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A Process Similar to Autophagy Is Associated with Cytocidal Chloroquine Resistance in *Plasmodium falciparum*

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Abstract

Resistance to the cytostatic activity of the antimalarial drug chloroquine (CQ) is becoming well understood, however, resistance to cytocidal effects of CQ is largely unexplored. We find that PFCRT mutations that almost fully recapitulate *P. falciparum* cytostatic CQ resistance (CQRCS) as quantified by CQ IC50 shift, account for only 10–20% of cytocidal CQR (CQRCC) as quantified by CQ LD50 shift. Quantitative trait loci (QTL) analysis of the progeny of a chloroquine sensitive (CQS; strain HB3) × chloroquine resistant (CQR; strain Dd2) genetic cross identifies distinct genetic architectures for CQRCS vs CQRCC phenotypes, including identification of novel interacting chromosomal loci that influence CQ LD50. Candidate genes in these loci are consistent with a role for autophagy in CQRCC, leading us to directly examine the autophagy pathway in intraerythrocytic CQR parasites. Indirect immunofluorescence of RBC infected with synchronized CQ-resistant trophozoite stage parasites reveals differences in the distribution of the autophagy marker protein PIATG8 coinciding with CQRCC. Taken together, the data show that an unusual autophagy-like process is either activated or inhibited for intraerythrocytic trophozoite parasites at LD50 doses (but not IC50 doses) of CQ, that the pathway is altered in CQR parasites, and that it may contribute along with mutations in PFCRT to confer the CQRCC phenotype.

Introduction

Resistance to quinoline and antifolate antimalarial drugs, as well as emerging tolerance to artemisinin–based drugs [1] threatens the health of over half of the world’s population. Understanding the molecular details of *P. falciparum* and *P. vivax* antimalarial drug resistance phenomena facilitates surveillance of resistance and rapid development of more effective treatment. Two approaches for monitoring antimalarial drug resistance for *P. falciparum* malaria are available. One is analysis of clinical data to assess efficacy of specific treatments, the second is *in vitro* analysis of parasite strains or patient isolates to quantify their susceptibility to specific drugs. With the discovery of key genetic mutations in *P. falciparum* that confer resistance to either antifolate [2,3] or quinoline–based [4] antimalarial drugs, rapid field-based surveillance of the geographic spread of existing drug-resistant malaria is now possible, as is personalized delivery of second-tier drug therapy to patients infected with a specific drug-resistant strain.

Many antimicrobial drugs are both cytostatic and cytocidal, including quinoline antimalarial drugs such as CQ [5–7]. That is, under certain conditions a drug slows the rate of cell growth or impairs cell division such that the rate of proliferation of a mass population of the microbe is reduced, and under other conditions the drug kills the microbial cell. Often, cytocidal (cell kill) activity requires higher dose of drug, longer drug exposure time, or both. Cytostatic potency is usually quantified via IC50 values (the dose of drug at which growth is inhibited by 50% relative to control), whereas cytocidal potency is quantified via LD50 values (the dose of drug that kills 50% of a microbial population).

However, to date, all laboratory-based quantification of antimalarial drug potency, and hence quantification of all antimalarial drug resistance phenomena, has been done with IC50 values alone. Again, IC50 assays quantify the concentration of drug required to inhibit proliferation of parasite populations by 50%. Drug IC50 for *P. falciparum* are typically measured in red blood cell culture suspensions in the continuous presence of serially diluted concentrations of the drug. Such quantification has proved critical for defining the genetics and biochemistry behind resistance to the cytostatic effects of CQ (CQRCS) [4,8–11] and for identifying new drug leads with excellent cytostatic potential vs CQR malaria [12]. It is sometimes assumed that IC50 values measure the “cell kill” effect of a drug. Although this can be true in specific cases, in many others this is not the case. Use of IC50 values alone can over-estimate the cytoidal activity of a drug and...
can under-estimate potential parasite survival in the presence of higher (clinically relevant) levels of the drug. Since laboratory CQ IC50 are in the 10^{-9}–10^{-8} M range, but peak plasma levels of CQ in patients are ~1000 times higher (10^{-6}–10^{-5} M; see [13–15]), clarification of these points is essential for fully understanding CQR.

That is, although the mechanism of CQRCS is becoming clear, much less is known about resistance to the cytocidal effects of CQ (CQRCC) (or cytocidal resistance vs any other antimalarial drug for that matter). This is a critical piece of missing information, given that parasite survival determines the rate of adaptation to selection by drugs. Only recently has it been possible to efficiently and reproducibly quantify LD50 for some antimalarial drugs, and rapid quantification of LD50 differences for drug sensitive vs drug resistant parasites are found in only one paper to our knowledge [6]. Formally, without additional information the ratio of drug IC50 values for drug sensitive vs drug resistant parasites expresses the degree of cytostatic resistance, whereas LD50 ratios express the degree of cytotoxic resistance [6].

For any drug (anti-tumor, anti-bacterial, anti-fungal, anti-parasitic) it is theoretically possible that the molecular mechanisms controlling cytostatic and cytocidal activities could overlap. If this is the case, then the molecular mechanisms of resistance to those two distinct layers of drug pharmacology would also presumably overlap. In other cases, particularly when significantly different concentrations of drug are needed to kill the relevant cell vs merely slow its growth, molecular targets for the two layers of drug activity may differ, and then the mechanisms of cytostatic vs cytocidal resistance could be distinct. This has been observed in some examples of multidrug resistance, for example, overexpression of P-glycoprotein and other plasma membrane events can be particularly relevant for antitumor drug cytostatic resistance, whereas altered induction or regulation of apoptosis (programmed cell death) is particularly important in antitumor drug cytotoxic resistance [16].

