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To kill or not to kill, that is the question: cytotoxic antimalarial drug resistance

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Abstract

Elucidating mechanisms of antimalarial drug resistance accelerates development of improved diagnostics and the design of new, effective malaria therapy. Recently, several studies have emphasized that chloroquine (CQ) resistance (CQR) can be quantified in two very distinct ways, depending on whether sensitivity to the growth inhibitory effects or parasite – kill effects of the drug are being measured. It is now clear that these cytostatic and cytotoxic CQR phenotypes are not equivalent, and recent genetic, cell biological, and biophysical evidence suggests how the molecular mechanisms may overlap. These conclusions have important implications for elucidating other drug resistance phenomena and emphasize new concepts that are essential for the development of new drug therapy.

Keywords

Plasmodium falciparum; cytostatic; QTL analysis; drug transport; autophagy

Altered drug transport in CQR *Plasmodium falciparum*: history and curiosities

All scientists have their favorite older research articles written by colleagues to which they refer often over the years. Sometimes they are particularly unforgettable because they perturb the scientist's pre-conceived sense that things are mostly understood for the topic at hand. One such personal favorite is a 1986 study of drug transport in *P. falciparum* infected red blood cells (iRBC) by Geary, Jensen, and Ginsburg [1], wherein the key question, "how does CQ accumulation differ for CQR versus CQ sensitive (CQS) malarial parasites?" is asked. In the study, iRBC are exposed to μM levels of external CQ. At μM levels, well known differences in CQ accumulation for CQS versus CQR parasites that are seen at nM levels of external CQ vanish, as recently confirmed [2].

Reading this paper today, any new student of antimalarial drug resistance phenomena would ask several pointed questions. It is now well established that at lower concentrations (1 – 10 nM), differences in CQ accumulation are easily measured for CQS versus CQR parasites, and that this is intricately linked to the mechanism of CQR [3,4]. This observation is in fact

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central to an entire field that begins with the proposal of Fitch, Macomber, and Spitz over 45 years ago [5,6], wherein free heme in the acidified parasite digestive vacuole (DV), released upon catabolism of red blood cell hemoglobin (Hb), is the molecular target of CQ and related quinoline antimalarial drugs. The implications of this proposal have guided malaria research for decades. Recent work indeed shows, in atomic detail, how these drugs react with free heme [7,8]. We now know that heme-drug interactions stall the growth of DV hemozoin (Hz), the formation of which is essential to parasite physiology [9–12]. We also know that the key determinant for *P. falciparum* CQR is an integral membrane protein localized to the DV membrane [13]. A series of studies have shown that mutated versions of this protein, PfCRT (*Plasmodium falciparum* chloroquine resistance transporter), catalyze increased efflux of CQ out of the DV for CQR parasites, and away from heme target [14 – 18]. Although the thermodynamics and kinetics of this transport are still debated [3] and additional questions remain for how CQR and multidrug resistance (MDR) overlap [19–21], overall this has led to a very satisfying model for CQ pharmacology and the mechanism of CQR. Namely, in CQR, increased efflux of CQ from the DV leads to decreased net accumulation of CQ because fewer complexes are formed between CQ and the target, DV localized heme, relative to what occurs in CQS parasites. In support of this, clearly reduced DV CQ accumulation is seen for CQR parasites at nM levels of external CQ [2]. Relatedly, easily measured 10 – 100 nM differences in CQ sensitivity are routinely seen for CQS versus CQR parasites, and many mutant *pfert* alleles, created by different drug selection histories in various regions, have been found around the globe [22,23]. A popular theory has been that these different alleles confer the different levels of CQR or MDR that exist.

Or do they? The above model for CQR has been elucidated primarily by studying how nM chloroquine concentrations ([CQ]) interact with iRBC. Dozens of drug transport studies have examined iRBC transport at nM levels, and thousands of CQ susceptibility measurements have calculated nM drug IC₅₀ for various strains and isolates. These IC₅₀ yield levels of CQR that are universally 5–15 fold, which are expressed by calculating CQR/CQS strain IC₅₀ ratios. The 2–10 fold differences in CQ accumulation measured for CQS versus CQR parasites seem entirely compatible with this ~ 10 – fold degree of resistance. Nonetheless, in re-reading Geary *et al.* [1] today, where differences in drug accumulation are not observed, one might ask which drug concentrations are relevant, or, what is the concentration of drug to which parasites in the human body are exposed?

