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Rapid Report

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 conducted at low nanomolar CQ concentrations, and definition of CQ resistance (CQR) has been via growth inhibition assays versus low-dose CQ (i.e., via IC_{50} ratios). These data have led to the nearly universally accepted idea that reduced parasite CQ accumulation is the underlying basis of CQR. Surprisingly, when quantifying CQR via cytocidal CQ activity and examining CQ accumulation at medically relevant LD_{50} doses, we find reduced CQ accumulation is not the underlying cause of CQR.

CQR[1] is a 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 a similar amount of time relative to CQ sensitive (CQS) (*2*). This crucial observation birthed an entire field concerned with elucidation of the molecular basis of this phenomenon (*2*−*13*), yet only very recently have growth inhibitory [cytostatic (CS)] versus toxic [cytocidal or cytotoxic (CT)] functions of CQ been quantified for different life stages of *Plasmodium falciparum* and compared for CQS versus 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 ≥1 μM (*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, the level of 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 the plasma CQ concentration decreases.

CQR parasites exhibit a ≥7-fold higher LD_{50} relative to CQS when CQ cytocidal 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 using IC_{50} ratios, that is, by comparing long-term (48−96 h) growth of CQS versus CQR parasites in the constant presence of low CQ concentrations (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 in live CQS versus CQR malarial parasites is almost always measured at low cytostatic doses (1−10 nM). At these concentrations, net cellular CQ accumulation differs 2−10-fold for CQR versus CQS parasites (*6*) (Figure S1 of the Supporting Information). Reduced cellular accumulation for CQR parasites at these fixed low doses has led to the nearly universally accepted conclusion that the biochemical basis of CQR is reduced CQ uptake and/or increased CQ efflux. Rigorously, however, this is valid for only CQR^{CS}.

As the external CQ concentration ($[CQ]_{ex}$) is increased in CQ accumulation experiments, the fold difference in cellular accumulation for CQR versus CQS parasites drops and is <2-fold as $[CQ]_{ex}$ approaches 1 μM (*2*, *3*). Since the plasma CQ concentration is ≥1 μM, 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 versus CS functions of CQ, quantified LD_{50} and IC_{50}, respectively, and showed that *P. falciparum* chloroquine resistance transporter (PfCRT) mutations confer both CQR^{CT} and CQR^{CS} (*7*). We found that an ∼750 nM bolus dose of CQ did not kill any strain Dd2 CQR parasites, whereas an ∼250 nM bolus dose killed ∼50% of strain HB3 CQS parasites. Similar observations were documented by others (*11*); however, quantification of LD_{50} has only been done recently (*7*). We compared accumulation of 750 nM CQ in Dd2 parasites versus accumulation of 250 nM CQ in HB3 and found similar levels of drug accumulated in Dd2 [not shown (see ref *6*)]. That is, the higher dose led to similar internal CQ concentrations for CQR parasites relative to CQS incubated at lower drug levels, yet substantially fewer CQR parasites were killed at this higher dose, relative to CQS treated at the lower dose. This appears to contradict the idea that CQR is due to reduced CQ accumulation. Moreover, at a $[CQ]_{ex}$ of 400 nM, CQS parasites accumulate to a $[CQ]_{in}$ of 55 μM and two-thirds of the parasites are killed, whereas at a $[CQ]_{ex}$ of 1.2 μM, CQR parasites accumulate to 65 μM yet only one-third 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 [^{3}H]CQ over a wide range of $[CQ]_{ex}$ for both HB3 and Dd2 parasites. Panel A of Figure 1 shows raw data, whereas panels B and C show calculated cellular accumulation ratios (CARs) for each strain and ΔCAR (HB3 CAR/Dd2 CAR). Similar data can be found in many publications (e.g., refs (*2*−*6*)), but with a few

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[1]Abbreviations: CQR, chloroquine resistance or resistant; CQ, chloroquine; CQS, chloroquine sensitive; LD_{50}, 50% lethal concentration; CQR^{CT}, resistance to CQ toxicity; CQR^{CS}, resistance to CQ growth inhibition; IC_{50}, 50% inhibitory concentration; PfCRT, *P. 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.