Perhaps relatedly, we recently reported that although decreased CQ accumulation within CQR P. falciparum is clearly related to elevated CQ IC50, it is not necessarily relevant for elevated CQ LD50 [17]. Surprisingly, much higher concentrations of CQ or fluorescent NBD - CQ can be found within parasites exhibiting cytocidal CQ resistance, even though reduced drug uptake is generally accepted to be the principle basis of CQR [17]. We have also recently found that, for both 4 amino quinolines similar to CQ and quinoline methanols similar to quinine (QN), IC50 is correlated with the ability of the drugs to inhibit hemoglobin crystallization under close to physiologic conditions, but LD50 for the same drugs is not [18,19]. Patterns of IC50 vs LD50 for a variety of quinoline drugs also suggest that the mechanisms for cytostatic vs cytocidal CQ resistance in P. falciparum are not entirely the same [6]. Taken together these data suggest that the cellular targets relevant for quinoline antimalarial drug cytotoxic activities may differ from targets for cytostatic activities. Drug DV localization and drug/heme binding is the likely basis of CQ cytostatic pharmacology, but perhaps not the entire basis of CQ cytocidal pharmacology [17–19]. Since drug resistance is due to disruption of drug/dv drug targets interactions, then different targets for cytostatic vs cytocidal effects predict distinct mechanisms of cytostatic vs cytocidal resistance, unless resistance is merely due to increased catalobilism of the drug (which is not the case for CQR or QNR in P. falciparum).

With respect to P. falciparum CQRCS, elevated CQ IC50 and reduced parasite CQ accumulation are well correlated with mutations in the DV membrane CQ transporter PICRT [11], suggesting that CQRCS is due to decreased drug accessibility to heme targets within the parasite DV [20]. However, resistance to the cytocidal effects of CQ is predicted to include alterations in additional targets, access to these additional targets [17], and/or to encompass mutations in key regulators of P. falciparum cell death pathways as recently hypothesized [21]. With regard to this last point, being a single celled organism, and due to the lack of caspase genes and other genes that encode key apoptosis regulators within the P. falciparum genome, it is questionable whether the canonical apoptosis pathway is the cause of drug - induced cell death for the malarial parasite [21]. Some evidence for an apoptotic - like cell death pathway for P. falciparum involving metacaspases has been presented [22–25] but there is disagreement on how relevant these observations are for P. falciparum death via different drugs [26]. More importantly, no molecular alterations in apoptosis have been found for CQR malaria.

These points led us to rank the progeny of the HB3 (CQS) x Dd2 (CQR) P. falciparum cross for CQ LD50 and to perform LD50 directed QTL analysis. Progeny of this genetic cross have proven invaluable to analysis of CQR phenomena [4,8,10,27]. By quantifying CQ IC50 values for these progeny, a single locus on chr7 was previously identified as controlling the difference between CQR and CQS strain CQ IC50 [4]. Subsequent sequencing, in vitro drug pressure, and transfection results showed that multiple amino acid substitution mutations within a single gene in the chr7 locus, pfcrt, causes the large shift in IC50 values that has historically defined CQR and CQS status [4,28,29]. Allelic exchange experiments that directly replaced the ‘wild type’ CQS associated pfcrt allele with mutant CQR associated pfcrt resulted in elevated CQ IC50 without the need to condition or select cells with CQ [30]. The degree to which CQ IC50 was elevated for these allelic exchange transfectants was very similar to that seen for highly drug selected CQR strains (70%–90% of the corresponding strain IC50 shift, see [30]), suggesting that the presence of mutant PICRT protein was in-and-of-itself sufficient (or nearly sufficient) for conversion to a CQR phenotype. However, subsequent QTL analyses suggested that additional genetic components, such as inheritance of different chr5 loci containing mutations and varying copies of pfmdr1, may combine with PICRT mutations in various isolates to confer the range of CQ IC50 and variable IC50 patterns for different drugs now known to exist across the globe [8–10,27]. In contrast, QTL analysis in this paper shows a complete lack of the key chr5 locus previously identified for CQRCS, and identifies additional and unique genomic loci specific to CQRCS. Examination of genes in these loci suggests candidate pathways that may contribute to CQRCS. Relatedly, LD50 analysis of pfcrt allelic exchange progeny further supports our overall conclusion that although some PICRT mutations in and of themselves confer nearly complete resistance to CQ cytocidal effects as defined by IC50 shift (see [30]), they are less important for cytoidal CQ resistance (CQRCS) as defined by LD50 shift. Using antibodies to the autophagy indicator protein ATG8 and high - resolution fluorescence microscopy, we find that a drug-induced autophagy-like cascade is dysregulated in CQRCS P. falciparum. Taken together, and considered alongside additional recent work with the related parasite T. gondii [31], our data suggest that a dysregulated autophagy-like process, combined with PICRT mutations, promotes elevated CQ LD50 in CQR P. falciparum.

Materials and Methods

Cell Culture

P. falciparum strains HB3, Dd2, and their genetic cross progeny were acquired from the Malaria Research and Reference Reagent Services.
LD50 values for laboratory strains analyzed in this paper are found [7]. Parasites were incubated at 37 °C for 10, and 52 hours using a solution of 5% sorbitol as described [6]. Parasites were incubated at 37 °C for 5% CO2 in either complete, starvation, or CQ containing media for 6 hours. When quantification of PATG8 puncta was desired, CQ pulse was done for 6 hours using highly synchronized parasites at the mid trophozoite stage, followed by washing to remove drug as described [6].

**IC50 and LD50 Quantification**

Our assay for IC50 has been published previously [32] and has been used extensively and validated by many other laboratories. Our semi-high-throughput assay for LD50 quantification has recently been published elsewhere [6]. In brief, for LD50 quantification asynchronous P. falciparum cultures at 2% hematocrit and 2% parasitemia were treated with CQ in bolus fashion for 6 hours and then the drug was completely washed away [6]. After 48 hours of further incubation at 37 °C under 5% CO2, Sybr Green I was added and fluorescence was measured at excitation and emission wavelengths of 485 nm and 538 nm, respectively [6]. For IC50 quantification initial parasitemia is reduced to 0.5% and cells remain in the constant presence of lower levels of drug [32]. In previous work [6] we defined differences in LD50 for synchronized vs asynchronous culture and vs other variables. LD50 and IC50 values for data individual assays (each assay performed with triplicate plating of parasites treated at a given dose) were fit to a sigmoidal function using SigmaPlot 9.0 (San Jose, CA), and IC50 or LD50 values were calculated from at least three individual assays (at least 9 determinations in total) and averaged. Outgrowth in the LD50 assay 48 hours after drug is washed away represents expansion of live cells that survived cytocidal dosages of drug, as supported by staining for outgrowth after different incubation times (see [6] for detailed discussion of this point and related issues). CQ IC50 and LD50 values for laboratory strains analyzed in this paper are found in Table 1, and CQ LD50 values for HB3 x Dd2 cross progeny can be found in Table S1.

