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Reduced Digestive Vacuolar Accumulation of Chloroquine is Not Linked to Resistance to Chloroquine Toxicity

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Abstract

Chloroquine (CQ) accumulation studies in live malaria parasites are typically done at low nM [CQ], and definition of CQ resistance (CQR) has been via growth inhibition assays vs low dose CQ (*i.e.* via IC₅₀ ratios). These data have led to the near universally accepted idea that reduced parasite CQ accumulation is the underlying basis of CQR. Surprisingly, when quantifying CQR via cytotoxic CQ activity and examining CQ accumulation at medically relevant LD₅₀ doses, we find reduced CQ accumulation is not the underlying cause of CQR.

CQR¹ is an horrific problem [1]. For 40 years it has been recognized that, when incubated at a fixed low dose, CQR malarial parasites accumulate less CQ in similar time relative to CQ sensitive (CQS) [2]. This crucial observation birthed an entire field concerned with elucidating the molecular basis of this phenomenon [2–13]. Yet, only very recently have growth inhibitory (cytostatic, CS) vs. toxic (cytotoxic or cytotoxic, CT) functions of CQ been quantified for different life stages of *Plasmodium falciparum*, and compared for CQS vs CQR parasites [7]. CT assays are still in their infancy [7,8] yet CT effects are more relevant for clearing parasites from the human host since plasma CQ concentrations are $\geq 1 \mu\text{M}$ [9,10]. Not coincidentally, a 1 μM , 1–2 h duration bolus dose of CQ kills >95% of CQS parasites [7]. Correspondingly, in a human receiving CQ parasitemia drops precipitously within hours [9,10]. Residual parasites left in distal vascular beds, the liver, and other sites are then presumably held in check by the cytostatic activity of CQ, as plasma [CQ] decreases.

CQR parasites show ≥ 7 fold higher LD₅₀ relative to CQS when CQ cytotoxic activity is quantified [7] and we define this as resistance to CQ cytotoxicity (CQR^{CT}). However, for whatever reasons CQR has almost always been quantified by ratioing IC₅₀; that is, by comparing long term (48–96 h) growth of CQS vs CQR parasites in the constant presence of low [CQ] (1–100 nM). In this format, CQR parasites typically show ≥ 10 -fold resistance. Formally, this is resistance to the cytostatic actions of CQ (CQR^{CS}). Presumably because CS measurements are so common, accumulation of CQ into live CQS vs CQR malarial parasites

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SUPPORTING INFORMATION PARAGRAPH

Experimental procedures and supplementary figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

¹Abbreviations: CQR, chloroquine resistance/resistant; CQ, chloroquine; CQS, chloroquine sensitive; LD₅₀, 50% lethal concentration; CQR^{CT}, resistance to CQ toxicity; CQR^{CS}, resistance to CQ growth inhibition; IC₅₀, 50% inhibitory concentration; PfCRT, *Plasmodium falciparum* chloroquine resistance transporter; CAR, cellular accumulation ratio; [CQ]_{ex}, external CQ concentration; [CQ]_{in}, internal CQ concentration; DV, digestive vacuole; SDCM, spinning disk confocal microscopy; HBSS, Hank's balanced salt solution.

is almost always measured at low cytostatic dose (1–10 nM). At these concentrations net cellular CQ accumulation differs 2–10 fold for CQR vs. CQS parasites ([6], Fig. S1). Reduced cellular accumulation for CQR parasites at these fixed low doses has led to the near universally accepted conclusion that the biochemical basis of CQR is reduced CQ uptake and/or increased CQ efflux. Rigorously however, this is valid only for CQR^{CS}.

As external CQ ($[CQ]_{ex}$) is raised in CQ accumulation experiments, the fold difference in cellular accumulation for CQR vs CQS parasites drops, and is <2 fold as $[CQ]_{ex}$ approaches 1 μ M [2,3]. Since plasma $[CQ]$ is $\geq 1 \mu$ M, since malaria chemotherapy kills parasites and does not merely prevent their growth, and since CQR evolved in the human host and not in the laboratory, we were curious to see if reduced CQ accumulation is associated with CQR^{CT}.

Recently we emphasized CT vs. CS functions of CQ, quantified LD₅₀ and IC₅₀, respectively, and showed that *P. falciparum* Chloroquine Resistance Transporter (PfCRT) mutations confer both CQR^{CT} and CQR^{CS} [7]. We found that a ~750 nM bolus dose of CQ did not kill any strain Dd2 CQR parasites, whereas a ~250 nM bolus dose killed ~50% of strain HB3 CQS parasites. Similar observations have been made by others [11], however, quantification of LD₅₀ has only been done recently [7]. We compared accumulation of 750 nM CQ in Dd2 parasites vs. accumulation of 250 nM CQ in HB3 and found Dd2 accumulated similar levels of drug (not shown, see [6]). That is, the higher dose led to similar internal $[CQ]$ for CQR parasites relative to CQS incubated at lower drug, yet substantially fewer CQR parasites were killed at this higher dose, relative to CQS treated at the lower. This appears to contradict the idea that CQR is due to reduced CQ accumulation. Moreover, at 400 nM CQ_{ex} , CQS parasites accumulate to 55 μ M $[CQ]_{in}$ and 2/3 of the parasites are killed, whereas at 1.2 μ M CQ_{ex} , CQR parasites accumulate to 65 μ M yet only 1/3 are killed [6,7].

