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Research paper

Artesunate-tafenoquine combination therapy promotes clearance and abrogates transmission of the avian malaria parasite *Plasmodium gallinaceum*

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ABSTRACT

Clinical manifestations of malaria infection in vertebrate hosts arise from the multiplication of the asexual stage parasites in the blood, while the gametocytes are responsible for the transmission of the disease. Antimalarial drugs that target the blood stage parasites and transmissible gametocytes are rare, but are essentially needed for the effective control of malaria and for limiting the spread of resistance. Artemisinin and its derivatives are the current first-line antimalarials that are effective against the blood stage parasites and gametocytes, but resistance to artemisinin has now emerged and spread in various malaria endemic areas. Therefore, a novel antimalarial drug, or a new drug combination, is critically needed to overcome this problem. The objectives of this study were to evaluate the efficacy of a relatively new antimalarial compound, tafenoquine (TQ), and a combination of TQ and a low dose of artesunate (ATN) on the *in vivo* blood stage multiplication, gametocyte development and transmission of the avian malaria parasite *Plasmodium gallinaceum* to the vector *Aedes aegypti*. The results showed that a 5-d treatment with TQ alone was unable to clear the blood stage parasites, but was capable of reducing the mortality rate, while TQ monotherapy at a high dose of 30 mg/kg was highly effective against the gametocytes and completely blocked the transmission of *P. gallinaceum*. In addition, the combination therapy of TQ + ATN completely cleared *P. gallinaceum* blood stages and sped up the gametocyte clearance from chickens, suggesting the synergistic effect of the two drugs. In conclusion, TQ is demonstrated to be effective for limiting avian malaria transmission and may be used in combination with a low dose of ATN for safe and effective treatment.

1. Introduction

Malaria is a mosquito-borne disease caused by parasite protozoa in the genus *Plasmodium*, and is commonly found in a wide range of terrestrial vertebrates, including reptiles, birds and mammals (Clark et al., 2014; Pattaradilokrat et al., 2015; Schall, 1996; Templeton et al., 2016). Human malaria diseases are considered major health problems due to their wide distribution in tropical and sub-tropical regions, mainly in Asia, Africa and South America, with estimates of 200–300 million clinical cases annually. Life-threatening complications, including coma and death, can develop during a malaria infection, which frequently occur in children under 5 years old and pregnant women (Wassmer et al., 2015). The emergence of drug resistant malaria parasites also impedes global malaria control and eradication efforts (Antony and Parija, 2016). It has been recommended that all existing antimalarial drugs should be evaluated for their transmission-blocking activities because this has been advocated as a way for limiting the expansion of drug resistant parasites (Teklehaimanot et al., 1985; White, 2008). Thus, the development and screening of new antimalarial drugs with gametocytocidal properties has a high research priority.

Like human malaria diseases, avian malaria in domestic chickens (*Gallus gallus domesticus*) is highly pathogenic (Williams, 2005).

Abbreviations: ATM, artemisinin; ATN, artesunate; BW, body weight; CQ, chloroquine; dpi, days post infection; MTD, maximum tolerant dose; PBS, phosphate buffered saline; PQ, primaquine; TQ, tafenoquine.

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The main causative agent, *Plasmodium gallinaceum*, is prevalent in a number of countries in Southeast and South Asia, where it often causes a high mortality rate, and contributes strongly to the economic loss in the poultry industry. The parasite itself can be transmitted by a number of mosquito species, including *Aedes* sp., *Anopheles* sp. and *Culex sp*., allowing malaria to spread rapidly to new areas (Alavi et al., 2003; Pruck-Ngern et al., 2015). The life cycle of *P. gallinaceum* in domestic chicken is complex and requires two rounds of pre-erythrocytic stage replication in the liver and endothelial cells. After that, the resulting parasites are released into the blood vessels and develop inside the erythrocytes. During this erythrocytic stage development, avian malaria shares characteristics with other malaria species (Macchi Bde et al., 2010; Nagao et al., 2008; Paulman and McAllister, 2005), providing an alternative model for *in vivo* antimalarial drug tests that could be used to complement human malaria research.

Chloroquine (CQ) and doxycycline are the two most frequently used drugs for the treatment of avian malaria (Sohsuebngarm et al., 2014). These drugs have been shown to target the erythrocyte stage of *P. gallinaceum*, thereby reducing the morbidity and mortality of the infected chickens. However, the drugs are poorly effective against the gametocytes, and so they do not affect the rate of malaria transmission. Identification of antimalarial drugs targeting both the blood stages and gametocytes would be preferable for long-term malaria control. To address this need, an *in vivo* transmission blocking assay has been established, in which *P. gallinaceum* is readily employed for screening gametocytocidal compounds (Kumnuan et al., 2013). Briefly, the blood stage malarias are grown in domestic chickens (*G. gallus domesticus*). Once the parasites have grown to approximate levels of 10% parasitemia and 1% gametocytemia, the infected chickens are treated with the compound(s) of interest prior to mosquito feeding. The transmission dynamics of the malaria parasites before and after treatment can be carefully monitored. Recent studies demonstrated that a 7-d administration of 10 mg/kg artesunate (ATN) cleared the blood stage parasites and gametocytes and also blocked transmission of the parasites to *Aedes aegypti* and *Culex quinquefasciatus* (Kumnuan et al., 2013; Pruck-Ngern et al., 2015). Hence, this assay has provided a platform for the rapid discovery of potential antimalarial drugs for avian and human malaria diseases.

