EFFECTS OF ANTIMALARIAL DRUGS ON MOVEMENT OF *PLASMODIUM FALCIPARUM*

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Abstract. *In vitro* antimalarial drug susceptibility is conventionally assessed by the concentration dependent growth inhibition of *Plasmodium* in an *in vitro* culture system. Inhibition of the kinetic properties of the parasites could provide an alternative method to assess *in vitro* antimalarial drugs sensitivity. In this study we used a novel real time microscopic technique, which does not require fixation and staining of the parasite, to study the effects of antimalarial drugs on the intracellular movement of *Plasmodium (P.) falciparum* trophozoites. Using real time microscopy movement of *P. falciparum* pigment within erythrocytes was investigated before and after antimalarial drugs exposure (artesunate, quinine, and piperaquine). For artesunate, the 50% inhibition concentration (IC_{50}) at which movement in half of the trophozoites was abolished was estimated by sigmoid curve fitting. Intra- and inter-observer agreements were also assessed. Healthy unexposed *P. falciparum* trophozoites in culture showed very active movement of malaria pigment. Quinine and piperaquine had no effect but artesunate did reduce pigment movement which started after 2.5 hours exposure to the drug. The mean (SD) IC_{50} for artesunate regarding abolishment of pigment movement was 54 (14) ng/ml. Assessments of intra- and inter-rater agreement showed good reproducibility of the technique (Kappa value 0.82 to 0.91). Abolishment of active movement of malaria pigment is an alternative approach to assess drug sensitivity for artesunate. Malaria pigment movement is abolished by artesunate early after exposure, but at concentrations higher than those inhibiting growth.

Keywords: *Plasmodium falciparum*, antimalarial drugs, pigment movement

INTRODUCTION

Malaria is one of the most important infectious diseases in the world. *Plasmodium falciparum* causes severe complications, including cerebral malaria. Each year 500 million cases of malaria occur worldwide, with an estimated annual mortality of up to 1 million (Greenwood *et al*, 2005). Antimalarial drug resistance is a major threat to global malaria control and to reduce the risks of emergence and spread of antimalarial drug resistance and

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to improve treatment outcome, the World Health Organization (WHO) now recommends combinations of antimalarials for the treatment of falciparum malaria, in particular artemisinin-based combination therapies (ACTs) (Ashley and White, 2005; Dondorp *et al,* 2005; WHO, 2006).

Assessment of antimalarial drug sensitivity is of utmost importance to monitor drug resistance. Most studies on *in vitro* drug susceptibility are based on growth inhibition of parasites after exposure to antimalarial drugs at different concentrations over a period of 48 hours. The effects on morphological and ultrastructural changes in the parasite after drug exposure using both light and electron microscopy have also been reported (Ono *et al,* 1991; Maeno *et al*, 1993; Orjih 1996). The effects of antimalarial drugs on kinetic processes within living parasites have not been addressed. In this study, we used Real Time Microscopy (RTM) to study the effects of antimalarial drugs on the intraparasitic movement of pigment in *P. falciparum* trophozoites. The RTM time-lapse imaging system allows assessment of dynamic motion within living parasites without fixation and staining of the parasite.

MATERIALS AND METHODS

Parasites

The chloroquine-resistant *P. falciparum* strain, TM267, was maintained in continuous culture as described previously (Trager and Jensen, 1976). Malaria parasites were cultured in 75 cm^2 sealed flasks containing 5% (v/v) blood group "O" Rh+ red blood cells (RBC) in complete malaria culture medium (MCM). MCM consisted of RPMI1640 culture medium (GIBCO, Carlsbad, CA) supplemented with 25 mM HEPES (Sigma, St Louis, MO), 2% $NaHCO_3$, 13% hypoxanthine (Sigma, St Louis, MO), 4.5% glucose, 0.04% gentamicin (Sigma, St Louis, MO), and 0.5% Albumax (GIBCO, Carlsbad, CA). Parasite cultures were maintained at 37°C with 90% N_2, 5% O_2 and 5% CO_2, and culture medium was changed daily. Parasites were synchronized to the ring stage using D-sorbitol as described previously (Lambros and Vanderberg, 1979).

