Pharmacokinetic and Pharmacodynamic Assessment of Hydroxychloroquine in Breast Cancer

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5-ethynyl-2'-deoxyuridine (EdU), area under the curve (AUC),
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Abstract

Hydroxychloroquine (HCQ) is being tested in a number of human clinical trials to determine the role of autophagy in response to standard anticancer therapies. However, HCQ pharmacodynamic responses are difficult to assess in patients and preclinical studies in mouse models are equivocal with regard to HCQ exposure and inhibition of autophagy. Here, pharmacokinetic (PK) assessment of HCQ in non-tumor bearing mice following intraperitoneal (IP) dosing established 60 mg/kg as the human equivalent dose of HCQ in mice. Autophagy inhibition, cell proliferation, and cell death were assessed in 2D cell culture and 3D tumor organoids in breast cancer. Mice challenged with breast cancer xenografts were then treated with 60 mg/kg HCQ via IP dosing and subsequent PK and pharmacodynamic (PD) responses were assessed. Although autophagic flux was significantly inhibited in cells irrespective of autophagy dependency status, autophagy dependent tumors had decreased cell proliferation and increased cell death at earlier time points compared to autophagy independent tumors. Overall, this study shows that 2D cell culture, 3D tumor organoids, and in vivo studies produce similar results and in vitro studies can be used as surrogates to recapitulate in vivo antitumor responses of HCQ.
Significance Statement

Autophagy dependent tumors, but not autophagy independent tumors, have decreased cell proliferation and increased cell death following single agent hydroxychloroquine treatment. However, hydroxychloroquine causes decreased autophagic flux regardless of autophagy status, suggesting its clinical efficacy in the context of autophagy inhibition.
Introduction

Macroautophagy (“autophagy”) is a normal cellular process in which cell components are taken up by vesicles called autophagosomes that fuse with lysosomes where components are degraded and recycled. Autophagy is induced by metabolic stress and in cells undergoing remodeling or differentiation (De Duve and Wattiaux 1966, Yang and Klionsky 2010). It plays important roles in many diseases including cancer by enhancing survival and therapy resistance (Duffy et al., 2015). Hydroxychloroquine (HCQ) is a lipophilic weak base that prevents lysosomal acidification by sequestering in lysosomes and raising their pH, in turn blocking the fusion of autophagosomes to lysosomes (Mauthe et al., 2018). HCQ is commonly used as an autophagy inhibitor in cancer clinical trials because it is already FDA approved, has a low toxicity profile, and is inexpensive (Cheong 2015, Thorburn et al., 2014, Ruiz-Irastorza et al., 2010, Manic et al., 2014).

HCQ pharmacokinetic (PK) parameters have been well characterized in humans. Following oral administration, HCQ is almost completely and rapidly absorbed in the gastrointestinal tract and is about 75% bioavailable (Lim et al., 2009, Estes et al., 1987, Carmichael et al., 2003). Approximately 50% of HCQ is bound to plasma proteins and extensively sequesters in tissue (Lim et al., 2009, Tett 1993, Ducharme and Farinotti 1996). It partitions into red blood cells and binds strongly to heme proteins, leading to a high volume of distribution and a prolonged half-life of 40-50 days (Tett 1993, Ducharme and Farinotti 1996, Furst 1996). HCQ is dealkylated in the liver by cytochrome P450 enzymes into active metabolites including desethylhydroxychloroquine (DHCQ) (Lim et al., 2009, Ducharme and Farinotti 1996, Cardoso and Bonato 2009). HCQ clearance is
divided nearly equally between hepatic and renal mechanisms (Ducharme and Farinotti 1996).

While HCQ PK is well described in humans, the pharmacodynamics (PD) associated with HCQ exposure is difficult to assess. There are over 90 cancer clinical trials (ClinicalTrials.gov) that currently or have previously investigated the effects of HCQ alone and in combination with chemo-, immuno-, and/or radiation therapies. Trials use between 200 and 1200 mg of HCQ daily but the number of patients with partial or stable responses is variable (Wolpin et al., 2014, Boehrer et al., 2008, Goldberg et al., 2012, Zeh et al., 2020). Further, when assessing patients for LC3-II or p62 accumulation as markers for autophagy inhibition, autophagy inhibition is achievable with HCQ but inconsistent across patient populations (Wolpin et al., 2014, Zeh et al., 2020, Kimmelman 2011). Monitoring HCQ-driven correlative PD endpoints has not been successful at doses less than the maximum tolerated dose (Mahalingam et al., 2014). Given the large number of clinical trials using HCQ to target cancer, it is important to understand how HCQ exposure modulates cellular responses.

