DISCOVERY BIOLOGY

Griffith UNIVERSITY

Continuous culture conditions: impact on *Plasmodium falciparum* intra-erythrocytic cell cycle duration and parasite age sensitivity to Artemisinin

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Hypothesis: Variations in culture media used for continuous in vitro culture of *Plasmodium falciparum* 3D7 (*Pf*3D7) alter the age specific sensitivity of the parasite to artemisinin but not to all anti-malarial compounds.

*Pf*3D7-----→ Environmental influences ------→Transcriptional variations -----→ altered phenotype?

Background: Culture of the entire intra-erythrocytic life cycle of *Plasmodium falciparum* is possible *in vitro*. With the identification of "ring stage" artemisinin tolerance reported [1] the requirement of age defined and highly synchronous parasites is recognized as essential for future research. Presented is a method for the isolation of age defined ring stage parasites and the effect of *in vitro* culture media on the artemisinin sensitivity profile of *Pf*3D7 in relation to parasite age post red blood cell invasion.

Methods:

Parasite culture conditions.

*Pf*3D7 was resuscitated from liquid nitrogen storage and cultured in RPMI 1640 supplemented with 25mM HEPES and 50µg/ml Hypoxanthine plus either 5mg/ml Albumax II (referred to as albumax only = AO) or 2.5mg/ml AlbumaxII plus 5% Human AB serum (referred to as serum/albumax II media = S/A). Both cultures were maintained in continuous culture utilizing a single sorbitol (noon) treatment every second intra-erythrocytic cycle to maintain a roughly synchronous culture. The cultures were adjusted with non infected red blood cells (RBCs)to maintain parasitaemia at between 1% trophozoites and 3-5%rings. Incubation was performed at 37°C, 5% CO_2, 5% O_2, 90% N_2 and 60% humidity. Culture protocol and parasite isolation is described in **Figure 1** and parasite age sensitivity determination **Figure 3.**

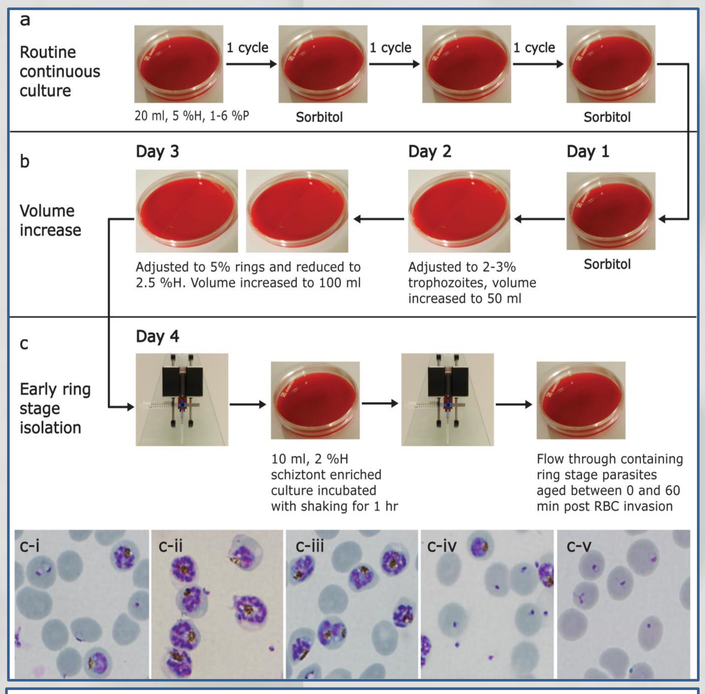


Figure 1. Age defined parasite isolation protocol.