Occasionally forgetting the laboratory and thinking only about the clinic is important

The answer to that question is “initially, μM , not nM” [24–26]. Yet, prior to 2009, only one study [1] compared CQ accumulation for CQS versus CQR parasites at levels corresponding to the initial clinical situation. At those higher levels of drug, differences in accumulation for CQS and CQR parasites vanish [1,2]. So what is really going on at the higher levels of drug and how might this affect our understanding of CQR? How resistant are CQR parasites to these higher concentrations? Since lowered drug accumulation does not appear to be involved at these concentrations, if they are resistant, what is the mechanism?

A straightforward explanation begins to reveal itself by recognizing that, for many drugs used to treat infectious diseases, low doses have growth inhibitory, or cytostatic effects, whereas higher doses often (but not always) have cell kill, or cytotoxic effects. This well-established concept has been central to elucidating multiple layers of resistance to anticancer and antibacterial drugs yet has not been highlighted for antimalarial drug resistance until recently. Why? This additional question is discussed below.

First, returning to Geary *et al.*, the reason those experiments are particularly unforgettable is because when a malaria patient enters a clinic, he/she will have up to 10^{11} – 10^{12} parasites in his/her body. Next, when CQ, artemether (ATM), or other potent antimalarials are administered, if the parasites are sensitive to the drug(s), then parasitemia drops by several logs (to 10^9) within hours. Plasma [CQ] is $1\text{ }\mu\text{M}$ [24–26], with C_{MAX} well above CQ LD_{50} for CQS parasites, but below CQR CQ LD_{50} . Correspondingly, patients infected with CQS malaria show fast reduction in parasitemia upon use of CQ, but patients infected with CQR malaria do not, which is why CQ therapy fails for these patients. The parasite intraerythrocytic life cycle is 2 days long, so it takes 2 days to observe growth inhibition by drug, as quantified by IC_{50} . However, in patients, parasites must be cleared within hours, not days. Clearly, an ideal antimalarial drug does not merely slow or hinder the growth of parasites but rather it kills parasites rapidly within the initial 48 h of therapy. Rigorously, IC_{50} quantifies growth inhibition potency, and not necessarily cell kill potency. LD_{50} quantifies cell kill potency. Thus in elucidating drug potencies and drug resistance phenomena, have we been measuring all that is necessary to quantify clinically relevant CQR?

We probably have not been measuring all that is necessary. Ten years ago, Fidock and colleagues performed an important experiment that asked if mutant PfCRT protein is necessary for elevated CQ IC_{50} in drug resistant *P. falciparum* [27]. When mutant PfCRT was expressed in a CQS parasite background without any CQ selection, CQ IC_{50} for the transfectants in fact matched CQ IC_{50} for highly drug-selected strains. Meaning, mutant PfCRT was sufficient, or very nearly sufficient, to recapitulate CQR as defined by CQ IC_{50} . In principle, the CQR problem seemed nearly solved, and the model was satisfyingly quite consistent with the Fitch, Macomber, Spitz hypothesis [5,6].

However, PfCRT is only responsible for 10–20% of the change in cytotoxic activity of CQ in the same CQR strains [28]. That is, mutant PfCRT confers nearly all CQ cytostatic resistance as defined by IC_{50} shift [13,27], but a much smaller component of CQ cytotoxic resistance as defined by LD_{50} shift [28]. This is not as radical as it might appear. Some cancer chemotherapeutics show cytostatic potential via inhibition of the cell cycle (e.g., by binding to tubulin) but exhibit cytotoxic potency via induction of programmed cell death (e.g., apoptosis). Accordingly, tubulin mutations can confer resistance to cytostatic doses of vinca alkaloids whereas mutations in apoptosis proteins are needed to confer cytotoxic resistance to the same drug [29–31].

Another prediction from the “nM IC_{50} model” for CQR is that if heme is the molecular target of quinoline drugs such as CQ, then the ability of this class of drugs to inhibit heme-to-hemozoin conversion should correlate with antiplasmodial potency. Indeed, for CQ and quinine (QN) analogues, it does, assuming the hemozoin formation assay is done under close-to-physiologic conditions (reviewed in [7]). Nevertheless, as recently learned, this is true only when potency is defined via IC_{50} [11]. When potency is defined by LD_{50} , the correlations vanish [11]. This evidence supports the notion that the mechanisms underlying the cytostatic versus cytotoxic actions of quinoline drugs are distinct. If mechanisms of action are distinct, resistance to those two mechanisms can be distinct.

