasites and stage IV 3D7$^{\alpha\text{-}tubIIGFP}$ gametocytes tested against ACT-partner drugs, CQ, and TFQ using the SYBR Green I method for the asexual stages and flow cytometry method for the gametocytes.
[a] Significantly different drug susceptibility (ANOVA $p < 0.001$), standard error of the mean (SEM).

Table 3
TFQ IC_{50} values in *P. falciparum* stage II-V gametocytes.

Gametocyte stage	TFQ (μM, mean ± SEM)
II	8.8 ± 2.4
III	8.1 ± 0.5
IV	9.8 ± 1.4
V	11.1 ± 1.7

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 similar role to PMQ (Llanos-Cuentas et al., 2014). Since ACTs are already the first-line treatment 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 C_{max} ratios were used (Bray et al., 2005), the latter used ratios of *in vitro* IC_{50} 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 C_{max} and $t_{1/2}$ 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 $t_{1/2}$ 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 IC_{50} 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 C_{max} in the gametocytes, which were in the micromolar range. Pharmacologically relevant concentrations based on C_{max} may not be enough to result in dose-effect curves necessary to reach the IC_{50} values for gametocytes (Cabrera and Cui, 2015). It is unclear why TFQ-ACT partner drug interactions in gametocytes are different

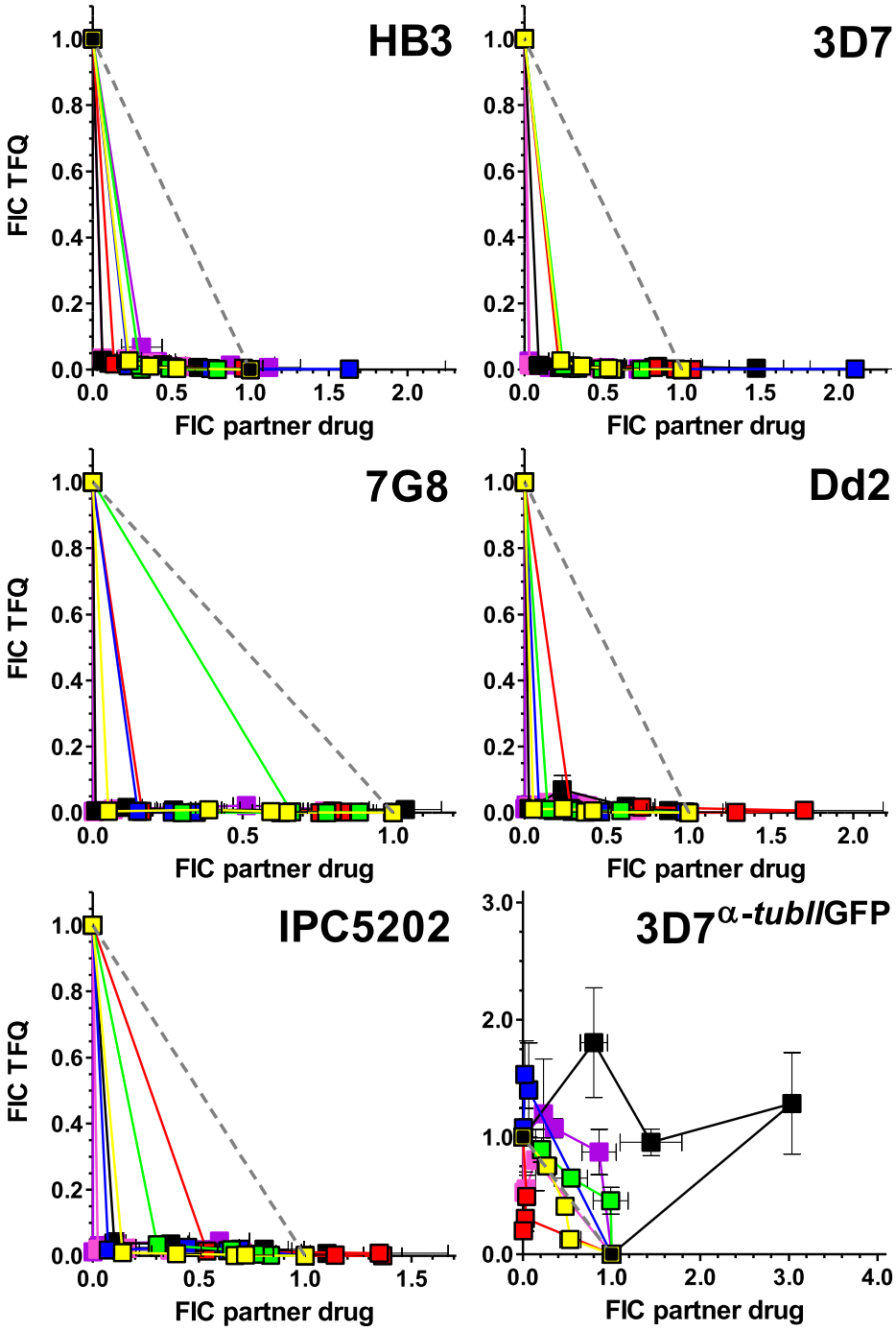

Fig. 1. Isobolograms of *in vitro* sensitivities of *P. falciparum* strains to the TFQ – ACT-partner drug combination at fixed molar ratios. Fractional inhibitory concentrations (FICs) of TFQ against various ACT partner drugs: AMQ (black), NQ (red), LMF (blue), MFQ (green), PND (yellow), PPQ (pink), CQ (purple); in asexual stages of HB3, 3D7, 7G8, Dd2, IPC5202, and stage IV gametocytes of 3D7$^{\alpha\text{-}tubIIGFP}$. Error bars indicate standard error of the mean (SEM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 organellar 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 drugs	HB3	3D7	7G8	Dd2	IPC5202	3D7$^{\alpha\text{-}tubIIGFP}$
AMQ	Syn	Syn[a]	Syn	Syn	Syn[a]	Ant
NQ	Syn	Syn[a]	Syn	Syn[a]	Ant[b]	Syn