Routine continuous culture **(a),** parasite synchronization to approximately 0-10 hours synchronicity and increasing culture volume **(b).** Magnetic isolation of schizonts and addition of non infected RBCs **(c).** Incubate for 1 hour then removed non ruptured schizonts, collecting newly infected RBCs in the column flow through, containing parasites aged between 0-1hours post RBC invasion. **(ci)** Culture prior to schizont isolation. **(cii)** Culture post schizont isolation before erythrocyte addition. **(ciii)** Schizont enriched culture after erythrocyte addition. **(civ)** Culture after 1 hr incubation prior to isolation showing merozoite release. **(cv)** Isolated 0 to 60 min post erythrocyte invasion rings. Average %P for standard process for *Pf*3D7 = 10.9 (± 2.8 n=4). Shortest time frame 15minutes and maximum %P 35-40% for 3hour incubation.

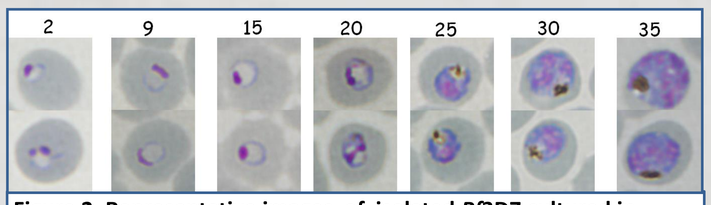


Figure 2. Representative images of isolated *Pf*3D7 cultured in serum/albumax II media over time from 2 to 35 hours

Cell cycle duration

Isolations were performed for *Pf*3D7 AO and *Pf*3D7 S/A within the same working day, obtaining 0-60 min ring stage parasites post erythrocyte invasion. Cell cycle duration was expressed as hours post RBC invasion where 50% of schizonts at a defined %P had ruptured. The AO culture demonstrated a cell cycle duration of 45hours for AO and 40hours for S/A cultures. i.e. growth in AO media extended the cell cycle duration by 5 hours a fact which has been reported previously by Frankland *et al.*[2]

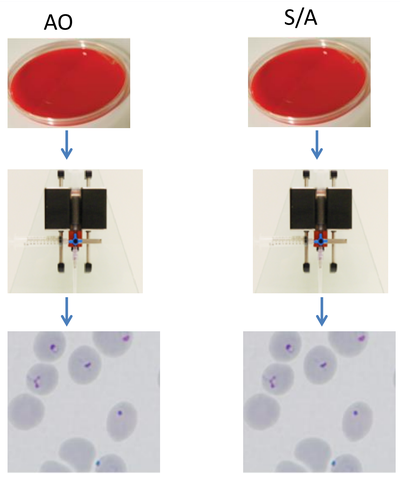
Two vials of *Pf*3D7 (S/A) were raised from Nitrogen storage and cultured as described in **Figure 1** in either AO or S/A for four months.

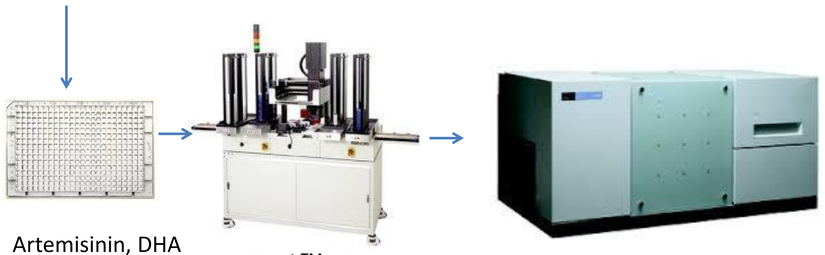
↓

Both cultures were simultaneously processed for 0-1hour age defined ring stage parasites as described in **Figure 1**.

↓

Parasites were adjusted to 3%P and 0.3% haematocrit in the respective medium and 45µl dispensed into **15** x 384 well imaging plates and incubated at 37°C, 5% CO_2, 5% O_2, 90% N_2 and 60% humidity .





Artemisinin, DHA and puromycin Compound IC_{50} plate

MiniTrack™ 384 to 384 well compound transfer to parasite containing Imaging plates at three hour intervals throughout parasite development.

