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A mechanistic model quantifies artemisinin-induced parasite growth retardation in blood-stage *Plasmodium falciparum* infection

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ABSTRACT

Falciparum malaria is a major parasitic disease causing widespread morbidity and mortality globally. Artemisinin derivatives—the most effective and widely-used antimalarials that have helped reduce the burden of malaria by 60% in some areas over the past decade—have recently been found to induce growth retardation of blood-stage *Plasmodium falciparum* when applied at clinically relevant concentrations. To date, no model has been designed to quantify the growth retardation effect and to predict the influence of this property on *in vivo* parasite killing. Here we introduce a mechanistic model of parasite growth from the ring to trophozoite stage of the parasite's life cycle, and by modelling the level of staining with an RNA-binding dye, we demonstrate that the model is able to reproduce fluorescence distribution data from *in vitro* experiments using the laboratory 3D7 strain. We quantify the dependence of growth retardation on drug concentration and identify the concentration threshold above which growth retardation is evident. We estimate that the parasite life cycle is prolonged by up to 10 hours. We illustrate that even such a relatively short delay in growth may significantly influence *in vivo* parasite dynamics, demonstrating the importance of considering growth retardation in the design of optimal artemisinin-based dosing regimens.

1. Introduction

Plasmodium falciparum malaria is a major parasitic disease which causes severe morbidity and mortality in approximately half a million people annually (World Health Organization, 2015). Artemisinin (ART) and its derivatives (e.g. artesunate, dihydroartemisinin and artemether), used in combination with partner drugs, provide front-line protection, and have been responsible for dramatic reductions in disease burden over the past few decades (World Health Organization, 2015). Despite their clinical and public health effectiveness, the emergence of ART resistance and lack of alternative treatments places current control programs at risk (Ariey et al., 2014; Ashley et al., 2014; Dondorp et al., 2009; Phyo et al., 2012). Development of a comprehensive understanding of ART's mechanism of action and associated effects on infected red blood cells (iRBCs) is therefore critical for development of optimised ART-based treatment regimens and maintenance of control program impact (Simpson et al., 2014).

Recent *in vitro* experiments (Dogovski et al., 2015; Klonis et al., 2013; Yang et al., 2016), combined with advances in pharmacokinetic–pharmacodynamic (PK–PD) modelling (Cao et al., 2016), have established a platform to probe the parasite's temporal response to antimalarial drugs. The key experimental advance underlying these *in vitro* studies was the application of short drug pulses, which enabled fine-scale measurement of the killing effect of drug (Cao et al., 2016). A normal life cycle of an iRBC for *P. falciparum* is approximately 48 h and is classified based on morphological appearance into three main stages: the ring stage (approximately 0–26 h post infection (h p.i.)), trophozoite stage (approximately 27–38 h p.i.) and schizont stage (approximately 39–48 h p.i.). Upon rupture at approximately 48 h p.i., iRBCs release merozoites, 8–12 of which successfully invade susceptible RBCs to initiate a new round of infection (Dietz et al., 2006; Simpson

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et al., 2002; Zaloumis et al., 2012). Dogovski et al. demonstrated that a short pulse of ART (or dihydroartemisinin (DHA)) can induce growth retardation, prolonging the 48 h life cycle (Dogovski et al., 2015). Importantly, they found that growth retardation did not stop parasite growth entirely and was thus considered to be distinct from parasite dormancy which "freezes" parasites for days to weeks (Codd et al., 2011; Teuscher et al., 2010).

Experimental identification of drug-induced growth retardation raises two questions: 1) By how much is the life cycle of a parasite prolonged in response to a short drug exposure pulse?; and 2) How influential is growth retardation when considering *in vivo* parasite killing using PK–PD models? These two questions are important because we expect any drug-mediated variation in the duration of one or more life stages to impact on the efficacy of the drug (given the well-established finding that drug can exert stage-specific killing effect to parasites (Cao et al., 2016; Klonis et al., 2013; Saralamba et al., 2011; Witkowski et al., 2013). The alteration in drug efficacy may be significant if the prolonged stage(s) covered by the drug pulse exhibit very distinct killing effects. Quantification of growth retardation and assessment of its potential effect on *in vivo* parasite killing is therefore important in guiding further experimental investigations into drug activity and strategies for optimising ART-based combination therapies.

To the best of our knowledge, no model has yet been designed to quantify the growth retardation effect and predict its influence on *in vivo* parasite killing. Here we construct a mechanistic model of parasite growth to explain drug-induced growth retardation. We model the relationship between parasite age and the fluorescence intensity of a parasite's RNA/DNA-binding dye. Through application to fluorescence data from *in vitro* experiments using the laboratory 3D7 strain by Dogovski et al. (2015), we provide the first quantification of growth retardation. We identify a concentration threshold above which the growth retardation is evident. Furthermore, the model accommodates two possible mechanisms of drug-induced growth retardation, which can be reliably identified in future experimental studies. Finally, by incorporating growth retardation into a PK–PD modelling framework, we simulate *in vivo* parasite killing upon exposure to a single dose of artesunate and demonstrate the importance of considering growth retardation in the design of optimal artemisinin-based dosing regimens.