| Strain | CQ IC50 (nM +/- [SEM]) | CQ LD50 (μM +/- [SEM]) |
|--------|------------------------|------------------------|
| GC03   | 221 [1.7]              | 0.031 [0.005]          |
| Dd2    | 206 [26.3]             | 15.7 [1.82]            |
| 7G8    | 161 [14.1]             | 3.99 [0.62]            |
| C2G03  | 24.4 [1.9]             | 0.96 [0.08]            |
| C4Dd2  | 187 [21.0] (90%)       | 1.10 [0.082] (7%)      |
| C67G8  | 128 [12.8] (79%)       | 0.91 [0.067] (23%)     |

Also shown for C4Dd2 and C67G8 are (% IC50 or % LD50, relative to Dd2 or 7G8 values, respectively). See [30] for description of the allelic exchange transfectants. Note IC50 values are done in the continuous presence of low dose CQ for >48 hrs [33] whereas LD50 assays require higher doses of CQ given as a 6 hr bolus [6].

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**QTL Analysis**

Using mean LD50 for each of the progeny of the HB3 × Dd2 cross, genome-wide scans were run using Pseudomarker 2.04 to detect quantitative trait loci (QTL) associated with the drug response. Genome-wide significance thresholds which correct for multiple testing errors were determined by permutation testing (n = 1000 permutations). The strength of the association between a given locus and the trait (LD50) is expressed as a logarithm of odds (LOD) score. Loci that exceeded the 99th percentile (p<0.01), the 95th percentile (p<0.05), and the 90th percentile (p<0.63) were identified respectively as highly significant, significant, and suggestive QTL. Two-dimensional linear regression genome scans were run to test for potential loci interactions and joint LOD scores were calculated to identify significant interactions.

Candidate genes within QTL loci were selected as described [33]. In brief, candidate genes were selected based on four selection criteria: (1) genomic position, (2) structural polymorphisms, (3) correlation between LD50 and expression phenotypes for each parasite, and (4) gene annotations and enrichment analysis. PlasmoDB version 9.3 was used for SNP density scoring (CDS), and gene annotations. Gene enrichment analysis for biological processes and molecular functions was performed using DAVID [34]. Expression phenotypes for genes within our loci were taken from [35]. Permutation testing (n = 1000) confirmed significance of GO enriched terms within the loci of interest.

**Preparation of Affinity Purified ATG8 IgG and ATG8 Monoclonal Antibody 2K19**

A RACE validated cDNA encoding *Toxoplasma gondii* TgATG8 (ToxoDB accession number TGGT1_003400) was used as template to amplify the full length coding region using the primers (CACCATGCCCATCGATCAGGAATGGTGGC and TTACCCAGAGTGTCTCTCAGAGTATTCCA CGTACA). The ampiclon was subcloned into pET100 establishing an in frame N-terminal hexa-His tag. Following expression in E. coli, the recombinant His-TgATG8 was purified on Ni-NTA magnetic beads (Invitrogen) and used as the immunogen to immunize a single rabbit (Cocalico, Reamstown, PA). The resulting antiserum was affinity purified using the bead immobilized crosslinked antigen and eluted using low pH.

Generation of a monoclonal antibody was contracted to AbMART.com (Shanghai, China) and accomplished using a synthetic polyprotein containing 6 tandemly arrayed epitopes. Epitopes were selected on the basis of the highest homology between TgATG8 and PATG8. Supernatants from clones yielding ≥3 fold signal in an ELISA at 1:128K dilution were screened by IFA on both *Toxoplasma* and *Plasmodium* organisms. The clone 2K19 was selected for detailed work as it was found to be an IgG1 isotype (data not shown) and gave the best defined signal in both organisms.

**Immunohistochemistry**

For starvation treatments, cells at the mid trophozoite stage were pelleted and resuspended in HBS supplemented with 0.1 mg mL−1 hypoxanthine, 25 mM HEPES (pH 7.3), and 20 mM sodium bicarbonate. Cells were gassed and incubated at 37 °C for a desired interval (typically 6 hours) before fixation. For CQ treatments, highly synchronized mid stage trophozoites were treated as described [6,7] using drug concentrations noted in the text. Resultant cell pellets were resuspended in complete media and treated as below.
Cells were washed 3 times with 25 mM HEPES pH 7.3, fixed with 4% formaldehyde/0.0075% glutaraldehyde in PBS for 30 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes, reduced with 0.3 mg mL⁻¹ sodium triacetoxysorbidehyde for 10 minutes, blocked with 5% goat serum for 1 hour, and sequentially treated with antibodies (1:500) diluted in 5% goat serum/PBS Tween-20 with PBS washes in between; antibody treatments lasted 1 hour at 37°C in the dark. For experiments involving mouse monoclonal 2K19, the primary antibody solution was prepared at 1:500 and the secondary (typically goat anti mouse DyLight488) at 1:500. For experiments involving antiserum or purified IgG from rat or rabbit, primary solutions were prepared at 1:500 and secondary solutions (goat anti rat AlexaFluor394 and goat anti rabbit DyLight488 or DyLight649) at 1:500. Cells were attached to #1.5 coverslips and mounted using “Fluororeg” mounting media. Samples were imaged using a customized Perkin–Elmer spinning disk confocal microscope with 405 and 491 nm laser lines, typically at 200 ns exposure and 35% laser power [36].

**Cell Fluorescence Data Analysis**

Images were iteratively deconvolved using a point spread function obtained under identical imaging conditions (via doping one sample with fluorescent beads) and running multiple iterations in AutoQuantX2 [36]. Images were further processed and overlaid using Imaris 7.4.2 software. Using the “spots” routine in Imaris 7.4.2, puncta were defined and distances were measured from each spot to a single point within the DV as defined by the center of hemozoin optical density (see [36] and Scheme S1). These distances were exported to Excel and the resulting data were plotted as number of puncta vs distance from hemozoin.