Back to the question ‘why’?

Differences in cytotoxic (LD_{50}) versus cytostatic (IC_{50}) activities of antimalarial drugs historically have not been emphasized for a very simple reason; namely, assays for easily quantifying LD_{50} have not been available. Even now, with two approaches recently published [32,33], these semi high-throughput methods have limitations. The only unambiguous assay for cytotoxic activity is a tedious “limiting dilution” approach alluded to

by Rathod and colleagues 20 years ago [34] that has recently been developed further [35]. It remains as tedious as it was 20 years ago and requires at least a month of live parasite culturing to even begin quantifying cidalty.

A much faster (2 – 3 day) and vastly less expensive LD₅₀ assay was recently published [32]. This assay relies on outgrowth of viable parasites after bolus dose of drug (Figure 1). However, the limitations include difficulty in unequivocally distinguishing parasites that are dead versus quiescent. Sometimes, a high dose of a cytotoxic drug does not kill a microbe, but induces a hibernatory or quiescent state that also prevents normal rates of outgrowth. One way to control for this with the Paguio assay is to examine multiple time points, and to examine cell cycle kinetics [32]. With these control measurements, meaningful LD₅₀ for well-understood diffusible drugs such as CQ can be obtained [28].

Initial results with this assay were quite striking but consistent with the questions raised by re-reading Geary *et al.* [1]. First, in contrast to the well-established notion that all CQR is ~10 fold (via ratios of CQR/CQS strain CQ IC₅₀), CQR parasites can in fact show 10 – 200 fold levels of resistance by LD₅₀ ratios [28,32]. Cytotoxic resistances can be higher and they span a wider range of values. Second, the rank order of resistance to drugs via IC₅₀ (e.g., resistance to CQ > QN > MQ > AQ for a CQR strain) is not necessarily the same rank order as defined by LD₅₀. Third, verapamil (VPL), which is well known to re-normalize IC₅₀ for some CQR parasites by negating altered drug accumulation, does not re-normalize LD₅₀ [32]; yet another clue that cytostatic and cytotoxic layers of CQ pharmacology must be molecularly distinct (Box 1).

Box 1

The summary presented in this opinion piece yields the following questions for future work

1. What are the molecular targets for the cytotoxic activity of chloroquine and other quinoline-based antimalarial drugs?
2. What is the range of cytotoxic chloroquine resistance in the field?
3. Do other antimalarial drugs have distinct cytostatic versus cytotoxic targets?
4. How are LD₅₀ versus IC₅₀ data for a candidate drug best leveraged in pre-clinical development?

The answers to these questions will have significant influence on more rapid development of new antimalarial therapy active against drug resistant malaria.

This assay was also recently used in a quantitative trait loci (QTL) analysis of the invaluable HB3 (CQS) x Dd2 (CQR) genetic cross [28]. As is well known, IC₅₀ – directed QTL of this cross has elucidated the primary role of PfCRT in *P. falciparum* CQR phenomena [13], as well as the important modulatory role of PfMDR1 (*Plasmodium falciparum* multidrug resistance protein 1) [19–21]. As recently reported [28], LD₅₀ – directed QTL of the same cross reveals another layer to CQR phenomena that is particularly relevant for the cytotoxic activity of CQ (Figure 2). Importantly, the chromosomal locus harboring *pfcr*t is also revealed in LD₅₀ – directed QTL, consistent with analysis of CQ LD₅₀ for PfCRT transfectants described above. However, two new chromosomal loci, not previously associated with CQR phenomena are also revealed. Analysis of genes within these loci (see [28]) suggests vesicle traffic, proteasome, and lipid metabolism pathways are potentially relevant for cytotoxic CQR. One pathway relevant to cell death that intersects with all three processes is autophagy. Interestingly then, autophagy has recently been implicated in cell

death for several cell types, including the related apicomplexan *Toxoplasma gondii* [36]. Cytocidal resistance (elevated LD₅₀) is resistance to cell death. This invokes altered control of cell signaling relevant for regulation of a cell death pathway. Perhaps not coincidentally then, alterations in an autophagy-like cascade have recently been quantified for CQR parasites [28]. Also, consistent with its partial effect on CQ LD₅₀, mutant *pfcr* alone is not responsible for these alterations [28]. Much work remains to be done, but one prediction is that mutations are likely to be found in multiple autophagy genes for CQR *P. falciparum* isolates that show high CQR^{CC} because there are multiple ways to regulate balance between the cell survival and cell death consequences of this cascade [37].

