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Stage independent chloroquine resistance and chloroquine toxicity revealed via spinning disk confocal microscopy☆,☆☆

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Abstract

We previously customized a Nipkow spinning disk confocal microscope (SDCM) to acquire 4D data for live, intraerythrocytic malarial parasites [Gligorijevic B, McAllister R, Urbach JS, Roepe, PD. Spinning disk confocal microscopy of live, intraerythrocytic malarial parasites. 1. Quantification of hemozoin development for drug sensitive versus resistant malaria. Biochemistry 2006;45:12400–10]. We reported that chloroquine (CQ) treatment did not appear to affect progress through the cell cycle, and suggested that toxicity may be manifested post-schizogony. We now use SDCM, synchronized cell culture and continuous *vs.* bolus drug dosing to investigate stage specific CQ effects in detail. We develop a novel, extremely rapid method for counting schizont nuclei in 3D. We then quantify schizont nuclei and hemozoin (Hz) production for live parasite cultures pulsed with CQ at different stages in the cell cycle and find that bolus treatment of rings affects the multiplicity of nuclear division. We quantify parasitemia and merozoite development in subsequent cycles following bolus CQ exposure and find that a portion of CQ toxicity is manifested post-schizogony as "delayed death". Using these methods and others we compare CQ sensitive (CQS) *vs.* resistant (CQR) strains as well as transfectants that are CQR via introduction of mutant PfCRT. Surprisingly, we find that PfCRT confers resistance to CQ administered at the very early ring stage of development, wherein a digestive vacuole is not yet formed, as well as at the schizont stage, wherein Hz production is thought to plateau. Taken together, these data force a rethinking of CQ pharmacology and the mechanism of CQR.

Keywords: Hemozoin; Merozoite production; Delayed death

Abbreviations: BR, (short) bolus exposure during ring stage; BS, (short) bolus exposure during schizont stage; BT, (short) bolus exposure during trophozoite stage; CE, continuous exposure; CQ, chloroquine; CQR, chloroquine resistance (resistant); CQS, chloroquine sensitive; DIC, differential interference contrast; DV, digestive vacuole; FPIX, ferriprotoporphyrin IX; Hb, hemoglobin; SDCM, spinning disk confocal microscopy; 4D, four dimensional (3D *vs.* time); Hz, hemozoin; IM, incomplete media; iRBC, infected red blood cell; LSCM, laser scanning confocal microscopy; *n*, cell cycle in which parasites were exposed to drug; *N*, number of nuclei inside one schizont; PBS, phosphate buffered saline; PfCRT, *Plasmodium falciparum* chloroquine resistance transporter; R, ring stage; RBC, red blood cell; Rf, resistance factor; S, schizont stage; SCP, single cell photometry; T, trophozoite stage.

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☆☆ Quicktime movies that rotate 3D data sets of the SYBR Green I labeled schizonts on the *x*, *y*, and *z* axes are available as supplemental data from the authors.

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1. Introduction

P. falciparum malarial parasites are now largely resistant to several key antimalarial drugs. Until recently chloroquine (CQ) was the drug of choice but globally >50% of *P. falciparum* infections are now chloroquine resistant (CQR). Elucidating the molecular mechanism of CQR is essential to the ongoing design of second line antimalarial drugs and new therapies [1]. In addition, the mechanism of action of CQ is not fully understood but is obviously also relevant for drug design.

Early on, parasite DNA was postulated to be the CQ target [2,3]. Cohen and Yielding further proposed that DNA synthesis is inhibited through an effect on DNA and RNA polymerase, due to the binding of CQ to DNA primer [4]. Hahn and co-workers investigated aminoquinoline action *vs*. nucleic acids and proposed that CQ stabilizes double-stranded DNA [5,6]. Meshnick and co-workers have also proposed that a portion of the CQ mechanism comes from binding to DNA, possibly by preventing formation of Z-DNA [7,8]. However, none of these models easily explained much lower inhibitory concentrations of CQ for *P. falciparum vs*. mammalian cells (∼1000–10,000 fold lower). Thus the nucleic acid hypotheses have been largely discounted, particularly after the discovery that the *P. falciparum* genome was highly AT-rich whereas CQ preferentially interacts with GC-rich DNA [9].