That is, we suspected CQR parasites may accumulate *more* drug yet still show CQR^{CT}.

To analyze this in depth we quantified accumulation of ³H-CQ over a wide range of $[CQ]_{ex}$ for both HB3 and Dd2 parasites. Fig. 1A shows raw data, whereas panels 1B and 1C show calculated Cellular Accumulation Ratio (CAR) for each strain and Δ CAR (HB3 CAR/Dd2 CAR). Similar data can be found in many publications (e.g.[2–6]), but with a few exceptions they are obtained at only one $[CQ]_{ex}$. To our knowledge, this is the first measurement of CAR and Δ CAR over this wide a $[CQ]_{ex}$ range using data from a consistent set of assay conditions in one laboratory. The data show that CAR is lower for CQR parasites at all values and that Δ CAR remains relatively constant over 3 orders of magnitude of $[CQ]_{ex}$. Plots of CQS or CQR CAR and Δ CAR, from many earlier experiments, are shown in Supplemental Information (Fig. S1), and demonstrate that data in Fig. 1 lie well within the range reported earlier.

Knowing $[CQ]_{in}$ vs $[CQ]_{ex}$ we then compared CS (Fig. 2A,C) and CT (Fig. 2B,D) data for CQS (circles) and CQR (triangles) parasites vs. external (A,B) and internal (C,D) levels of drug. As shown earlier [7] CQR parasites are resistant via both assays. When growth or survival are plotted vs *internal* $[CQ]$ it is clear that CQ pharmacology differs for CQS vs CQR parasites. Meaning, the CT dose effect curves have conspicuously different slope for CQS (circles) vs CQR (triangles) parasites (~2-fold different, cf. Fig 2D). In C and D, we show multiple x axes that denote $[CQ]_{in}$ for CQS and CQR parasites (top x axis) and then the $[CQ]_{ex}$ at which these plateau $[CQ]_{in}$ are obtained for CQS (middle x axis) and CQR (bottom x axis) parasites.

CS assays require several days of growth in the presence of drug, so it is difficult to extrapolate measured plateau $[CQ]_{in}$ to what is present intracellularly days later. Also, $[CQ]_{in}$ differences at a given level of CQS vs CQR parasite growth are relatively small. However, the plateau $[CQ]_{in}$ we measure correspond precisely to $[CQ]_{in}$ used in the CT assay [7] so direct comparison between CQS vs CQR parasite CQ toxicity at a given $[CQ]_{in}$ is possible.

Clearly then, CQR parasites can accumulate more drug yet still show CQR^{CT}. For example, 40% of CQR parasites with [CQ]_{in} = 325 μ M survive once the drug is washed away (see methods and [7]) whereas only 10% of CQS parasites with [CQ]_{in} = 125 μ M survive (Fig. 2D).

Recent work shows that PfCRT transports CQ [6,12,13] and that this likely contributes to CQR^{CS}. But even if reduced CQ accumulation is part of the explanation for CQR^{CS} at low levels of drug, reduced accumulation is not necessarily the explanation for resistance to the cytotoxic effects of CQ (CQR^{CT}). However, since a chief target for CQ is heme within the digestive vacuole (DV), at high doses of CQ used in shorter term exposure CT assays, perhaps less CQ is still found within the DV for CQR parasites due to PfCRT drug transport [6,12,13]. That is, perhaps “effective dose” at the site of action is still reduced. To test this, we measured DV concentrations of a recently validated fluorescent CQ reporter (NBD-CQ; see [6,12]) using rapid Spinning Disk Confocal Microscopy (SDCM) for live parasites under perfusion [7]. In parallel to similar cellular accumulation, Dd2 parasites perfused with 750 nM NBD-CQ accumulate similar drug probe within the DV relative to HB3 parasites perfused with 250 nM (Fig. 3G).

Targeting cell cycle regulatory proteins leads to cytostatic effects, whereas promoting apoptotic or necrotic pathways is the basis of cytotoxicity. The initial distinction between static vs toxic effects is made by comparing low dose continuous drug exposure (via a growth inhibition assay to determine IC₅₀) to high(er) dose bolus exposure (via a cytostatic or cytotoxic assay to determine LD₅₀). For tumor cells, clear molecular markers for distinguishing between cytostasis vs cytotoxicity are available. For malarial parasites, we can find only one previous laboratory study that distinguishes cytostatic vs cytotoxic drug effects [8], even though discussion of this is relatively common in the clinical malaria literature [9,10]. We can only speculate as to why this essential distinction has not been emphasized at all when studying CQR pathways, but perhaps it is related to the fact that current cytotoxic assays for malarial parasites are enormously tedious, time consuming, and relatively expensive [7,8].