2. Materials and methods

2.1. Experiment and data

We first summarise the *in vitro* experiment in which parasite growth retardation was identified. We provide sufficient information for the purposes of model development and evaluation. For full details on the experimental implementation we refer the reader to the original publication (Dogovski et al., 2015).

Fig. 1 presents the experimental process. A culture containing tightly synchronised rings (3D7 strain; over 80% of the population within a one-hour age window) with an average age of 6 h p.i. was equally divided into a number of small cultures, each of which was treated with a different concentration of drug (ART or DHA) for 4 h. Two cultures unexposed to drug acted as the control. The cultures were stained with SYTO-61 and examined by flow cytometry (Fu et al., 2010). SYTO-61 is a nucleic acid stain which stains both DNA and RNA and allows the distinction of infected RBC from uninfected RBC, as well as distinguishing between parasite-infected RBC of different ages due to an increase in nucleic acid content as the parasite ages. SYTO-61 signals from all cultures were collected simultaneously at approximately 72 h post drug administration (indicated in Fig. 1), corresponding to the period in which parasites transition from the ring to trophozoite stage (during the second cycle). As trophozoites express significantly more nucleic acid, this period of transition exhibited a bimodal SYTO-61 fluorescence distribution. Quantitative analysis of this bimodal distribution is the key to our approach as it enables us to identify the relative populations of parasites in the two successive life stages (Dogovski et al., 2015). As indicated in Fig. 1, if the administration of drug slows parasite growth then the first life cycle would be prolonged and the second life cycle would start later. In consequence, we would observe an increase in the ring population (i.e. the mode with the lower fluorescence) and a corresponding decrease in the trophozoite population (i.e. the mode with the higher fluorescence) in the SYTO-61 fluorescence histogram. Note that the underlying processes of iRBC rupture and merozoite release and re-infection were not observable in the experiment.

The SYTO-61 fluorescence histograms often contain a small population of dead or dormant parasites (due to drug activity or otherwise) that are not involved in the second life cycle. To account for this non-viable population, a *ground experiment* in which supermaximal drug concentration ($>10\times$ the 50% Lethal dose (3 days)) was applied for over 48 h was also performed (Dogovski et al., 2015). The high drug concentration and long exposure time guaranteed that all parasites became non-viable. Hence, denoting the SYTO-61 fluorescence frequency histogram under the *background* condition by f_b and the SYTO-61 fluorescence frequency histogram under a 4 h drug pulse by f, the corrected SYTO-61 fluorescence frequency histogram, f_c, is given by

$$f_c = f - (1 - V) f_b, \tag{1}$$

where V represents the viability (the fraction of parasites entering the second life cycle; see Dogovski et al., 2015; Klonis et al., 2013 for details).

The corrected histogram data is shown in Fig. 2 (for various ART concentrations) and Fig. S1 in the *Supplementary material* (for various DHA concentrations). Experiments were performed in technical replicates for each drug concentration. The histograms in Fig. 2 present all available SYTO-61 fluorescence intensity data. Also note that for each histogram, the samples with fluorescence intensity less than 3000 (indicated by the vertical dashed lines) were considered to include fluorescence signals from uninfected RBCs and were thus excluded in the analysis. Each histogram was generated by distributing log-transformed SYTO-61 fluorescence intensity samples (with magnitude >3000) into 40 equally spaced bins. Raw SYTO-61 fluorescence intensity data is provided in Dataset S1.

2.2. The model

Since the *in vitro* experiment measures the SYTO-61 fluorescence data in the second life cycle, our model is designed to reproduce the underlying process of parasite growth over the period of the ring-to-trophozoite transition in the second life cycle (see Fig. 1).

Over the period of the ring-to-trophozoite transition, the growth of individual parasites is modelled by two sequential stages — an "immature" ring stage during which the ring-to-trophozoite transition cannot occur (due to incomplete cellular development) followed by a "mature" ring stage where the ring-to-trophozoite transition is possible — inspired by the classic model for the mammalian cell cycle (Brooks et al., 1980; Smith and Martin, 1973). We introduce A_r, the *ready-for-change* age (which is assumed to be the same for all parasites). Parasites of age $a < A_r$ are, by definition, rings. For parasites of age $a > A_r$, the waiting time before entering the trophozoite stage follows a Poisson distribution with transition rate λ (denoted as $Pois(\lambda)$).

We assume that the age distribution of viable parasites (i.e. the parasites able to asexually reproduce in the second life cycle) at the time of data collection is Gaussian ($\sim N(\mu, \sigma^2)$). This is reasonable given that the parasites are tightly synchronised and drug