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Invited article

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.

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

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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 (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 $3D7^{\alpha\text{-}tubII\text{GFP}}$ 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