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Using a genome-scale metabolic network model to elucidate the mechanism of chloroquine action in *Plasmodium falciparum*

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ABSTRACT

Chloroquine, long the default first-line treatment against malaria, is now abandoned in large parts of the world because of widespread drug-resistance in *Plasmodium falciparum*. In spite of its importance as a cost-effective and efficient drug, a coherent understanding of the cellular mechanisms affected by chloroquine and how they influence the fitness and survival of the parasite remains elusive. Here, we used a systems biology approach to integrate genome-scale transcriptomics to map out the effects of chloroquine, identify targeted metabolic pathways, and translate these findings into mechanistic insights. Specifically, we first developed a method that integrates transcriptomic and metabolomic data, which we independently validated against a recently published set of such data for Krebs-cycle mutants of *P. falciparum*. We then used the method to calculate the effect of chloroquine treatment on the metabolic flux profiles of *P. falciparum* during the intraerythrocytic developmental cycle. The model predicted dose-dependent inhibition of DNA replication, in agreement with earlier experimental results for both drug-sensitive and drug-resistant *P. falciparum* strains. Our simulations also corroborated experimental findings that suggest differences in chloroquine sensitivity between ring- and schizont-stage *P. falciparum*. Our analysis also suggests that metabolic fluxes that govern reduced thioredoxin and phosphoenolpyruvate synthesis are significantly decreased and are pivotal to chloroquine-based inhibition of *P. falciparum* DNA replication. The consequences of impaired phosphoenolpyruvate synthesis and redox metabolism are reduced carbon fixation and increased oxidative stress, respectively, both of which eventually facilitate killing of the parasite. Our analysis suggests that a combination of chloroquine (or an analogue) and another drug, which inhibits carbon fixation and/or increases oxidative stress, should increase the clearance of *P. falciparum* from the host system.

1. Introduction

Malaria, which is caused by a protozoan parasite of the *Plasmodium* genus, represents a serious global health concern given that nearly half of the world population is at risk of infection (WHO, 2014). Although several anti-malarial drugs for treating the symptomatic stage (or blood stage) of malarial infection are commercially available (Antony and Parija, 2016), their efficacy has declined appreciably in the last few decades owing to widespread drug resistance developed by the parasite (Breman et al., 2004).

Plasmodium falciparum—the most lethal species of malaria—causes approximately 50% of all malarial infections. Chloroquine was the first-line malaria treatment for many decades until drug-resistant *P. falciparum* strains became common. The drug causes a dose-dependent decrease in hemozoin formation (Chou and Fitch, 1992; Slater and Cerami, 1992) and an associated increase in toxic free heme in the food vacuole of the parasite (Combrinck et al., 2013; Loria et al., 1999). Over the past few decades, researchers have proposed many different mechanisms for chloroquine action, including *1*) DNA intercalation (Meshnick, 1990), *2*) alteration of digestive food vacuole pH (Yayon et al., 1985), *3*) inhibition of heme polymerase (Yayon et al., 1985), and *4*) formation of a toxic chloroquine-ferriprotoporphyrin IX complex (Sugioka et al., 1987). Yet, there is no consensus on the exact mechanisms by which chloroquine kills the parasite and under

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what circumstances they operate. Shedding light on the possible actions of chloroquine is critical for developing more potent drugs or combination drug treatments against the parasite and for understanding how the parasite can develop resistance against them.

Here we used systems biology model and transcriptional profiles of *P. falciparum* to obtain information about metabolic and biological precursors that determine the physiological or pathophysiological state of the parasite. Specifically, we used the *P. falciparum* transcriptomic data obtained by Hu and colleagues during the intraerythrocytic development cycle (IDC) at different concentrations of chloroquine (Hu et al., 2010). Our primary goal was to use these data to investigate the effect of chloroquine on *P. falciparum* DNA replication. We chose to study this effect because it has been proposed as a mechanism of chloroquine action in different *Plasmodium* species (Gutteridge et al., 1972; Meshnick, 1990; Polet and Barr, 1968a, b), in bacteria (Ciak and Hahn, 1966), and in bioassays examining the effect of chloroquine on RNA and DNA polymerases (Cohen and Yielding, 1965; O'Brien et al., 1966b; Whichard et al., 1972).

We first developed a method that integrates the information embedded in the transcriptome data with the whole-genome metabolic network model for *P. falciparum* (Fang et al., 2014) to predict the metabolic phenotype corresponding to those transcription data. We validated the developed method by using a data set from an independent study that jointly capture transcriptomic and metabolomic data from *P. falciparum*, to correctly predict the metabolic phenotype tied to genetic perturbation of two Krebs cycle enzymes (Ke et al., 2015). We then focused on the inhibition of DNA replication in *P. falciparum* (as a consequence of chloroquine treatment) a critical metabolic phenotype (Ciak and Hahn, 1966; Cohen and Yielding, 1965; Polet and Barr, 1968a), and used our method to simulate the effect of chloroquine on the metabolic fluxes of *P. falciparum* during the IDC. We identified genes that were substantially altered in response to chloroquine treatment and linked to the inhibition of *P. falciparum* DNA replication. The cohort of identified genes suggests that DNA replication is inhibited by the downstream effect of heme accumulation. Specifically, our analysis suggests that continuous accumulation of heme inhibits redox metabolism, carbon fixation, and pyrimidine metabolism, which leads to inhibition of DNA replication and facilitates death of the parasite. Our results provide a mechanistic explanation for why parasites with an efficient redox metabolism may have a lower sensitivity to chloroquine (Kasozi et al., 2013; Lehane et al., 2012; Meierjohann et al., 2002).