Antimalarial drug exposure

Stock solutions of all antimalarial drugs were prepared in RPMI 1640 medium in a concentration of 1 mg/ml. Artesunate powder (Guilin Pharmaceutical Factory, Guilin, China) was dissolved in $NaHCO_3$ to a stock solution of 60 mg/ml and then diluted in RPMI 1640 medium shortly prior to use. Stock solutions of quinine dihydrochloride (300 mg of base/ml; Government Pharmaceutical Organization, Bangkok, Thailand) were prepared in RPMI 1640 medium in a concentration of 1 mg/ml. Stock solution of piperaquine phosphate (Guangzhou University of Traditional Chinese Medicine, Guangzhou, China) was prepared in 0.1 M phosphoric acid. The stock solutions were added to culture wells to give final concentrations of 550 ng/ml, 1.5 g/ml, and 15 ng/ml for artesunate, quinine, and piperaquine, respectively. *P. falciparum* strain TM267 with an initial parasitemia of 4 to 7% at a 2% hematocrit was exposed to the antimalarial drugs for a maximum time of 12 hours (h). A wet preparation of the culture was mounted on a glass slide for real-time microscopic assessment after 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 10, and 12 h incubation with artesunate; and after 2, 4, 6, 8, 10, and 12 h for quinine and piperaquine. In order to assess the 50% inhibition concentration (IC_{50}) of artesunate for inhibition of parasite movement, parasites were cultured in culture medium contain-

ing artesunate at varied concentration for 4 h, then the number of parasite movement was counted for 30 trophozoites.

Preparation of specimens for RTM

Since the nucleus of the parasite, which is a key determinant for stage identification, cannot be recognized in the unstained fresh preparations, parasites were stained with the DNA-binding fluorescent dye Hoechst 33342 in a final concentration of 5 g/ml in PBS for 5 minutes (min) at room temperature. The wet preparation consisted of 5 ml parasite culture in the middle of a microscopic glass slide and covered with a standard 22 mm x 22 mm glass cover slip allowing the fluid suspension to spread out evenly to an approximately 10 m thick fluid layer. The slides were assessed using a 100x objective lens requiring immersion oil.

Image acquisition and storage

Fresh samples were assessed by RTM at a stable temperature of 37°C. The RTM is equipped with a temperature control system. Assessment of each sample was within 15 min. Unstained parasites were observed using Richardson Contrast™ with a halogen lamp as the light source. The RTM microscope is equipped with a standard set of excitation filters transmitting light with a specific excitation wavelength which is directed through the preparation by a dichroic mirror. Hoechst 33342-stained parasites were observed using Richardson Contrast Fluorescent™ mode with EXFO X-Cite 120 unit as the light source. The images were captured as still images in a digital format (TIFF files) and time-lapse sequences were stored using Openlab software version 3.1.2 (Improvision, Coventry, UK). Images were also recorded as full color video with 30 frames per second (fps), and stored on digital tape using a DVCPRO50 recorder (Panasonic, Osaka, Japan). Digital movie data were then edited into a movie format using QuickTime, version 6.5.3 Apple computer Inc, USA and Final Cut Pro software version 5 (Apple Computer, Cupertino, CA).

Assessment of parasite movement

Movement was assessed in 30 trophozoites per sample, which were observed for 5 seconds (sec) under a 100x oil-immersion objective lens. Movement was scored as present if rapid random movement of pigment was observed. In case of slow or no motion, movement was scored as being absent. The proportion of moving trophozoites was plotted against the concentration of artesunate in the culture medium (1.5-400 ng/ml) in order to estimate the 50% inhibition concentration (IC_{50}) of artesunate regarding abolishment of movement. Data were analysed using WinNonlin software (Pharsight, Mountain View, CA).

Intra- and inter-rater agreement

The movement of 100 trophozoites from both control and artesunate-treated samples was rated by 3 observers. Using Richardson Contrast™ mode, trophozoites were recorded on digital tape and presence of movement was judged from the real time video. Each trophozoite was graded 3 times by 3 different observers. Intra-rater agreement was analyzed by calculating the chance to get perfect agreement among 3 observations in an individual observer. Inter-rater agreement for multiple observers was analyzed by the Cohen-Kappa statistic (Cohen, 1960) using STATA, version 9.0 (StataCorp LP, College Station, TX). Agreement was graded by the method of Landis and Koch (1977), in which 0.00 denotes poor agreement, 0.01-0.20 slight agreement, 0.21-0.4 fair agreement, 0.41-0.6 moderate agreement,