LMF	Syn[a]	Syn[a]	Syn	Syn	Syn	Ant
MFQ	Syn	Syn	Syn	Syn	Syn	Ant[b]
PND	Syn	Syn	Syn	Syn	Syn	Syn
PPQ	Syn	Syn	Syn	Syn	Syn	Syn
CQ	Syn[b]	Syn	Syn	Syn	Syn	Ant

Asexual stages of different strains and 3D7$^{\alpha tubIIGFP}$ 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 FIC_{index} 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 scenario.

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 C_{max}, 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 C_{max} 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 (ClinicalTrials.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 gametocytogenesis 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 (Brueckner et al., 1998; 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 that show 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	Naphthoquine
PMQ	Primaquine
PND	Pyronaridine
PPQ	Piperaquine
TFQ	Tafenoquine
MCM	Malaria Complete Medium
G6PD	Glucose-6-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
IC_{50}	Median inhibitory drug concentration
C_{max}	Maximum plasma concentration following a single drug dose
FIC	Fractional Inhibitory Concentration
*αtubII*GFP	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.

Acknowledgments

This research was supported by the National Institutes of Health (U19AI089672). We are grateful to Xiaolian Li for assisting with the parasite culture.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2017.03.002.

References

Ali, S., Najmi, M.H., Tarning, J., Lindegardh, N., 2010. Pharmacokinetics of artemether and dihydroartemisinin in healthy Pakistani male volunteers treated with artemether-lumefantrine. Malar. J. 9, 275.

Baird, J.K., 2009. Resistance to therapies for infection by *Plasmodium vivax*. Clin. Microbiol. Rev. 22, 508–534.

Baird, J.K., Hoffman, S.L., 2004. Primaquine therapy for malaria. Clin. Infect. Dis. 39, 1336–1345.

Beaudoin, R.L., Aikawa, M., 1968. Primaquine-induced changes in morphology of exoerythrocytic stages of malaria. Science 160, 1233–1234.

Bell, A., 2005. Antimalarial drug synergism and antagonism: mechanistic and clinical significance. FEMS Microbiol. Lett. 253, 171–184.