A new “two-tiered” model for CQR, and a new paradigm for antimalarial drug discovery

We are left with a more complex, but more satisfying understanding, which may begin to reconcile numerous observations that were not necessarily compatible with a “nM IC₅₀” view of CQR phenomena. Meaning, effects of CQ seen at concentrations closer to μ M peak plasma [CQ]. For example, CQ DNA intercalation [38], particularly potent induction of oxidative damage [39], accumulation of odd Hb-containing vesicles [40], altered multiplicity of schizogony [41], and changes in additional important proteins for CQR parasites (e.g., glutathione [42] and PfATG8 [28]). These phenomena have been seen in a variety of experiments but are often not emphasized with respect to CQR or CQ pharmacology, presumably because they do not necessarily correlate with CQ IC₅₀, DV CQ accumulation, or PfCRT status. Nevertheless, perhaps they are more relevant for CQ cytocidal potency, which is not correlated with CQ IC₅₀, DV CQ accumulation, or PfCRT status either. PfCRT changes are necessary but not sufficient for high levels of CQR^{CC}, and additional facets to CQ pharmacology are necessary for parasite kill. A common thread connecting these facets could be generation of, or response to, oxidative damage. Relatedly, to explain the only partial (10 – 20 %) effect of PfCRT on raising CQ LD₅₀, perhaps some CQ-heme complexes formed at higher, cytostatic [CQ] promote cell death via oxidative damage as proposed [42], whereas CQ-heme interactions at lower, cytostatic [CQ] are merely growth inhibitory because they stall hemozoin production but do not produce sufficiently toxic levels of heme or heme-drug conjugate. This might explain yet another puzzling observation, wherein multiple CQ-heme complexes with different solubility characteristics and oxidative potential are now known to be formed at different [CQ] and heme concentration [43,44]. Like many transporters, PfCRT is likely better able to kinetically compete with passive diffusion of its substrate at lower concentrations (e.g., IC₅₀ levels of CQ) but not at much higher concentrations (e.g., LD₅₀ levels of CQ), making it more relevant for cytostatic resistance. This broader view may also help explain why the CQ transport activity of different mutant PfCRT isoforms does not appear to correlate with CQR measured for the cognate strains of *P. falciparum* [45].

The broader view drives home a key principle recognized for some time [46], but that has been somewhat neglected in antimalarial drug discovery efforts. Namely, the cytocidal potency of candidate antimalarial drugs is just as important, if not more important, than the cytostatic potency. This is because malaria patients may enter the clinic with such a high parasite burden that hundreds of billions parasites must be killed quickly. Preventing parasite growth does not do this, regardless how low the drug IC₅₀ is. A corollary is that understanding cytocidal resistance is just as necessary as understanding cytostatic resistance. In some cases, drug discovery gets “lucky” because conventional IC₅₀ assays might reveal some degree of cytocidal potential. This is the case for artemisinin and its relatives. A very good way to slow proliferation of a mass population of parasites versus time is to kill some of them, which 10 nM levels of ATM do quite quickly [33], hence ATM IC₅₀ and LD₅₀ lie

close together. However, for other drugs (e.g., CQ), this is not the case at IC₅₀ dose, because the drug can be washed away during the IC₅₀ assay and none or very few of the parasites are found to be dead [32,41]. In these cases, higher doses or much longer exposure times are required to reveal cytotoxic activity. A simple conclusion is that, notwithstanding progress [32,33,35], there is a real need for even better and more convenient cytotoxic assays. Ideally, these assays should be able to inspect both C_{MAX} and T_{MIC} regimens and have the capability to examine different stages of parasite development, since cytotoxic potency can vary for these different stages [41,47]. At a given dose, some drugs might even show cytostatic activity for one stage, but cytotoxic for another.