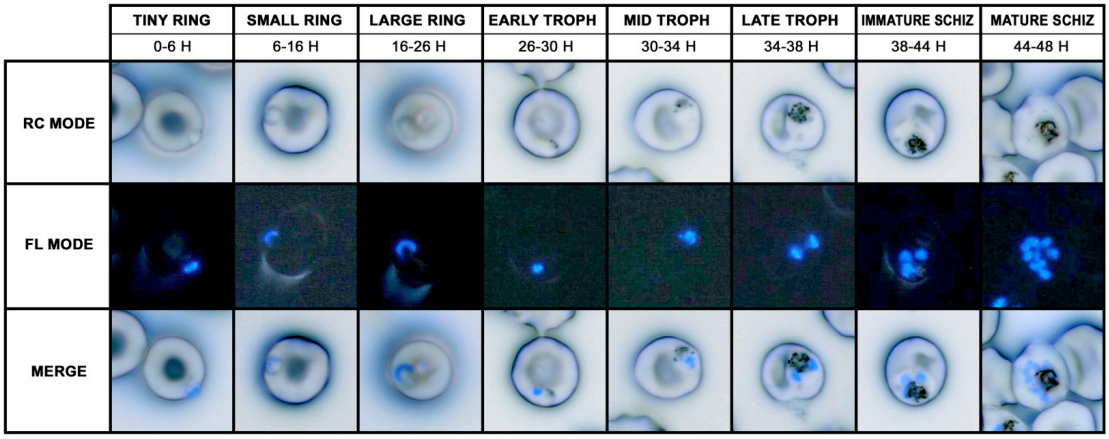

Fig 1 –Morphology of *Plasmodium falciparum*-infected red blood cells assessed by real time microscopy. Morphology of *P. falciparum* within the erythrocyte in an unstained preparation using Richardson Contrast (RC) mode. Staining with Hoechst stain 33342 gives the nucleus a blue color in Richardson Contrast Fluorescent (FL) mode (middle panel), but the nucleus can also be localized in RC mode (lower panel). Light microscopy of Field's stained preparations were used as the reference technique for assessment of parasite stages. TROPH, trophozoite; SCHIZ, schizont.

0.61-0.8 substantial agreement, and 0.81-1.00 = almost perfect agreement.

RESULTS

Inter- and intra-rater agreement

One hundred trophozoites from either untreated or artesunate-treated samples were assessed for movement of pigment within the food vacuole. Intra-rater agreement was perfect (100%) in untreated samples. In drug-treated samples median (range) intra-rater agreement was 90% (82% to 91%).

Inter-rater agreement for assessment of movement in untreated parasites was perfect with a multiple-rater Kappa score of 1.00. For observations in drug-exposed parasites, the Kappa score was 0.83 which translates to almost perfect agreement between the 3 raters.

Assessment of *P. falciparum* under RTM

Morphology without staining. The RTM image data can be presented in 4 display modes: Richardson Contrast™ (RC), Richardson Contrast Fluorescent™ (RCFL), Richardson Contrast Colour Translating™, and Richardson Contrast Fluorescent Colour Translating™. Using RC mode, red blood cells (RBC) could be observed as biconcave discs or discocytes and their plasma membrane could be clearly identified as a dark outline. The parasite plasma membrane varied from a light to dark outline within the parasitized red blood cell (PRBC). Without staining it was easy to distinguish non-pigmented and pigmented parasites (Fig 1). Non-pigmented ring stage parasites appeared as a doughnut or circular disc shape. The parasite could be recognized as a light grey area, but other organelles within the parasites could not be

identified and no malarial pigment was observed in ring stage parasites. Sub-staging of rings into tiny, small, and large ring was possible based on the size of the ring.

In pigmented parasites, including trophozoite and schizont stages, the edge of the parasite could only be poorly identified. The nucleus, cytoplasm, and other organelles within parasites were not recognizable. Pigment within the food vacuole was clearly visible and the only feature available for parasite stage differentiation. Pigment in early trophozoites appeared as fine grains with a black or dark brown color scattered throughout the digestive vacuole of the trophozoite. As parasites matured into the mid- and late trophozoite stage, pigment granules increased in size and became organized in clumps or rods (Silamut *et al*, 1999).

In the early schizont stage, pigment grouped in a similar way as late trophozoites, but with further maturation the clusters became smaller in number but larger in size until a single mass with a golden color appeared in late stage schizonts. Some schizonts showed just before schizont rupture the appearance of numerous grey marks in the cytoplasm presumably corresponding to merozoites.