Benjamin, J., Moore, B., Lee, S.T., Senn, M., Griffin, S., Lautu, D., Salman, S., Siba, P., Mueller, I., Davis, T.M., 2012. Artemisinin-naphthoquine combination therapy for uncomplicated pediatric malaria: a tolerability, safety, and preliminary efficacy study. Antimicrob. Agents Chemother. 56, 2465–2471.

Berenbaum, M.C., 1978. A method for testing for synergy with any number of agents. J. Infect. Dis. 137, 122–130.

Bray, P.G., Deed, S., Fox, E., Kalkanidis, M., Mungthin, M., Deady, L.W., Tilley, L., 2005. Primaquine synergises the activity of chloroquine against chloroquine-resistant P. falciparum. Biochem. Pharmacol. 70, 1158–1166.

Brueckner, R.P., Coster, T., Wesche, D.L., Shmuklarsky, M., Schuster, B.G., 1998. Prophylaxis of *Plasmodium falciparum* infection in a human challenge model with WR 238605, a new 8-aminoquinoline antimalarial. Antimicrob. Agents Chemother. 42, 1293–1294.

Buckling, A., Ranford-Cartwright, L.C., Miles, A., Read, A.F., 1999. Chloroquine increases *Plasmodium falciparum* gametocytogenesis in vitro. Parasitology 118 (Pt 4), 339–346.

Cabrera, M., Cui, L., 2015. In vitro activities of primaquine-schizonticide combinations on asexual blood stages and gametocytes of *Plasmodium falciparum*. Antimicrob. Agents Chemother. 59, 7650–7656.

Carvalho, L., Luque-Ortega, J.R., Manzano, J.I., Castanys, S., Rivas, L., Gamarro, F., 2010. Tafenoquine, an antiplasmodial 8-aminoquinoline, targets leishmania respiratory complex III and induces apoptosis. Antimicrob. Agents Chemother. 54, 5344–5351.

Carvalho, L., Martinez-Garcia, M., Perez-Victoria, I., Manzano, J.I., Yardley, V., Gamarro, F., Perez-Victoria, J.M., 2015. The oral antimalarial drug tafenoquine shows activity against Trypanosoma brucei. Antimicrob. Agents Chemother. 59, 6151–6160.

Crockett, M., Kain, K.C., 2007. Tafenoquine: a promising new antimalarial agent. Expert Opin. Investig. Drugs 16, 705–715.

Duffy, S., Avery, V.M., 2013. Identification of inhibitors of *Plasmodium falciparum* gametocyte development. Malar. J. 12, 408.

Eastman, R.T., Fidock, D.A., 2009. Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. Nat. Rev. Microbiol. 7, 864–874.

Edstein, M.D., Nasveld, P.E., Kocisko, D.A., Kitchener, S.J., Gatton, M.L., Rieckmann, K.H., 2007. Gender differences in gastrointestinal disturbances and plasma concentrations of tafenoquine in healthy volunteers after tafenoquine administration for post-exposure vivax malaria prophylaxis. Trans. R. Soc. Trop. Med. Hyg. 101, 226–230.

Gorka, A.P., Jacobs, L.M., Roepe, P.D., 2013. Cytostatic versus cytocidal profiling of quinoline drug combinations via modified fixed-ratio isobologram analysis. Malar. J. 12, 332.

Green, J.A., Mohamed, K., Goyal, N., Bouhired, S., Hussaini, A., Jones, S.W., Koh, G.C., Kostov, I., Taylor, M., Wolstenholm, A., Duparc, S., 2016. Pharmacokinetic interactions between tafenoquine and dihydroartemisinin-piperaquine or artemether-lumefantrine in healthy adult subjects. Antimicrob. Agents Chemother. 60, 7321–7332.

Green, J.A., Patel, A.K., Patel, B.R., Hussaini, A., Harrell, E.J., McDonald, M.J., Carter, N., Mohamed, K., Duparc, S., Miller, A.K., 2014. Tafenoquine at therapeutic concentrations does not prolong Fridericia-corrected QT interval in healthy subjects. J. Clin. Pharmacol. 54, 995–1005.

Hawley, S.R., Bray, P.G., Mungthin, M., Atkinson, J.D., O'Neill, P.M., Ward, S.A., 1998. Relationship between antimalarial drug activity, accumulation, and inhibition of heme polymerization in *Plasmodium falciparum* in vitro. Antimicrob. Agents Chemother. 42, 682–686.