The key antimalarial drug ATN, a derivative of artemisinin (ATM), is employed globally for malaria treatment (Noubiap, 2014), but parasites resistant to ATM and its derivatives have now emerged and spread in various malaria endemic areas (Ashley et al., 2014; Dondorp et al., 2009). Critically, a novel antimalarial drug, or a new drug combination, is needed to overcome this problem. Tafenoquine (TQ), also known as WR238605 or etaquine, is regarded as the most advanced candidate compound. It has been tested in Phase III clinical trials for treatment of the hypnozoite stages of *Plasmodium vivax*, which are responsible for the relapse of malaria (Campo et al., 2015). Although TQ belongs to the group of 8-aminoquinolines, which also includes primaquine (PQ) (Crockett and Kain, 2007), TQ has a better therapeutic index and greater activities against liver stages of *Plasmodium* than PQ and so has the potential to replace PQ as a prophylactic agent (Li et al., 2014). In a pre-clinical study, TQ was well tolerated, with only mild, transient gastrointestinal side effects in healthy human volunteers (Brueckner et al., 1998a). Within animal models, including humans, TQ has a long half-life compared with that of PQ (Brueckner et al., 1998b; Charles et al., 2007; Li et al., 2014). While the greater *in vitro* potency and longer half-life of TQ may be attributed to its higher *in vivo* efficacy, TQ monotherapy may provide selective pressure for the development of drug resistance in areas with a high level of malaria transmission. Accordingly, an appropriate combination of TQ with a more potent and fast acting blood schizontocidal drug would yield a more rapidly acting and effective regimen. A combination therapy of TQ and other antimalarial drugs, such as CQ, mefloquine and ATM-lumefantrin, has been extensively tested for prophylactic activities against pre-erythrocytic stages of human malarias with promising results (Dow et al., 2011; Llanos-Cuentas et al., 2014; Ramharter et al., 2002). Whereas only a few studies have reported gametocytocidal activities of TQ, there has still been no report on the gametocytocidal activities of TQ in combination with other drugs (Coleman, 1990; Coleman et al., 1992; Ponsa et al., 2003). It is also not known whether a combined treatment with ATN + TQ will have a synergistic effect. Thus, this study aimed to evaluate the inhibitory effects of TQ monotherapy and ATN + TQ combination therapy on the blood stage development, gametocytes and transmission of *P. gallinaceum* to *A. aegypti* mosquitoes. The outcome of the present study would likely be beneficial for designing an effective treatment for avian malaria and may be applicable for human malaria studies.

2. Materials and methods

2.1. Malaria parasite and laboratory hosts

A line of the blood stage *P. gallinaceum* strain Pg01/2013MUPH was used. The parasite isolate originated from an infected chicken at a local farm in Chachoengsao, Thailand, in 2013 (Pruck-Ngern et al., 2015), and has been maintained by serial blood passage and occasionally through mosquito feeding at the Department of Parasitology and Entomology, Faculty of Public Health, Mahidol University, Thailand. The malaria species was confirmed by microscopic examination of Giemsa's stain thin blood smears and DNA sequencing analysis of the cytochrome oxidase subunit I and cytochrome b genes (Pattaradilokrat et al., 2015). Experimental hosts were female domestic chickens. Chickens were allowed to feed on commercial food and water *ad labitum* and were reared in cages covered with nets to prevent entry of insects. The mosquito vectors were female adults *A. aegypti*, 5–6 d post emergence from pupae. The adults fed on 10% (w/v) sucrose in water and were starved for 6 h before use in mosquito feeding experiments. All laboratory animals were maintained in the animal facility in the Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Thailand. The room was lit on a 12 h light: 12 h dark cycle, with a temperature of 25–30 °C and relative humidity of 40–60%. Animal use protocol was reviewed and approved by the Chulalongkorn University Animal Care and Use Committee (Approval No. 1431067).

2.2. In vivo *drug test in chickens*

Infected blood, with a parasitemia level of 5–10%, was withdrawn from an infected chicken donor and diluted in an appropriate volume of phosphate buffered saline (PBS) to prepare an inoculum containing 10^6 blood stages of *P. gallinaceum* per 100 μl. The inoculum was injected intravenously into a jugular vein of recipient 3-d old chickens. The blood stage infection was monitored daily by microscopic examination of thin blood films stained with Giemsa's stain according to standard protocols. The numbers of parasitized erythrocytes, gametocytes and total number of erythrocytes were counted from at least five microscopic fields (at 1000× magnification) and the data were expressed as the % parasitemia and% gametocytemia, respectively.

To evaluate the efficacy of the TQ treatment, the infected chickens were divided into four groups with four chickens per group. One group (Control) was injected intramuscularly with 50 μl of PBS, while the other groups were injected intramuscularly with 50 μl of TQ (Sigma-Aldrich) in PBS at a dose of 3, 10 and 30 mg/kg body weight (BW), respectively. The treatments were given once a day