**Western Blot**

Western blots were done as previously described [7] with slight modification. Fractionation of synchronized iRBC was as described [7]. SDS-PAGE gels (15% acrylamide) were pre loaded with lysed trophozoite-infected RBCs, electrophoresed and transferred to nitrocellulose over night at 4°C. Blots were blocked with 10% dry milk (Biorad)/PBS, washed×3 with PBS/0.1% Tween-20, labeled with rabbit anti-ACTG8 antiserum (1:10,000), washed again, and incubated with anti-rabbit HRP secondary antibody (1:5000).

**Results**

Using a more rapid SybrGreen assay [32] in place of traditional 3H-hypoxanthine incorporation, our quantification of CQ IC₅₀ for pfcrt transfectants agrees with that published previously (Table 1). Clones C4Dd2 and C6G7G8 [30] show approximately 8-fold and 6-fold shifted CQ IC₅₀ relative to control transfectants (C2GCO3) or the parental CQS strain GCO3 (Table 1; e.g. 187 nM/24 nM, C4Dd2 vs C2GCO3). That is, cytostatic CQ resistance for these clones is very close to that seen for laboratory strains Dd2 and 7G8 (Table 1 and see also [37]).

Regardless, recently we showed that when expressed as a ratio of LD₅₀ (dose required to kill 50% of parasites), “cytotoxic” CQR was not the same as “cytostatic” CQR defined by IC₅₀ ratios [6].
Figure 1. LD<sub>50</sub> vs IC<sub>50</sub> directed QTL analyses for CQR HB3 × Dd2 cross progeny. A) IC<sub>50</sub> QTL scan for CQR progeny shows a peak on chr5 (asterix) that encompasses pfmdr1 as previously described [10]. Notably, the chr6 locus that is pertinent for the LD<sub>50</sub> scan (see Fig. 1B) does not pass the suggestive threshold on this IC<sub>50</sub> scan. B) LD<sub>50</sub> QTL scan for CQR progeny shows a peak on chr6 (asterix; L.O.D. = 2.5, passing the suggestive threshold). The locus that includes pfmdr1 does not pass the suggestive threshold for this scan (see also Fig. 1C). C) To more clearly highlight the differences in genetic architecture for LD<sub>50</sub> vs IC<sub>50</sub> phenotypes, an overlay of the two QTL scans is shown. The CQR progeny LD<sub>50</sub> QTL scan is shown in blue, while the CQR progeny IC<sub>50</sub> QTL scan is in black. The overlay shows quite clearly that the pfmdr1 locus does not factor at all into the LD<sub>50</sub> phenotype, and that the chr6 locus does not factor at all into the IC<sub>50</sub> phenotype. Thus the IC<sub>50</sub> and LD<sub>50</sub> phenotypes are genetically distinct. D) Similarly, the interaction locus between chr6 & 8 (see text) does not appear on a IC<sub>50</sub> pair-wise scan. However, additive effects between chr5 and chr7 loci are seen, as previously reported [10]. E) Pair-wise scan of the CQR progeny shows that chr6 and chr8 loci (circle) have additive effects on LD<sub>50</sub> (L.O.D. = 4.3).

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to identify genes or pathways that are most likely relevant to LD50. Thus we used four previously vetted methods wondered if altered autophagy might be related to LD50.

pathways often overlap mechanistically (see Discussion), we proteolysis'', ''lipid metabolism'', ''oxidative stress'' and autophagy distinct from apoptosis. Since apoptosis genes were not found in related apicomplexan parasite

HB3 (CQS) and strain Dd2 (CQR), and the similarity of strain HB3 (CQS) and strain Dd2 (CQR), and the inheritance in the HB3 × Dd2 cross, the chr6 × chr8 additive effect defines a much smaller region of the chr6 segment. This segment harbors only 20 genes, with 7 of those encoding proteins involved in lipid metabolism (see Discussion).

Elevated LD50 indicates resistance to cell death. Cell death is often mediated by signal transduction that controls a programmed cell death (PCD) pathway [21]. Importantly then, we find that the chr6 and chr8 loci do not harbor any candidate Pf metacaspases [23] or other molecules that typically regulate apoptotic PCD. That is, we find no genetic evidence from the HB3 × Dd2 cross for atypical apoptosis related to CQRCC. Autophagy ("self eating" upon starvation or stress) is an alternate pathway that has been linked to cell death for several cell types [42–44], including the related apicomplexan parasite T. gondii [31,45]. It is an orchestrated, vesicle mediated, proteolysis/degradative pathway that is distinct from apoptosis. Since apoptosis genes were not found in chr6 or chr8 loci, and since "vesicle traffic", "proteolysis/ proteolysis", "lipid metabolism", "oxidative stress" and autophagy pathways often overlap mechanistically (see Discussion), we wondered if altered autophagy might be related to LD50.

However, no experiments to our knowledge have been done to test if autophagy occurs in intraerythrocytic P. falciparum. The universal stimulus for autophagy is starvation, which also unambiguously induces cell death. An unambiguous feature of induction of autophagy in eukaryotes is redistribution of ATG8 protein (called LcIII in mammals) from a more localized and diffuse pattern to a more widely dispersed, more punctate pattern that defines the sites of autophagosome formation and/or recruitment of autophagosomal "cargo" [46]. Specific ATG8 antisera raised vs. T. gondii ATG8 protein (anti TgATG8) show excellent cross reactivity vs P. falciparum ATG8 because PIA(TG8 is ~70% identical to TgATG8 [21]. We easily identify a PIA(TG8 doublet at predicted masses of 15 & 17 kDa in immunoblots of fractionated parasites (Fig. 2D) that is consistent with well known de-lipidated and lipilated forms of ATG8 [46]. IFA analysis using ATG8 antisera suggests an autophagy-like process is active in intraerythrocytic P. falciparum trophozoites (Fig. 2,3). As expected, control IRBC trophozoite parasites grown in complete media show a cytosolic PIA(TG8 distribution (Fig. 2A, green) that appears somewhat punctate, perhaps due to at least partial localization to the apicoplast (see below and [47]). However, notably, when highly synchronized trophozoites are placed in starvation medium for 6 hrs, PIA(TG8 is redistributed in a much more expanded punctate fashion (Fig. 2B, green), as recently reported [21]. Closer inspection reveals puncta at the parasite periphery, possibly near the RBC membrane. Co – staining with a marker for Maurer’s cleft (anti - PIREX1, red) shows that some PIA(TG8 appears to be routed to very near Maurer’s cleft (MC) (yellow dots, Fig. 2B middle). The well-recognized inhibitor of autophagy, 3-methyl adenine (3-MA) partially reverses the starvation induced PIA(TG8 puncta redistribution (Fig. 2C), similar to what has recently been found for T. gondii [31]. Affinity purified IgG from the antisera as well as monoclonal antibody 2K19 raised against a highly conserved Apicomplexan ATG8 motif (see methods) yield results similar to polyclonal TgATG8 antisera (Fig. 3). Another recent report [47] presents data consistent with some localization of PIA(TG8 to the parasite apicoplast for control schizonts growing in normal media. We also obtain data consistent with partial (but not exclusive) localization of PIA(TG8 to the apicoplast for control late trophozoites/early schizonts, by co – staining for apicoplast – specific PIACP protein (see Fig. S1). We note that the trophozoite (feeding) and schizont (nuclear division/parasite replication) stages of parasite development would be expected to utilize autophagy machinery in different ways [48,49] and that further study of PIA(TG8 in trophozoites vs schizonts is warranted.