Staining with Hoechst 33342. In RC and RCFL display mode using excitation with UV light with a wavelength of 366 nm, the Hoechst 33342-stained nuclei showed an intense blue fluorescence (Fig 1), which could be indentified throughout the parasite asexual lifecycle. Using blue light (437 nm) nuclei of parasites showed a pale green fluorescence. No fluorescence was detected with a green (547 nm) excitation filter. The cytoplasm and other organelles were not stained by the Hoechst dye and could not be detected by fluorescence.

Ring stage parasites had a single nucleus showing as a dot or a curve. The usual singular nucleus of early trophozoites had a circular or arch shape with fine grains of pigment. The nucleus of mid trophozoites was either circular or irregular in shape and the irregularity increased with further maturation. The nucleus divided into 3 or more nuclei with maturation into the schizont stage. Immature schizonts were defined as containing 3 to 5 nuclei, whereas parasites with more than 5 nuclei were classified as mature schizont. Unlike the conventional thin blood film preparation there was a 3-dimensional appearance of parasitized red blood cells (PRBC) in the wet preparations assessed by RTM. There was no change in the relative position of the parasite within the RBC during parasite development.

Movement of parasites. Movement of parasites was determined by movement of pigment within the food vacuole of the parasite thus only observed in pigment containing trophozoites and schizonts. Movement could not be detected in ring stage parasites. Pigment within the food vacuole of healthy trophozoites moved rapidly impressing as Brownian motion with a random direction. Movement of fine grain pigments was more rapid than of larger clumps of pigment. With increasing clumping of pigment into the schizont stage, movement became slower and more variable; the single clump of pigment observed in the mature schizont was usually static.

Effects of antimalarial drugs on trophozoite movement

A synchronous culture of trophozoites (> 90% synchronicity of early and mid trophozoites, estimated age 30 ± 2 h) was exposed to 3 different antimalarial drugs for up to 12 h. Antimalarial drugs included artesunate, quinine, and piperaquine in a

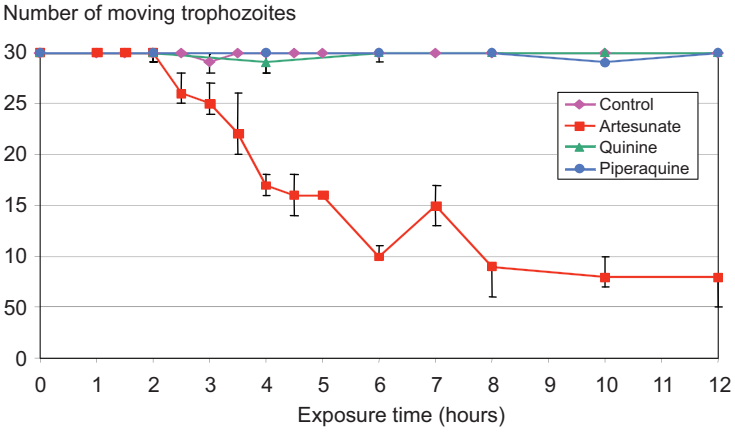

Fig 2–Number of moving trophozoites after antimalarial drugs exposure. The final drug concentrations were 1.5 g/ml 550 ng/ml, and 15 ng/ml for quinine, artesunate, and piperaquine, respectively. Every time point a total of 30 trophozoites were assessed. The results are shown as median and range (n=3).

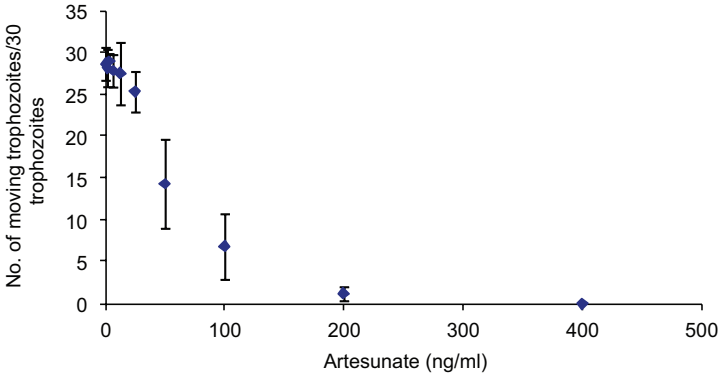

Fig 3–Number of moving trophozoites according to artesunate concentration in the culture medium (n=5). Incubation was for 4 hours. Every assessment a total of 30 trophozoites were scored. Bars represent mean-SD.

final concentration of 550 ng/ml, 1.5 g/ml, and 15 ng/ml, respectively. All trophozoites in the control group without drug exposure showed active movement of pigment at every time point. Artesunate decreased the number of moving trophozoites in a time dependent manner (Fig 2). Reduction in movement started early, after 2.5 h, and there was a 50% reduction in the number of trophozoites showing active movement after 5 h exposure to artesunate and this increased to 70% after 8 h exposure. Quinine and piperaquine did not affect pigment movement in trophozoites at any time point. In parasites without drug exposure, movement was stable over time (Fig 2).