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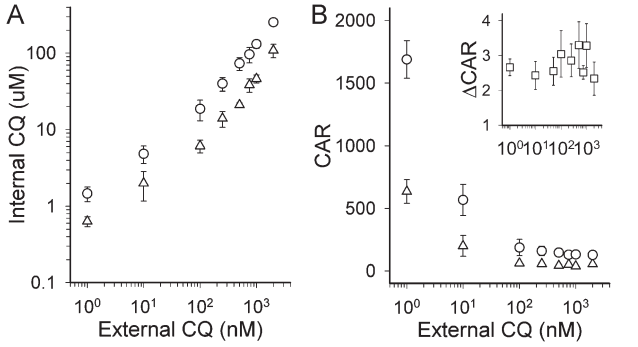

FIGURE 1: (A) Internal CQ concentrations for synchronized HB3 (○) and Dd2 (△) iRBC incubated in varying external [^{3}H]CQ concentrations. (B) Cellular accumulation ratio (CAR) calculated from the $[CQ]_{in}$ and $[CQ]_{ex}$ values for each strain at each concentration. The inset gives the ΔCAR calculated from the ratio of HB3 CAR to Dd2 CAR (□). See the Supporting Information for methods.

exceptions, they are obtained at only one $[CQ]_{ex}$. To the best of our knowledge, this is the first measurement of CAR and ΔCAR over a $[CQ]_{ex}$ range this wide 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 ΔCAR remains relatively constant over a $[CQ]_{ex}$ range of 3 orders of magnitude. Plots of CQS or CQR CAR and ΔCAR, from many earlier experiments, are shown in the Supporting Information (Figure S1) and demonstrate that data in Figure 1 lie well within the range reported earlier.

Knowing $[CQ]_{in}$ versus $[CQ]_{ex}$, we then compared CS (Figure 2A,C) and CT (Figure 2B,D) data for CQS (circles) and CQR (triangles) parasites versus external (Figure 2A,B) and internal (Figure 2C,D) levels of drug. As documented previously (*7*), CQR parasites are resistant via both assays. When growth or survival is plotted versus internal [CQ], it is clear that CQ pharmacology differs for CQS versus CQR parasites, meaning that the CT dose effect curves have conspicuously different slopes for CQS (circles) and CQR (triangles) parasites [~2-fold different (cf. Figure 2D)]. In panels 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 $[CQ]_{in}$ values are obtained for CQS (middle *x* axis) and CQR (bottom *x* axis) parasites.

CS assays require growth for several days in the presence of drug, so it is difficult to extrapolate measured plateau $[CQ]_{in}$ values to what is present intracellularly days later. Also, $[CQ]_{in}$ differences at a given level of CQS versus CQR parasite growth are relatively small. However, the plateau $[CQ]_{in}$ values we measure correspond precisely to $[CQ]_{in}$ values used in the CT assay (*7*), so direct comparison between CQS and 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 a $[CQ]_{in}$ of 325 μM survive once the drug is washed away (see methods and ref *7*), whereas only 10% of CQS parasites with a $[CQ]_{in}$ of 125 μM survive (Figure 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 higher CQ doses used in shorter-term exposure CT assays, perhaps less CQ is still found within the DV for CQR

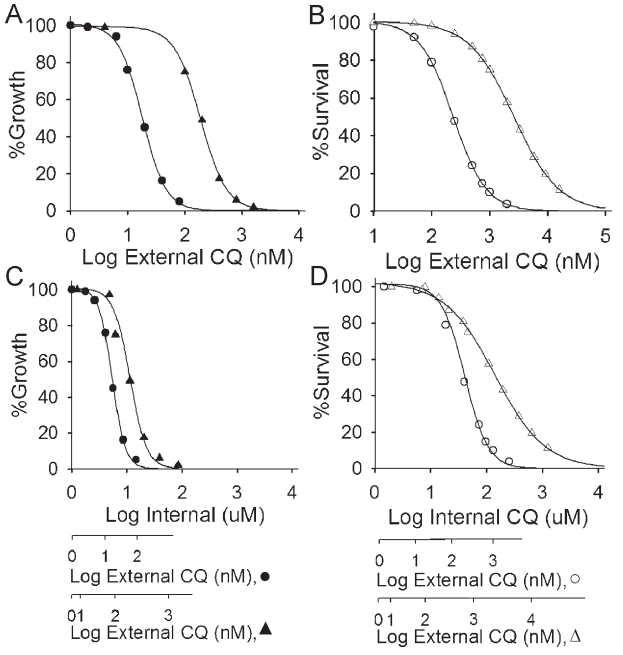

FIGURE 2: (A) Sensitivity of HB3 (●) and Dd2 (▲) parasites to the CS effect of CQ measured in a growth inhibition assay with varying incubating $[CQ]_{ex}$ values with continuous drug exposure for 65 h. (B) CT effect of CQ measured as survival of HB3 (○) and Dd2 (△) parasites at 65 h after a 2 h bolus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