Integrating these concepts into design of combination drug therapy is also essential. Most now agree that combination drug therapy will remain the standard of care for malaria for quite some time, but as more delayed clearance phenomena for patients treated with artemisinin combination therapies (ACTs) are found [48,49], it is clear that development of new drug combinations effective against CQR malaria are desperately needed. Ideally, these should be additive or synergistic at both IC₅₀ and LD₅₀ levels of quantification. Importantly then, recent evidence indicates that synergy as quantified by IC₅₀ potency does not necessarily correlate with synergy as quantified by LD₅₀ for the same drug pairs [50]. Experimental quantification of both is required. Such analyses will probably result in better correlation between *in vitro* predictions and measurement of drug activity in animals.

With regard to this last point, the above analysis yields yet another provocative question: if common *in vitro* assays have not quantified cytotoxic potency, then what is the real predictive ability of typical animal studies? Do they account for distinction between cytostatic versus cytotoxic potency? As described elsewhere [51], there are important distinctions between mouse models of malaria. Cytostatic drug activity in a mouse model is revealed by the standard Peter's suppression test, wherein drug is administered along side or shortly after inoculation with a rodent-infectious *Plasmodium* sp. By contrast, the Batty approach [52] allows parasitemia in the mouse to increase before administration of drug, similar to what occurs in a human patient. Results from this format more closely reflect the initial clinical situation, wherein 10¹¹ – 10¹² parasites must be reduced to < 10⁹.

Concluding remarks

The best antimalarial drugs show relatively fast kill of large numbers of parasites, along with excellent growth inhibitory (cytostatic) activity. The best antimalarial drug combinations will work together to achieve those two goals at lower doses (lower toxicity) and with improved PK/ PD, versus MDR drug resistant strains of *P. falciparum* and *P. vivax*. CQ and related quinolines have been highly successful drugs that show excellent cytostatic and cytotoxic activity, and that remain effective partner drugs in combination therapy. PfCRT mutations that result in decreased DV concentrations of CQ are sufficient for most, but not all, of the shift in nM CQ IC₅₀ observed in CQR parasites. However, cytotoxic CQR is not correlated with DV accumulation of CQ or inhibition of heme-to-hemozoin conversion; correspondingly, PfCRT mutations are only responsible for 10 – 20 % of the shift in μM CQ LD₅₀ for these same strains [28]. Further elucidating cytotoxic targets and cytotoxic resistance mechanisms are key remaining pieces to the antimalarial drug resistance puzzle.

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Glossary

Amodiaquine (AQ)	a common antimalarial drug related to chloroquine.
Artemether (ATM)	a methyl ether derivative of artemisinin and currently a preferred treatment for malaria, particularly in combination with lumefantrine.
Artemisinin combination therapy (ACT)	two antimalarial drugs used together, with one partner drug being artemisinin or one of its common derivatives (i.e., artemether).
Bolus	a dose of drug given in such a way as to rapidly achieve the desired effect, often in pulse fashion.
Batty approach	refers to a method for testing efficacy of antimalarial drugs in mouse models of malaria, developed by Kevin Batty and colleagues, wherein drug is administered after the number of malarial parasites in the mouse has climbed to a high level [51].
Chloroquine (CQ)	the most widely used antimalarial drug to date, a 4 amino quinoline derivative of the natural product quinine (QN).
Chloroquine sensitive (CQS)	refers to the natural sensitivity to chloroquine for wild type strains of <i>Plasmodium falciparum</i> .
Chloroquine resistance (CQR)	decreased sensitivity to chloroquine in malarial parasites.
C_{MAX}	maximum or peak concentration of drug achieved after administration.
Cytocidal chloroquine resistance (CQR^{CC})	CQR phenotype as quantified by the shift in LD ₅₀ or cytotoxic potency.
Cytostatic chloroquine resistance (CQR^{CS})	CQR phenotype as quantified by the shift in IC ₅₀ or cytostatic potency.
Digestive vacuole (DV)	lysosomal – like organelle within mature red blood cell stages of <i>Plasmodium falciparum</i> involved in digestion of red cell hemoglobin.
Fifty percent lethal dose (LD₅₀)	dose of a drug required to kill 50 percent of a cell population.
Fifty percent inhibitory concentration (IC₅₀)	dose of a drug required to slow the rate of growth of a cell population by 50 percent.
HB3 (CQS) x Dd2 (CQR) genetic cross	as described in [13] and references within, a library of clonal progeny from mating these two strains of <i>P. falciparum</i>
Hemozoin (Hz)	crystalline form of heme found within the parasite digestive vacuole (DV).
Multidrug resistance (MDR)	resistance to multiple, structurally divergent drugs.
Mefloquine (MQ)	a quinoline methanol relative of quinine, developed by the Walter Reed Army Institute of Research as a potent antimalarial drug substitute for chloroquine.