Lambros, C., Vanderberg, J.P., 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. 65, 418–420.

Lanners, H.N., 1991. Effect of the 8-aminoquinoline primaquine on culture-derived gametocytes of the malaria parasite *Plasmodium falciparum*. Parasitol. Res. 77, 478–481.

Li, Q., O'Neil, M., Xie, L., Caridha, D., Zeng, Q., Zhang, J., Pybus, B., Hickman, M., Melendez, V., 2014. Assessment of the prophylactic activity and pharmacokinetic profile of oral tafenoquine compared to primaquine for inhibition of liver stage malaria infections. Malar. J. 13, 141.

Llanos-Cuentas, A., Lacerda, M.V., Rueangweerayut, R., Krudsood, S., Gupta, S.K., Kochar, S.K., Arthur, P., Chuenchom, N., Mohrle, J.J., Duparc, S., Ugwuegbulam, C., Kleim, J.P., Carter, N., Green, J.A., Kellam, L., 2014. Tafenoquine plus chloroquine for the treatment and relapse prevention of *Plasmodium vivax* malaria (DETECTIVE): a multicentre, double-blind, randomised, phase 2b dose-selection study. Lancet 383, 1049–1058.

Miao, J., Wang, Z., Liu, M., Parker, D., Li, X., Chen, X., Cui, L., 2013. *Plasmodium falciparum*: generation of pure gametocyte culture by heparin treatment. Exp. Parasitol. 135, 541–545.

Morris, C.A., Duparc, S., Borghini-Fuhrer, I., Jung, D., Shin, C.S., Fleckenstein, L., 2011. Review of the clinical pharmacokinetics of artesunate and its active metabolite dihydroartemisinin following intravenous, intramuscular, oral or rectal administration. Malar. J. 10, 263.

Muangnoicharoen, S., Johnson, D.J., Looareesuwan, S., Krudsood, S., Ward, S.A., 2009. Role of known molecular markers of resistance in the antimalarial potency of piperaquine and dihydroartemisinin in vitro. Antimicrob. Agents Chemother. 53, 1362–1366.

Nguyen, T.C., Nguyen, N.Q., Nguyen, X.T., Bui, D., Travers, T., Edstein, M.D., 2008. Pharmacokinetics of the antimalarial drug piperaquine in healthy Vietnamese subjects. Am. J. Trop. Med. Hyg. 79, 620–623.

Okamoto, N., Spurck, T.P., Goodman, C.D., McFadden, G.I., 2009. Apicoplast and mitochondrion in gametocytogenesis of *Plasmodium falciparum*. Eukaryot. Cell 8, 128–132.

Peatey, C.L., Leroy, D., Gardiner, D.L., Trenholme, K.R., 2012. Anti-malarial drugs: how effective are they against *Plasmodium falciparum* gametocytes? Malar. J. 11, 34.

Pelfrene, E., Pinheiro, M.H., Cavaleri, M., 2015. Artemisinin-based combination therapy in the treatment of uncomplicated malaria: review of recent regulatory experience at the European Medicines Agency. Int. Health 7, 239–246.

Price, R., Nosten, F., Simpson, J.A., Luxemburger, C., Phaipun, L., ter Kuile, F., van Vugt, M., Chongsuphajaisiddhi, T., White, N.J., 1999. Risk factors for gametocyte carriage in uncomplicated falciparum malaria. Am. J. Trop. Med. Hyg. 60, 1019–1023.

Price, R.N., Nosten, F., Luxemburger, C., ter Kuile, F.O., Paiphun, L., Chongsuphajaisiddhi, T., White, N.J., 1996. Effects of artemisinin derivatives on malaria transmissibility. Lancet 347, 1654–1658.

Price, R.N., von Seidlein, L., Valecha, N., Nosten, F., Baird, J.K., White, N.J., 2014. Global extent of chloroquine-resistant *Plasmodium vivax*: a systematic review and meta-analysis. Lancet Infect. Dis. 14, 982–991.