2. Materials and methods

2.1. Transcriptome data used for simulating metabolic flux profile during IDC

Metabolic flux profiles of *P. falciparum* strains 3D7 (Pf3D7) and Dd2 (PfDd2) during the IDC were estimated by using transcriptome data previously reported by Llinas and colleagues (Llinas et al., 2006), who extracted cDNA from parasites cultured in erythrocytes and hybridized them to DNA microarrays to yield hourly levels of gene expression for the Pf3D7 (or PfDd2) strain during the IDC. The temporal resolution of these hourly gene expression profiles allowed us to make high-resolution time-dependent metabolic flux predictions during the IDC.

We used previously reported gene expression data (Hu et al., 2010) to simulate the effect of chloroquine on the time-dependent metabolic fluxes of Pf3D7 and PfDd2 during the IDC. Briefly, Hu and colleagues (Hu et al., 2010) administered chloroquine 18 h after erythrocyte invasion at concentrations of 41, 72, and 144 nM for Pf3D7 and at 43 nM for PfDd2, and obtained gene expression profiles every hour for 8 h during the IDC following the chloroquine treatment.

2.2. Metabolic network model and data processing

The metabolic network model used involved 1045 reactions and 376 genes. The model was identical to a previously published model (Fang et al., 2014), with one minor modification: we added reactions explicitly carried out by Pdx2 (PF11_0169) and Pdx1 (MAL6P1.215). This was done by *1*) modifying an old reaction that incorrectly assumed that pyridoxal 5-phosphate (PLP) was synthesized from ribulose 5-phosphate and *2*) adding a new (and separate) reaction for Pdx1 because experiments suggest that it can synthesize PLP independent of the ammonia generated by Pdx2 (Hanes et al., 2008). In the updated model, Pdx2 hydrolyzes L-glutamine to yield glutamate and ammonia while Pdx1 incorporates the ammonia generated by Pdx2 to yield PLP from the substrates ribose 5-phosphate and glyceraldehyde 3-phosphate (Gengenbacher et al., 2006), with the isomerization of ribose 5-phosphate to ribulose 5-phosphate as a side reaction.

We used gene-to-reaction mappings available in the metabolic network model to obtain reaction expressions r_{exp}, which depend on the expression of a gene (or genes) catalyzing a particular metabolic reaction. For a reaction catalyzed by a single enzyme, r_{exp} was equal to the expression of the gene catalyzing that reaction. However, if a reaction was associated with more than one gene, then their gene-to-reaction mappings were defined by using the Boolean operators “AND” and “OR.” We implemented these operations by taking the minimal (for the AND operator) and maximal (for the OR operator) values of the associated gene expression data (Song et al., 2014).

2.3. Estimating metabolic flux changes in response to external stress

We recently developed a method (Fang et al., 2014) that integrates hourly gene expression data with a whole-genome metabolic network model to estimate the hourly metabolic flux profiles of *P. falciparum* during the 48-h long IDC period. To take advantage of additional studies that collect gene expression data as a function of genetic or chemical perturbations, we modified the method because such studies usually do not provide the same granularity and density of gene expression data to fully cover the entire IDC. Therefore, we aimed to develop a method that could utilize a reduced transcriptomic data set to predict alterations in metabolic flux profiles during the IDC. Briefly, We used the following steps to estimate metabolic fluxes under no-stress conditions:

Step 1: Estimate nutrient fluxes sufficient for the nominal growth rate (40% of the maximum growth rate).
Step 2: Estimate reaction fluxes v_i^N for metabolite i corresponding to the nominal growth rate constrained by the nutrient fluxes obtained in *Step 1.*
Step 3: Estimate reaction fluxes v_i^t for reaction i at any time point t during the IDC by incorporating time-dependent transcriptomic profiles under stoichiometric and nutrient uptake constraints.

The last step was achieved by globally minimizing for all reactions and time points the value of t that minimized the weighted absolute difference, $\sum_{i \in G} \left| v_i^t - \tilde{r}_i^t v_i^N \right|$, where G represents the set of unidirectional reactions with known gene-to-reaction mapping. The factor $\tilde{r}_i^t = \frac{r_{exp,i}^t - \min(r_{exp,i}^t)}{\text{mean}(r_{exp,i}^t) - \min(r_{exp,i}^t)}$; where, $r_{exp,i}^t$ represents reaction expression of ith reaction at time t obtained from the gene-to-