We next tested if cytosidal levels of CQ induced similar PIA(TG8 redistribution. Indeed, CQS parasites show similar extensively distributed PIA(TG8 puncta when they are treated at 2×LD50 cytocidal dose of CQ (Fig. 4A), but not when they are treated with 2×IC50 cytostatic dose (Fig. S2). Thus the autophagy – like cascade induced by starvation is also involved in the response to cytosidal levels of CQ, but not response to cytostatic levels. When CQR parasites are treated with the same absolute dose (2×CQS LD50 dose; 250 nM), we do not observe the punctate redistribution of PIA(TG8 (see below). However, when CQR parasites are treated with 2×CQR parasite LD50 dose (e.g. a similar effective pharmacologic dose, ~32 μM for strain Dd2, see [6]) some PIA(TG8 redistribution is observed (Fig. 4B), but the ATG8 response appears somewhat muted.

To quantify this behavior, we devised a method based on spinning disk confocal microscopy and 3D Imaris rendering of z stacks to plot radial distributions of PIA(TG8 puncta relative to hemozoin optical density (Fig. 5). Essentially, very optically dense hemozoin within the DV is used to define a "center" point of reference for the IRBC parasite (Fig. 5 left) and distinct, clearly defined spots of ATG8 fluorescence (Fig. 5 middle, see also Scheme S1) are then measured for their relative distance (x,y,z) from the center of hemozoin optical density (white lines, Fig. 5 right). Using these methods, we quantified PIA(TG8 puncta abundance at >3.5 μm for CQS HB3, CQR Dd2 and C4ATG8 parasites +/- starvation and across a range of bolus CQ dosages (Fig. 6). These puncta distributions show that starvation produces similar number and similar radially distributed patterns of peripheral PIA(TG8 puncta for CQS and CQR parasites (compare far left and far right bars in each panel Fig. 6), but that 2×CQS LD50 dose of CQ (250 nM) only produces high numbers of distal radially - distributed puncta for CQS strain HB3 (Fig. 6A top), not for CQR strain Dd2 (Fig. 6B middle). C4ATG8 transfectants, wherein CQR is mediated solely by allelic exchange with CQR associated mutant pfext, show PIA(TG8 behavior that is intermediate relative to CQS HB3 and CQR Dd2 (Fig. 6C bottom), and again, Dd2 shows a muted ATG8 response (hashed bars indicate 2×LD50 dose for each strain).

We further tested the association between peripherally distributed PIA(TG8– positive puncta and CQR status by examining 4 other well-characterized strains, two CQS (strains 3D7 and Sudan 106) and two CQR (strains FCB and 7G8). Upon starvation for 6 hr, all 4 strains distribute PIA(TG8– positive puncta in a peripheral fashion similar to starred HB3, Dd2, and C4Dd2.
Figure 2. PfATG8– positive puncta. Shown are puncta for (A) control HB3 iRBC grown under normal culture conditions (B) HB3 iRBC grown for 6 hours under starvation conditions (see Methods) and (C) HB3 iRBC grown under starvation conditions plus the autophagy inhibitor 3 methyl adenine (3 MA). Shown are transmittance (left), immunofluorescence vs antiPfREX1 (Maurer’s cleft marker; red; second column), immunofluorescence vs antiTgATG8 (cross reacts with PfATG8; green, third column) and overlays (right). Bar = 5 μm. Also shown (D) are western blot data for iRBC harboring HB3 (CQS) and Dd2 (CQR) trophozoites grown under control culture conditions. Two separate gels for two independent sets of samples (two iRBC isolations for each culture) are shown. We note our data show a clear doublet at 15 and 17 kDa, similar to all other studies of eukaryotic ATG8 protein of which we are aware except one [47], which resolves only a single band instead of the usual doublet with a polyclonal antisera raised against a recombinant GST-PfATG8 fusion. We suggest three possible reasons for the discrepancy: 1) we use higher density [15% acrylate] gels relative to [47] in order to resolve the low mass doublet, 2) we do not solubilize parasites with saponin as in [47] which would release de-lipidated ATG8 into wash supernatant, 3) perhaps abundance of one PfATG8 species (presumably de lipidated) is higher in trophozoites relative to schizonts examined in [47].

Figure 3. Comparison of antisera vs monoclonal Ab staining. Shown are results for TgATG8 antisera (bottom row) vs staining using a monoclonal antibody raised vs a highly conserved Apicomplexan ATG8 epitope (top row, see methods). Panels A,G,D,J are transmittance, B,H,E,K are ATG8 fluorescence, and C,I,F,L are overlay, respectively. Left side is control intraerythrocytic HB3 P. falciparum; right side is HB3 starved for 6 hrs as described in methods. Bar = 5 μm.

Cytocidal Chloroquine Resistance in P. falciparum
Figure 4. PfATG8 (green) re-distribution, CQ vs time. CQS (strain HB3; top panel A) and CQR (strain Dd2; bottom panel B) parasites were grown in control media (left column) or in media plus 2×LD₅₀ dose of CQ (250 nM for HB3 Fig. 4A, 30 μM for Dd2, Fig. 4B) for 2, 4 or 8 hrs (column 2, 3, 4 respectively). The top row in panels A and B is transmittance, the second (green) is staining for PfATG8, the third is DAPI to visualize the single trophozoite nucleus, and the bottom row for each panel presents the overlay. Fluorescence acquired at 35% power, 200 ms, 642 nm; emission 700/75 nm dichroic, 690 nm cut-off. Bar = 5 μm.