Ramharter, M., Noedl, H., Thimasarn, K., Wiedermann, G., Wernsdorfer, G., Wernsdorfer, W.H., 2002. In vitro activity of tafenoquine alone and in combination with artemisinin against *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. 67, 39–43.

Rochford, R., Ohrt, C., Baresel, P.C., Campo, B., Sampath, A., Magill, A.J., Tekwani, B.L., Walker, L.A., 2013. Humanized mouse model of glucose 6-phosphate dehydrogenase deficiency for in vivo assessment of hemolytic toxicity. Proc. Natl. Acad. Sci. U. S. A. 110, 17486–17491.

Roobsoong, W., Tharinjaroen, C.S., Rachaphaew, N., Chobson, P., Schofield, L., Cui, L., Adams, J.H., Sattabongkot, J., 2015. Improvement of culture conditions for long-term in vitro culture of *Plasmodium vivax*. Malar. J. 14, 297.

Shanks, G.D., Kain, K.C., Keystone, J.S., 2001. Malaria chemoprophylaxis in the age of drug resistance. II. Drugs that may be available in the future. Clin. Infect. Dis. 33, 381–385.

Smilkstein, M., Sriwilaijaroen, N., Kelly, J.X., Wilairat, P., Riscoe, M., 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob. Agents Chemother. 48, 1803–1806.

Smithuis, F., Kyaw, M.K., Phe, O., Win, T., Aung, P.P., Oo, A.P., Naing, A.L., Nyo, M.Y., Myint, N.Z., Imwong, M., Ashley, E., Lee, S.J., White, N.J., 2010. Effectiveness of five artemisinin combination regimens with or without primaquine in uncomplicated falciparum malaria: an open-label randomised trial. Lancet Infect. Dis. 10, 673–681.

Snounou, G., White, N.J., 2004. The co-existence of Plasmodium: sidelights from falciparum and vivax malaria in Thailand. Trends Parasitol. 20, 333–339.

St Jean, P.L., Xue, Z., Carter, N., Koh, G.C., Duparc, S., Taylor, M., Beaumont, C., Llanos-Cuentas, A., Rueangweerayut, R., Krudsood, S., Green, J.A., Rubio, J.P., 2016. Tafenoquine treatment of *Plasmodium vivax* malaria: suggestive evidence that CYP2D6 reduced metabolism is not associated with relapse in the Phase 2b DETECTIVE trial. Malar. J. 15, 97.

Tallarida, R.J., 2011. Quantitative methods for assessing drug synergism. Genes Cancer 2, 1003–1008.

Udomsangpetch, R., Somsri, S., Panichakul, T., Chotivanich, K., Sirichaisinthop, J., Yang, Z., Cui, L., Sattabongkot, J., 2007. Short-term in vitro culture of field isolates of *Plasmodium vivax* using umbilical cord blood. Parasitol. Int. 56, 65–69.

Vennerstrom, J.L., Nuzum, E.O., Miller, R.E., Dorn, A., Gerena, L., Dande, P.A., Ellis, W.Y., Ridley, R.G., Milhous, W.K., 1999. 8-Aminoquinolines active against blood stage *Plasmodium falciparum* in vitro inhibit hematin polymerization. Antimicrob. Agents Chemother. 43, 598–602.

Vuong, C., Xie, L.H., Potter, B.M., Zhang, J., Zhang, P., Duan, D., Nolan, C.K., Sciotti, R.J., Zottig, V.E., Nanayakkara, N.P., Tekwani, B.L., Walker, L.A., Smith, P.L., Paris, R.M., Read, L.T., Li, Q., Pybus, B.S., Sousa, J.C., Reichard, G.A., Smith, B., Marcsisin, S.R., 2015. Differential cytochrome P450 2D metabolism alters tafenoquine pharmacokinetics. Antimicrob. Agents Chemother. 59, 3864–3869.

Wang, Z., Liu, M., Liang, X., Siriwat, S., Li, X., Chen, X., Parker, D.M., Miao, J., Cui, L., 2014. A flow cytometry-based quantitative drug sensitivity assay for all *Plasmodium falciparum* gametocyte stages. PLoS One 9, e93825.

White, N.J., 2013. Primaquine to prevent transmission of falciparum malaria. Lancet Infect. Dis. 13, 175–181.