When CQR parasites were found to accumulate substantially less CQ *vs.* CQ sensitive (CQS) [10], and a lysosomal-like organelle called the digestive vacuole (DV) was found to be the principle site of CQ accumulation [11,12], focus began to shift towards investigation of DV processes. Importantly, Orjih and Fitch found that mid-stage trophozoites with fully formed DVs accumulate more CQ than any other stage [13]. Morever, recently, CQR was shown to segregate with mutations in the *pfcrt* gene which encodes a protein found in the DV membrane, which is only fully developed during the early trophozoite stage [14]. Also much physical–chemical data support direct interaction between CQ and various forms of non-crystalline heme released in the DV upon hemoglobin (Hb) digestion (e.g. [15,16]). These interactions inhibit conversion of toxic heme to non-toxic crystalline hemozoin (Hz) and are thus believed to be central to CQ (and related quinoline antimalarial drug) pharmacology [17,18]. Taken together, this is why it is now widely assumed that CQ acts primarily on the trophozoite stage of intraerythrocytic parasites, and that this is the stage wherein the CQR mechanism must operate. Indeed, mutant PfCRT causes CQR, directly binds CQ [19] and is postulated to be a transporter that either directly or indirectly alters CQ–heme interaction within the DV during the trophozoite stage [19,20].

Further testing of this model and possibly others would benefit from additional close inspection of the stage specificity of CQ effects, using recently perfected imaging tools. Only a handful of studies report on the stage-specificity of CQ and related aminoquinolines, and many questions remain. Early on Peters demonstrated that CQ is not active against liver stages of infection, but is active *vs.* the erythrocytic stages that actively degrade Hb [21]. While describing gametocytogenesis, Smalley et al. found that early gametocytes are sensitive to CQ along with trophozoites that are actively digesting Hb [22]. Slater later summarized these results and others, noting that ". . . early rings, late schizonts and late gametocytes are innately resistant to concentrations of CQ that would kill trophozoites and rings on the border of trophozoite stage . . ." [23].

However, Zhang et al. [24] performed a detailed study on stage-specificity of CQ and reported that ring stages are the most sensitive, a conclusion also consistent with the work of Orjih [25], but in direct contrast to other studies by Yayon et al. [26] as well as others summarized in [23]. Interestingly, Zhang et al. also proposed that the effect of CQ on trophozoites and schizonts is not seen until the cycle following exposure to drug (after reinvasion and development of new rings; a phenomenon referred to as "delayed death"), a conclusion also reached by Krishna and co-workers [27]. Perhaps disagreement in the literature regarding stage specific CQ toxicity is due in part to measurement of survival at different endpoints (wherein different degrees of delayed death have occurred). In any case, collectively, these studies typically only report data for one parasite strain, do not quantify precise IC_{50} values for various dose schedules (i.e. continuous *vs.* bolus pulse for various time), and were performed well before our current level of molecular understanding of CQR and so do not compare CQS *vs*. CQR parasites.

In this study we investigate the stage specificity of CQ in detail using recently developed imaging methods [28,29] to measure parameters that are central to various models for toxicity, but that have not previously been measurable for live parasites. Our results are in agreement with previous observations that the effect of CQ is both schedule and dose-dependent, but also clearly show that ring stage parasites are affected by CQ long before the mature DV is formed, and that schizont stage parasites are similarly sensitive to CQ long after Hz formation has plateaued. In addition, quite surprisingly, we find that the CQR mechanism mediated by mutant PfCRT is equally active in all stages of intraerythrocytic growth. Our results are consistent with the Hz crystallization process being an important, but likely not the only, target of CQ. We propose that both CQ toxicity and the CQR mechanism controlled by mutant PfCRT involve multiple stage-specific molecular pathways.

2. Materials and methods

2.1. Materials

Fresh stocks of *P. falciparum* strains Dd2, HB3 and GCO3 were obtained from the Malaria Research and Reference Reagent Resource Center (MR4). The allelic exchange lines $C4^{Dd2}$ and $C2^{GCO3}$ were kindly provided by Dr. David Fidock (Albert Einstein College of Medicine, Bronx, NY). Custom 5% O_2/5% CO_2/90% N_2 gas blends were purchased from Robert's Oxygen (Rockville, MD). Off-the-clot, heat-inactivated, pooled O+ human serum and pooled O+ human whole blood were purchased from Biochemed Services (Winchester, VA). Albumax and SYBR Green I were purchased from Invitrogen Corporation (Carlsbad, CA). CQ phosphate, Poly-L-lysine, RPMI 1640, hypoxanthine, and Giemsa were from Sigma–Aldrich (St. Louis, MO). No. 1.5 coverslips were from Fisher Scientific (Pittsburgh,