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value = 1
orthologue, based on its very high identity to ScVps34 (E-
P. falciparum
Discussion). For example, a recently identified
in Dd2 (CQR) vs HB3 and 3D7 (CQS) parasites (see
of putative orthologues of key autophagy proteins are mutated (see also [50]) using available genomes, and note that a number
of autophagy pathway proteins that we can identify at this time
serve as a marker to quantify sensitivity to cytocidal CQ.

show statistically significant differences in PfATG can
be responsible for 10–20% of the shift in CQ LD50 for the
same strains of CQR parasites. PfCRT is thought to confer
CQRCS by reducing access of CQ to DV – localized heme
target and thereby reduce the ability of the drug to inhibit
heme – hemozoin biomineralization [11,29]. However, recent
data [17] shows that DV levels of CQ are not necessarily
correlated with CQ LD50. In addition, other recent work [18,19]
shows that the ability of CQ or QN analogues to
inhibit hemozoin formation correlates with their IC50 activity,
but not with their LD50 activity. Thus our observations are
not as surprising as they might initially appear, and one
important implication is that targets in addition to inhibition
of hemozoin crystallization are likely relevant for the cytocidal
activity of quinoline antimalarial drugs.

We next inventoried sequences of all P. falciparum orthologues
of autophagy pathway proteins that we can identify at this time
(see also [50]) using available genomes, and note that a number
of putative orthologues of key autophagy proteins are mutated
in Dd2 (CQR) vs HB3 and 3D7 (CQS) parasites (see Discussion). For example, a recently identified P. falciparum
PI3' kinase (PF3D7_0515300; [51]) appears to us to be a Vps34
orthologue, based on its very high identity to ScVps34 (E-
value = 1 × 10^-73; homology index score = 567). A similar
conclusion was also reached by Kitamura et al. [47]. During
autophagy in other eukaryotic cells, Vps34 produces copious
PI3P at the developing autophagosome, where ATG8 protein
localizes. PfVps34 from CQR strain Dd2 shows point
mutations and deletions relative to CQS strains HB3 and 3D7
that could conceivably affect function or regulation, as described
in Discussion. Quite interestingly then, this enzyme has recently
been localized to near Maurer’s cleft organelles [51], very
similar to the PIATG8 relocalization upon starvation that is
reported here. It remains to be determined if these additional
autophagy gene mutations uniformly segregate with CQR
phenomena for geographically distinct isolates of CQR P. falciparum. Importantly, LD50 quantification for these isolates
will need to be performed, which will require the ability to culture
these isolates over long time periods.

Discussion

Data in this paper reveal three important concepts:

1) Mutant PfCRT protein, while responsible for the majority of
the shift in CQ IC50 that has traditionally characterized CQR
P. falciparum strains Dd2 and 7G8 ([30], and present data), is
only responsible for 10–20% of the shift in CQ LD50 for the
same strains of CQR parasites. PfCRT is thought to confer
CQRCS by reducing access of CQ to DV – localized heme
target and thereby reduce the ability of the drug to inhibit
heme – hemozoin biomineralization [11,29]. However, recent
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inhibit hemozoin formation correlates with their IC50 activity,
but not with their LD50 activity. Thus our observations are
not as surprising as they might initially appear, and one
important implication is that targets in addition to inhibition
of hemozoin crystallization are likely relevant for the cytocidal
activity of quinoline antimalarial drugs.

2) LD50-directed Quantitative trait loci (QTL) analysis in the
well-characterized HB3 × Dd2 genetic cross shows that the
chr5 segment previously identified as modulating PfCRT
mediated CQRCS does not contribute to CQRCS. Other
distinct differences in genetic architecture include two novel
loci, on chr6 and chr8, associated with elevated LD50. These
loci are enriched for genes that encode proteins linked to
proteasome and autophagy pathways, but no genes encoding
metacaspases or other proteins that regulate apoptosis are
found in the loci (Tables S2– S7). A number of recent
observations suggest interesting cross talk between proteasome
and autophagy pathways (e.g. [32,33]), thus enrichment for
both processes in the LD50 loci is intriguing.

3) Staining for a definitive marker of autophagy (ATG8 protein
puncta) shows cell death from LD50 CQ treatment is linked to
an autophagy – like process and that this process is altered in
CQR parasites. Interestingly, we find that starvation and CQ
– induced death lead to accumulation of PIATG8– positive
puncta to near Maurer’s clefts (MC). These poorly understood
organelles are known to be involved in export of proteins from
the parasite to the red cell plasma membrane but they are also

Figure 5. Semi automated computational method for quantifying the distribution of ATG8 puncta relative to hemozoin crystals
within the DV. (A) Hemozoin within the DV is detected by transmittance, the outline of <10% transmittance is defined (labeled "DV", left panel)
and the center of this outline then defined by voxel analysis using Imaris software. (B) The center of distinct ATG8 puncta (bright green dots) are
labeled using the option 'Add new measurement points' in the Imaris „spots“ subroutine. (C) Distances from the hemozoin center to the ATG8
puncta centers are then computed (white lines) and data collated in excel.
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To our knowledge, only three studies have attempted to distinguish CQ cytostatic and cytolic activities \textit{in vitro} [5,6,55] but only recently have LD\textsubscript{50} been quantified [6], allowing QTL analyses as presented here. All previous cellular quantification of antimarial drug resistance phenomena of which we are aware has been solely via IC\textsubscript{50} ratios. IC\textsubscript{50} does not \textit{a priori} define cytocidal (parasite kill) potency; formally it defines growth inhibition potency vs mass populations of parasites. It is certainly true that killing some parasites over time with a drug prevents growth seen in controlled cultures, but it can also be true that a drug in continuous culture does not kill parasites, but merely slows the cell cycle, changes multiplicty of schizogony, or has other effects that yield IC\textsubscript{50} of a given value for mass populations of parasites [7]. CQ appears to exhibit both dose dependent (“C\textsubscript{max}”) [7] and this work) and time dependent (“T\text{max}”) [56] parasite killing. In studies that report IC\textsubscript{50} data there may be greater “mixing” between CQ cytostatic and cytolic effects relative to studies that report IC\textsubscript{50} data. In this paper, we have made the first attempt to our knowledge to more fully separate CQ IC\textsubscript{50} and LD\textsubscript{50} phenomena for progeny of the HB3\times Dd2 cross.