Peter's suppression test	refers to a commonly used method to test the efficacy of an antimalarial drug in a mouse model of malaria, initially described by Wallace Peters of the London School of Hygiene and Tropical Medicine [52], wherein drug is administered alongside, or shortly after, inoculation of the mouse with malarial parasites.
<i>P. falciparum</i> chloroquine resistance transporter (PfCRT)	integral membrane protein found within the digestive vacuolar (DV) membrane that is mutated in chloroquine resistant (CQR) <i>Plasmodium falciparum</i> .
<i>P. falciparum</i> infected red blood cell (iRBC)	a human red blood cell infected with a <i>Plasmodium falciparum</i> malarial parasite; usually only one parasite is found within the RBC.
<i>P. falciparum</i> multidrug resistance protein 1 (PfMDR1)	integral membrane protein found within the digestive vacuolar (DV) membrane that is often, but not always, either mutated or overexpressed in chloroquine resistant (CQR) <i>Plasmodium falciparum</i> .
Proteasome	large cylindrical protein complexes whose purpose is to degrade damaged or misfolded proteins.
Quantitative Trait Loci (QTL)	DNA segments linked to a quantifiable phenotypic trait.
Quinine (QN)	natural product antimalarial drug derived from the cinchona tree that was the preferred antimalarial treatment until the 1940s.
T_{MIC}	aggregate time for which a drug exceeds the minimum inhibitory concentration (MIC).
Verapamil (VPL)	a calcium channel blocker used to treat hypertension and cardiac arrhythmia that is also well known for "resistance reversal" effects in some strains of CQR <i>Plasmodium falciparum</i> .
Vinca alkaloids	class of anticancer drugs originally derived from <i>Catharanthus roseus</i> . Well known for antimetabolic effects via inhibition of tubulin polymerization.

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Highlights

1. Cytostatic vs Cytocidal Drug Resistance
2. IC_{50} vs LD_{50} Drug Susceptibility Assays
3. Genetics of 'static vs 'cidal chloroquine resistance

Figure 1.

Schematic of LD₅₀ versus IC₅₀ assays. In the conventional IC₅₀ assay (top), asynchronous *P. falciparum* cultures at 2% hematocrit and 1% parasitemia are continuously treated with serial dilutions of drug for 72 h at 37 °C under 5% CO₂ atmosphere. After 72 h, Sybr Green I is added and fluorescence measured at excitation and emission wavelengths of 485 nm and 538 nm, respectively, to quantify relative growth. In the LD₅₀ assay (bottom; see [32]), *P. falciparum* cultures at 2% hematocrit and 2% parasitemia are treated with serial dilutions of CQ in bolus fashion for 6 h and then the drug is completely washed away. After 48 h of additional incubation in the absence of drug, Sybr Green I is added and fluorescence measured as above.

Figure 2.

Re-routing of PfATG8 positive vesicles in iRBC treated with cytocidal dose of CQ. Different cellular effects are seen upon treating malarial parasites with cytostatic (IC_{50}) versus cytocidal (LD_{50}) dosages of CQ. From left to right these panels show transmittance, PfATG8 staining (green), DAPI nuclear staining (blue), and overlay of the three images for methanol fixed iRBC harboring CQS strain HB3 *P. falciparum* (see [28] for methods). CQS *P. falciparum* treated with IC_{50} or 2 x IC_{50} levels of CQ (top two rows) exhibit somewhat punctate cytosolic distribution of the autophagy marker protein ATG8 that partially overlaps with the apicoplast (not shown, see [28]), whereas parasites treated at higher cytocidal dose (bottom two rows) show more intense and expanded punctate PfATG8 staining, including near the periphery of the parasite [28]. CQR parasites show a dampened PfATG8 response upon treatment with LD_{50} levels of CQ that has recently been linked to CQR^{CC} and that is not due to PfCRT mutations [28]. Scale bar = 5 μ m.