Concentrations of artesunate in the culture medium were varied in order to assess the 50% inhibition concentration (IC_{50}) for inhibition of parasite movement. The mean (SD) IC_{50} of artesunate to stop trophozoites movement was 54 ng/ml (14 ng/ml, range 39 to 78 ng/ml, Fig 3).

DISCUSSION

The RTM live cell imaging system allows long term observation of unstained living parasites in real time without disturbing their activity. The procedure causes minimum impact on the living parasite using several techniques, including a temperature control system and removal of infrared and ultraviolet energy from the illuminating light. The extreme-dark-field condenser and a high-quality camera ensure high contrast

with a full color image output in a picture or video format.

Without staining, parasites could be easily differentiated into ring stage versus trophozoite and schizont stages based on the appearance of malarial pigments in the later stages. Pigmented parasites could be roughly sub-categorized based on the number, color and clumping pattern of the pigment, with an increasing amount of pigment and increased clumping with further maturation. Compared to staging based on Field's-stained thin blood films using light microscopy it is difficult to differentiate between late trophozoite and immature schizonts since their pigment have similar characteristics. In the latest stages of parasite asexual development, pigment clumped further together to a single mass, which can be interpreted as a hallmark of the mature schizont. Further staging was possible after staining of the parasite nucleus with Hoechst 33342 fluorescent vital stain. This stain binds preferentially to AT rich regions of DNA (Shapiro, 2003) , which makes it ideal for the study of AT rich *P. falciparum* in fluorescence microscopy and flow cytophotometry (Howard *et al*, 1979; Jacobberger *et al*,1992; Jouin *et al*, 1995; Salmon *et al*, 2001; Haynes *et al*, 2002).

The movement of the malarial pigments within the digestive vacuole was observed in all living pigmented parasites. Pigment or hemozoin formation is the major process for heme detoxification in the malaria parasite. Movement of ring stage parasites could not be detected because of the absence of pigment in these young parasites. Intra- and inter-rater agreement regarding the detection of parasite movement was high, mounting to 100% for assessing unexposed parasites. Limiting the observation time to 5 seconds excluded that small movements were classified as positive movement. The movement of pigment was random and Brownian in character, which would imply that this is not an active energy dependent process. Further studies are under way to further characterize the nature of these kinetic properties.

This study is the first report on the effects of antimalarial drugs on the kinetic properties of living parasites. The drug concentrations were chosen based on free drug concentrations calculated from *in vivo* studies (White *et al*, 1983; Silamut *et al*, 1985; Hien *et al*, 2004; Hung *et al*, 2004; Li *et al*, 2006; Lindegardh N, personal communication). We found that of the tested antimalarial drugs, only artesunate interrupted the movement of trophozoite pigment. The mean (SD) IC_{50} artesunate concentration causing inhibition of movement in 50% of the trophozoites was 54 (14) ng/ml, which is higher than the IC_{50} concentration for the inhibition of parasite (schizont) maturation (0.8-1.3 ng/ml). Inhibition was time dependent, starting early after only 2.5 h drug exposure, mounting to 80% inhibition after 8 h. Changes in the efficacy of artesunate to reduce parasite movement over time could be a useful additional *in vitro* test for the assessment of drug sensitivity in artemisinin resistant parasites, but this needs further study. Artemisisin resistance has recently been identified in western Cambodia, and conventional *in vitro* sensitivity tests have shown to be relatively insensitive to identify this new phenotype (Dondorp *et al*, 2009).

In conclusion, this study describes the stage dependent morphological changes and kinetic properties of unstained and unfixed *P. falciparum in vitro* using RTM, a novel microscopic technique. Movement of pigment is prominent in trophozoite stage parasites, which becomes slower

with increasing clumping of pigment as the parasite matures. Abolishment of pigment movement is an early effect of artesunate, but not of quinine or piperaquine.

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