We previously found that some strains are much more CQR\textsubscript{CC} than CQR\textsubscript{CS} [6], and that the rank order of LD\textsubscript{50} and IC\textsubscript{50} for different drugs varies (meaning the drugs to which a parasite shows the highest IC\textsubscript{50} are not necessarily the drugs to which it shows the highest LD\textsubscript{50} [6]). Plotting CQ IC\textsubscript{50} [4] vs CQ LD\textsubscript{50} (Table S1) for HB3\times Dd2 progeny yields poor correlation ($r^2$<0.4; not shown). Also, a recent study shows that CQ transport ability for 13 mutant PfCRT isoforms found in 13 different CQR strains does not correlate with CQ IC\textsubscript{50} for those strains [57]. Considering these observations along with the current data suggests that parasite resistance to cytolic effects of CQ is influenced by additional genetic or physiological events, along with PfCRT mutations. Our initial analysis suggests these events include alterations in a novel pathway showing some similarities to autophagy.

In all eukaryotic cell types examined, the re distribution of ATG8 protein to more widely disbursed puncta marks the induction of autophagy by starvation [46]. In all other examples, the membranes to which ATG8 is routed are synthesizing double membraned autophagosomes and copius phosphatidyl inositol 3’ phosphate (PI3P) via Vps34. It is striking then that previous work completely unrelated to the present study places Vps34 near MC, similar to our localization for some re – routed PIATG8 [51]. In all eukaryotic cell types examined, autophagosomes containing high levels of ATG8 and PI3P lipids then engulf cytosolic or organellar targets, fuse with lysosomes, and the contents are then degraded, serving as nutrient pools that temporarily keep the starving cell alive. In the case of \textit{P. falciparum}, the parasite trophozoite undergoes heightened accumulation of PIATG8–associated vesicles at or near the MC upon starvation and cytolic CQ treatment. The parasite trophozoite does not appear to engulf and degrade its sole mitochondrion to provide additional food upon starvation. Indeed, starvation induced mitochondrial fragmentation by autophagy in \textit{T. gondii} causes cell death [31]. Instead, the unique properties of the RBC, which is devoid of de novo biosynthetic activity as a host cell, necessitates enhanced endocytosis to acquire extracellular food. We suggest that under starvation pressure the parasite up regulates additional endocytosis from the red cell cytosol using \textit{at least in part} the vesicle formation and fusion functions of encoded autophagy machinery. This might be consistent with PIATG8 vesicles eventually interacting with the parasite DV, analogous to ATG8 positive vesicle fusion to lysosomes in other eukaryotes after they recruit nutritional “cargo”.

In yeast and higher eukaryotes, membrane association of ATG8 is believed to have several other functions, including vesicle fusion [54].

To our knowledge, these three studies have attempted to distinguish CQ cytostatic and cytolic activities \textit{in vitro} [5,6,55] but only recently have LD\textsubscript{50} been quantified [6], allowing QTL analyses as presented here. All previous cellular quantification of antimarial drug resistance phenomena of which we are aware
proteolysis to reveal G as in other species is absent, suggestive of constitutive membrane association [49]. In reality however, PfATG8 exists in both the unlipidated and lipidated forms (Fig. 2D) suggesting mechanisms other than ATG4 regulate PfATG8 dynamics. We propose that a low level of constitutively activated autophagy is present in iRBC parasites, and that CQR parasites have developed resistance to CQ induced perturbations in autophagy. Accumulation of PfATG8 puncta upon toxic CQ treatment is consistent with either upregulation of puncta formation or inhibition of autophagosome fusion, so CQR parasites could in theory have perturbations in either (or both) steps of the autophagy pathway.

Interestingly then, CQ is a known potent inhibitor of autophagy in other cell types. Its diprotic weak base character promotes profound accumulation in acidic compartments such as lysosomes, autophagosomes, and vacuoles. At doses that correspond to these LD50, CQ is known to block the fusion of autophagosomes with lysosomes/vacuoles and also raises the pH of these compartments, thereby inhibiting processes that require acidic pH (e.g. intra lysosomal proteolysis [42,44] and references within). A few molecular possibilities specific to P. falciparum are that LD50 dosages of CQ (i) block the fusion of endocytic vesicles carrying hemoglobin to the DV (in fact, a somewhat overlooked paper [58] shows that CQ LD50 doses do indeed lead to a buildup of undigested Hb trapped within arrested parasite vesicles), (ii) inhibit falcipain and plasmepsin activity by raising the pH in endocytic vesicles and/or the DV, (iii) inhibit fusion of autophagosomes and/or other vesicles with their target organelles. Other lysosomotropic agents would be expected to mimic this CQ pharmacology. Interestingly then, certain alkaloids that inhibit autophagy also show antimalarial activity [59,60]. One example is voacamine, a tertiary alkaloid isolated from Peschiera fuchsiaefolia stem bark that shows good antimalarial activity (238 ng/mL vs strain D6 and 290 ng/mL vs strain W2), and which has also been reported to chemosensitize MDR cancer cells in an autophagy-dependent manner [61]. Overall, since P. falciparum has been subjected to decades of cytocidal CQ selective pressure, it is logical that the parasites would evolve resistance to CQ autophagy inhibition.

With regard to the starvation effects that we observe, work in the Goldberg laboratory has shown that P. falciparum meets its amino acid requirements by a combination of hemoglobin degradation and uptake of free amino acids from the medium [62–64]. When some extracellular amino acids are removed, the parasite responds by up – regulating additional hemoglobin transport and degradation; hemoglobin, however, lacks the essential amino acid Ile, so parasite survival is conditional under these circumstances [63]. Conversely, if the hemoglobin pathway is inhibited, the parasite survives by acquiring additional amino acids from the extracellular medium. If Ile is withdrawn the parasite can enter a hibernatory state [64]. These observations suggest that (i) malaria parasites are able to sense amino acid levels
in the medium and (ii) they possess a system that can respond to the lack of some extracellular amino acids by regulating intracellular transport to the vacuole. During starvation induced autophagy, other eukaryotic cells respond to low amino acid levels in the medium by trapping cytosolic material in transport vesicles, which will eventually fuse and release cargo into a lysosome or vacuole to then be digested to amino acids. Although for intraerythrocytic *P. falciparum* the “cargo” is presumably within the host cell cytosol, starvation induced autophagy reported here could be somewhat reminiscent of elevated hemoglobin endocytosis in *P. falciparum*.