Induction of apoptosis in malarial parasites is a controversial topic [14,15] and no agreed upon quantification of apoptotic death is available [16–17]. Direct quantification of necrotic death is essentially impossible in merozoite red cell culture since necrotized cells disappear from culture. We have quantified cytotoxic effects of CQ for live malarial parasites by eliminating cytostatic effects as an explanation for reduced propagation following bolus administration of CQ [7]. Although extremely tedious, this allowed us to conclude that a 1 – 2 h bolus dose of 750 nM CQ is not at all cytotoxic to CQR (strain Dd2) parasites, but that the CQ LD₅₀ is 250 nM for CQS (HB3) parasites. Here, we show that at 750 nM external ³H-CQ, Dd2 parasites accumulate the same amount of drug relative to that accumulated by HB3 incubated at 250 nM drug. Using a validated fluorescent CQ probe [6,12] and fast 4D imaging via SDCM (see [7] and Supplemental), we show that intra-DV accumulation is the same as well. This leads to the surprising yet simple conclusion that CQR parasites do not need to accumulate less CQ in order to show CQR^{CT}. Further inspection of the data show that CQR parasites can even accumulate *more* drug relative to CQS and *still* exhibit CQR^{CT}. We propose that a full understanding of the CQR mechanism requires careful comparison of CQ accumulation at higher (plasma) levels and that comparing accumulation at only one fixed, sub-IC₅₀ dose can be misleading.

Peak plasma [CQ] is frequently $\geq 1 \mu$ M but is also variable among malaria patients. Individuals infected with either CQS or CQR parasites may achieve several fold different plasma concentration of CQ (e.g. 400 nM vs 1.2 μ M). It is imperative then to recognize that this could be the circumstance under which resistance to CQ evolved. That is, variable CQ toxicity pressure, not only variable CQ growth inhibitory pressure, could have contributed to the emergence of CQR (see also [9]), which is now recognized to be a multi-genetic trait. If this notion is entertained, then elucidating the mechanism that confers CQR^{CT} is at least as relevant

for circumventing CQR in the field, relative to the mechanism that confers CQR^{CS}. Reduced drug accumulation may be central to CQR^{CS}, but it is not the explanation for CQR^{CT}. For the past 20 years, have we been studying all that is particularly relevant? To quote [3], "...higher intracellular [CQ] are needed to kill resistant strains." Data in this paper extends and underscores that prophetic yet infrequently cited conclusion.

We propose distinction between the molecular details of CQR^{CS} vs CQR^{CT} is essential. We predict this will uncover additional CQ targets that may or may not reside within the parasite DV. Indeed, previously [7] we found that schizonts (wherein DV metabolism of heme to hemozoin has stopped) are nearly as susceptible to CQ cytotoxicity as are trophozoites with active DV heme metabolism. If additional, non-DV, non-heme targets are indeed relevant for CQ toxicity, then non-DV mediated mechanisms of resistance must be relevant for CQR^{CT}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Internal CQ concentrations for synchronized HB3 (○) and Dd2 (Δ) iRBC incubated in varying external ^3H -CQ concentrations. (B) Cellular Accumulation Ratio (CAR) was calculated from the $[\text{CQ}]_{\text{in}}$ to the $[\text{CQ}]_{\text{ex}}$ for each strain at each concentration. (Inset) Δ CAR was calculated from the ratio of HB3 CAR to Dd2 CAR (□). See supplemental file for methods.

Figure 2.

(A) The sensitivity of HB3 (●) and Dd2 (▲) parasites to the CS effect of CQ was measured in a growth inhibition assay with varying incubating $[CQ]_{ex}$ for 65 h of continuous drug exposure. (B) The CT effect of CQ was measured as survival of HB3 (○) and Dd2 (Δ) parasites at 65 h after a 2 h bolus exposure at the late trophozoite stage with different $[CQ]_{ex}$. Growth inhibition (C), or survival (D) vs. CQ_{in} is shown with multiple x-axes that denote the $[CQ]_{ex}$ at which $[CQ]_{in}$ were obtained for CQS and CQR parasites (calculated from Fig.1A and $[CQ]_{in}$).

Figure 3.

NBD-CQ accumulation in the DV via SDCM. DIC (A), fluorescence (B), and fluorescence overlay (C) images in Dd2 parasites. Cells were perfused with 750 nM NBD-CQ in gas-balanced HBSS. X-, y-, z-axes in (B) correspond to the same axes in (D–F) which show 3-D reconstruction of DV fluorescence for 1 parasite. (G) Internal NBD-CQ in HB3 and Dd2 DV was calibrated as in [6] and represents the mean from >15 parasites (error bars were calculated from the standard error of the mean). Scale bar= 1 μm .