parasites due to PfCRT drug transport (*6*, *12*, *13*). That is, perhaps the "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 refs *6* and *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 a similar level of drug probe within the DV relative to HB3 parasites perfused with a concentration of 250 nM (Figure 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 and toxic effects is made by comparing low-dose continuous drug exposure (via a growth inhibition assay to determine IC_{50}) to higher dose bolus exposure (via a cytotoxic or cytocidal assay to determine LD_{50}). For tumor cells, clear molecular markers for distinguishing between cytostasis and cytotoxicity are available. For malarial parasites, we can find only one previous laboratory study that distinguishes cytostastic and cytocidal drug effects (*8*), even though discussion of this is relatively common in the clinical malaria literature (*9*, *10*). We can only speculate about why this essential distinction has not been emphasized at all when CQR pathways have been studied; perhaps it is related to the fact that current cytocidal 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 consensus 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

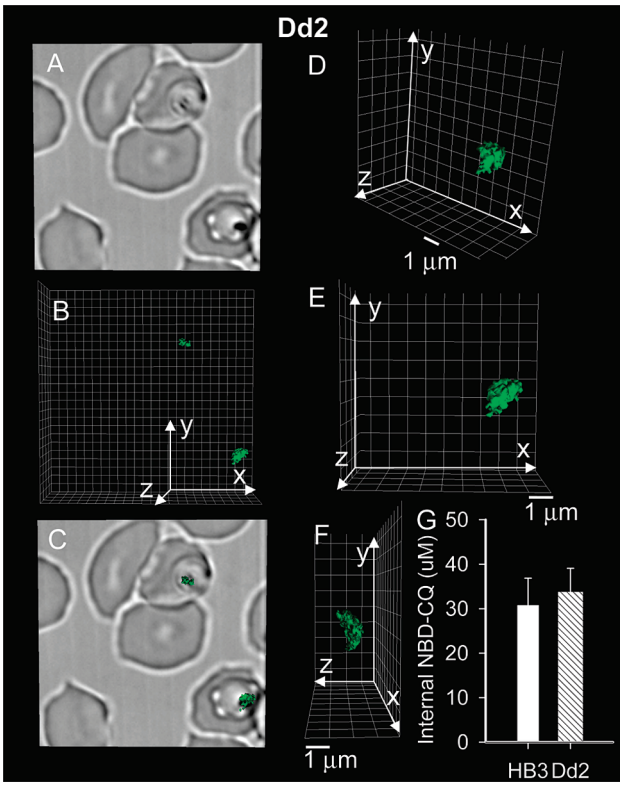

FIGURE 3: Accumulation of NBD-CQ in the DV via SDCM. DIC (A), fluorescence (B), and fluorescence overlay (C) images in Dd2 parasites. Cells were perfused with 750 nM NBD-CQ in gas-balanced HBSS. *x*, *y*, and *z* axes in panel B correspond to the same axes in panels D−F which show three-dimensional reconstruction of DV fluorescence for one parasite. (G) Internal NBD-CQ in HB3 and Dd2 DV calibrated as described in ref *6* representing the mean from >15 parasites (error bars were calculated from the standard error of the mean). The scale bar is 1 μm.

effects as an explanation for reduced propagation following bolus administration of CQ (*7*). Although extremely tedious, this process 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 LD_{50} is 250 nM for CQS (HB3) parasites. Here, we show that at an external [^{3}H]CQ concentration of 750 nM, 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 four-dimensional imaging via SDCM (see ref *7* and the Supporting Information), 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 to show CQR^{CT}. Further inspection of the data shows 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-IC_{50} dose can be misleading.

The peak plasma CQ concentration is frequently ≥1 μM but is also variable among malaria patients. Individuals infected with either CQS or CQR parasites may achieve several-fold different plasma concentrations of CQ (e.g., 400 nM vs 1.2 μM). 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 ref *9*), which is now recognized as a multigenetic 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 ref *3*, "... higher intracellular [CQ] are needed to kill resistant strains." Data in this paper extend and underscore that prophetic yet infrequently cited conclusion.

We propose a distinction between the molecular details of CQR^{CS} and 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 (in which 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}.

SUPPORTING INFORMATION AVAILABLE

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

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