We wondered if autophagy genes in the identified chr6/chr8 loci might be hinting at mutations in other Pf autophagy gene (PIATG gene) orthologues for CQR parasites. A partial inventory of PIATG gene orthologues has been published [50] but does not include important co factors such as Vps34, Pex14, Vps15, etc. We re – queried the Pf genome with a more complete set of 42 autophagy related genes. Remarkably, a number of candidate orthologues (16 of 42) lie within QT loci previously associated with drug resistance phenomena, or within “eQTl” gene sets that are up/down regulated in trans by resistance associated eQTl [35]. Also, after alignment of Dd2 (CQR) vs HB3 and 3D7 (CQS) alleles via Broad Institute data (http://www.broadinstitute.org/), we find that many candidate PIATG gene orthologues are mutated in the Dd2 CQR genome relative to 3D7 or HB3 CQS. One in particular is candidate PVps34 (PF3D7_0515300; E-value 10^-73 and homology index score 367 relative to ScVps34) which encodes K at codon 423 for Dd2 in place of Q found for HB3 and 3D7, and a deletion of 24 codons relative to HB3 and 3D7 (from codon 931 to 955). Interestingly, this deletion is within what we predict is a C2 regulatory domain that presumably binds Ca2+; a similar C2 domain prediction for this protein has also been made by two other groups [51,65]. Since a clear TOR orthologue is missing within the *P. falciparum* genome [50] altered regulation via mutant PVps34 is one possibility for the altered cascade seen in the CQR strain Dd2.

Regardless, we predict that multiple routes to dysregulated autophagy will be found for various strains and isolates of CQRCC *P. falciparum*, analogous to how multiple routes to dysregulated programmed cell death are found in various multidrug resistant tumor cell lines [16]. At LD50 doses, via its well known lysosomotropic behavior [58,66] CQ will inhibit vesicle formation and vesicle fusion, two processes that are essential to autophagy. Since these processes are controlled by a number of proteins, many mutations are possible for altering CQ response.

In sum, we find that mutant PfCRT protein confers the majority of CQRCC as defined by CQ IC50 shift, but only partial CQRCS as defined by LD50 shift. Not surprisingly then, our data also show that distinct genetic architecture is associated with CQRCS vs CQRCC, that a specialized *P. falciparum* autophagy cascade is induced by LD50 doses of CQ but not IC50 doses of the drug, and that altered regulation of this unique autophagy – like process is present in CQRCC parasites. Consistent with a clear role for autophagy in cell death for other apicomplexa [31,43], altered PIATG8 redistribution indicative of a novel autophagy – like cascade appears to be associated with CQRCC for intraerythrocytic *P. falciparum* trophozoites. Elucidating additional molecular events controlling perturbation of autophagy signaling for CQR *P. falciparum*, potentially involving at least two dozen autophagy gene orthologues [50], should prove to be a fertile area of future research. Among additional data needed to elucidate the pathway further are precise measurements of ATG8 puncta flux, meaning the rate of ATG8 vesicle production vs presumed fusion with either MC and perhaps the DV.

Finally, regardless the exact role of the novel autophagy – like process for iRBC trophozoites that we describe here, we propose that CQRCC requires prior acquisition of CQRCS, meaning that parasites must first be able to survive lower IC50 dose via PICRT mutations, before they can survive higher LD50 dose via acquiring additional autophagy mutations. Precise measurements of IC50 vs LD50 phenomena among geographically diverse isolates of CQR *P. falciparum* will further test this hypothesis. Also, since PvCRT mutations have not been found for CQR *P. vivax*, it should prove interesting to investigate the status of genes in the chr6 and chr8 loci for CQR *P. vivax* isolates, as well as the formation of PvATG8 positive structures in response to CQ treatment.

Supporting Information

Figure S1 Late trophozoite/early schizont stained with anti-ATG8 (green) and anti-apicoplast (red) antibodies. Some overlap between ATG8 and apicoplast localized ACP (D) suggests partial (but not complete, ~25%) co – localization of PIATG8 and apicoplast as previously suggested [46]. Scale bar = 5 μm.

Figure S2 CQS (HB3, A) and CQR (Dd2, B) parasites treated with IC50 (top row each panel) and 2×IC50 (bottom row) doses of CQ (10, 20 nM and 125, 250 nM, respectively) for 48 hr. Parasites were then stained for ATG8 (green) and with DAPI (blue), as described in Methods. Very few peripherally disposed PIATG8 puncta are observed, supporting the conclusion that puncta formation is associated with LD50, but not IC50 doses of CQ. Scale bar = 5 μm.

Table S1 LD50 values for the HB3×Dd2 cross progeny.

Table S2 All genes within the chr6 LD50 locus.

Table S3 All genes within the LD50 chr6×chr8 interaction.

Table S4 GO enriched molecular functions for the chr6 LD50 locus.

Table S5 GO enriched biological processes for the LD50 chr6 locus.

Table S6 GO enriched molecular functions for the LD50 chr×chr8 interaction.

Table S7 GO enriched biological processes for the LD50 chr×chr8 interaction.

Scheme S1 Cartoon representation of 3D puncta quantification method used in the present work. Hemozoin density (red “x”) is used as the origin, and distances to PATG8 positive puncta are defined for the reconstructed confocal z stack of images, relative to hemozoin, using 3D Cartesian (x,y,z) coordinates. The cartoon shows an abbreviated depiction of 3 SDCM “slices”, but as described in methods the z stack data set for each cell is a fully assembled, iteratively deconvolved 3D image.
constructed from approximately 15–20 z slices (see [35] for additional detail).

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Author Contributions

Conceived and designed the experiments: PDR APS MTF D. Gavria LB. MTF performed the experiments. D. Gavria MTF LB T. APS D. Ghosh.

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