After 65 hours post RBC invasion all the plates were stained with DAPI (4',6-diamidino-2-phenylindole) and imaged on a OPERA high content imaging system. Using Acapella image analysis software the number of classified "spots" (parasites) per image are quantified.[3]

Figure 3. Parasite age sensitivity determination process

Data analysis

IC_{50} values were determined for each compound at all time points and culture media conditions. The fold change in IC_{50} value in relation to that obtained for the first time point ie 2hours post RBC invasion was calculated for each compound and media composition. The fold change in IC_{50} was plotted against parasite age in hours.

Figure 4 and 5.

Results

Both *Pf*3D7 cultures (AO and S/A) demonstrated distinctly different age sensitivity profiles to Artemisinin and DHA, but were comparable for Puromycin as demonstrated in **Figure 4**.

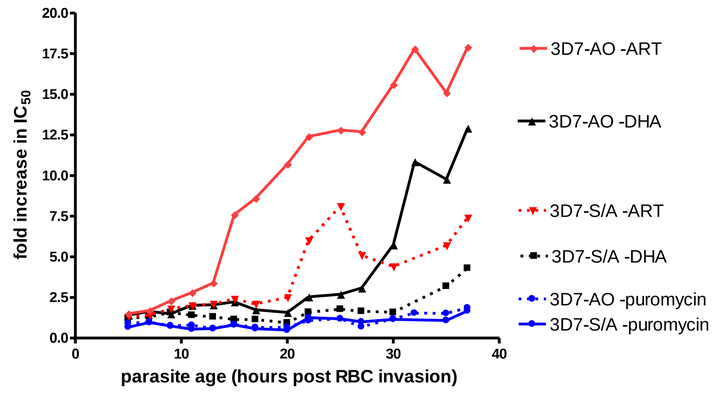


Figure 4. Comparison of the fold increase in IC_{50} for Artemisinin (ART), DHA and puromycin against AO *Pf*3D7 and S/A *Pf*3D7 at increasing age post RBC invasion.

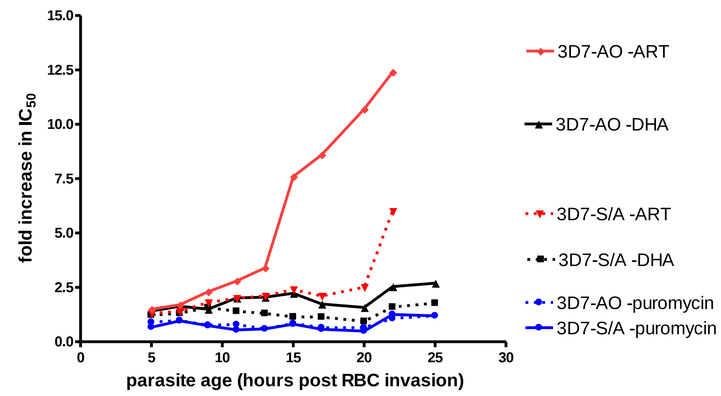


Figure 5. Age sensitivity profile for *Pf*3F7 AO and S/A cultures and the first 25 hours of parasite development.

The AO cultured parasite is demonstrating a greater tolerance to artemisinin occurring after 13hours post RBC invasion in comparison to greater than 20 hours for the S/A *Pf*3D7 culture. At 20hours post RBC invasion the AO culture demonstrates an almost 8 fold greater tolerance to artemsinin than that cultured in S/A.

Conclusion

Simple alterations to *Pf*3D7 culturing conditions, specifically the use of AO or S/A media can result in alteration of the phenotypic profile of the cultured parasite. In this case altering the cell cycle duration and tolerance of the parasite to artemisinin and its metabolite DHA, but not to a general non specific compound (puromycin) , which is equally active against all intra-erythrocytic stages of plasmodium, irrespective of the culture media employed. It is therefore possible that certain environmental conditions can alter the parasites response to particular compounds depending on their mechanism of action.

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References

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3) Sandra Duffy* and Vicky M. Avery. Development and Optimization of a Novel 384-Well Anti-Malarial Imaging Assay Validated for High-Throughput Screening. Am J Trop Med Hyg 2012 vol. 86 no. 1 84-92