Mice are a widely used pre-clinical cancer research model system and have been used in numerous autophagy-modulation studies (Frese and Tuveson 2007, Amaravadi et al., 2007, Komatsu et al., 2007, Duran et al., 2008, Yang et al., 2011). However, many human tumors do not grow in immunodeficient mouse models which makes it impossible to study certain cancers using this model. For instance, triple negative breast cancers grow in immunodeficient mouse models, but most luminal estrogen receptor positive and human epidermal growth factor receptor 2 positive breast xenografts cannot grow (Sflomos et al., 2016, Dobrolecki et al., 2016). However, there are reports of growing
these subtypes in three-dimensional cell culture as organoids (Li et al., 2019, Campaner et al., 2020) which is important since organoids have been shown to more closely mimic \textit{in vivo} responses compared to 2D cell culture (Bleijs et al., 2019, Yang et al., 2020). In addition, tumor organoids can accurately recapitulate \textit{in vivo} drug responses, simplify experiments, and save time and money (Bleijs et al., 2019, Fan et al., 2019, Sasmita and Wong 2018).

Although HCQ is used clinically as an autophagy inhibitor to prevent cancer cell survival and therapy resistance, the efficacy of HCQ is not known in humans and has not been established in mouse models. Here, the human equivalent dose (HED) of HCQ reflected in human clinical trial data was determined in non-tumor bearing mice and the response to autophagy inhibition was assessed. Cell proliferation, death, and autophagic flux following HCQ were then evaluated in breast cancer cell lines that are variably dependent on autophagy \textit{in vitro}. Mice with human breast xenografts were treated with the HED to characterize pharmacodynamic responses \textit{in vivo}. Understanding HCQ PK and PD allows for comparison to human PK reported in recent trials and subsequently the PD associated with autophagy inhibition.
Materials and Methods

Mouse treatments

Protocols for animal studies were approved by the Institutional Animal Care and Use Committee at Colorado State University. Six- to eight-week-old non-tumor bearing female BALB/c mice (Charles River NCI, Frederick, MD) were treated with a single intraperitoneal dose (IP) of 20, 40, or 80 mg/kg HCQ (Sigma-Aldrich, H0915). Tissues and whole blood were collected at 3, 6, 12, 24, 48 and 72 hours. For tumor-bearing mice, MDA-MB-231 and MDA-MB-468 breast cancer cell lines were implanted in the third mammary fat pads of 6- to 8-week-old female athymic nude mice (Charles River NCI, Frederick, MD). MCF7 breast cancer cells were implanted in the third mammary fat pads of 6- to 8-week-old ovariectomized female athymic nude mice (Charles River NCI, Wilmington, MA) and supplemented with 0.18 mg 17β-estradiol 60-day slow-release tablets (Innovative Research of America, SE-121). Tumors were grown to between 150 and 400 mm$^3$ then treated with a single IP dose of 60 mg/kg HCQ or once daily with a 60 mg/kg HCQ IP dose for one week. Tissues, tumors, and whole blood were collected at 3, 6, 12, 24, 48, and 72 hours following the single dose or 24 hours following the last daily dose.

Drug Measurements

Levels of HCQ and DHCQ in whole blood and tissues were determined via a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay as previously described (Barnard et al., 2014). Pharmacokinetic parameters were calculated by non-compartmental analysis (NCA) using Phoenix WinNonlin (v 8.3.3.33) (Certara, Princeton, NJ).
Western blot analysis

Tissues and tumors were flash frozen in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.5, 100 mM Na-orthovanadate [Alexis Biochemicals, 400-032-G025], 34.8 µg/mL PMSF [Fluka Biochemica, 78830], and 1x protease inhibitor cocktail [Roche, 11836153001]). Samples were homogenized for 20 s and sonicated with three 3 second bursts on ice then centrifuged at 14,000 rpm for 10 min at 4°C. Supernatant was collected and protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, 23225). Twenty µg of protein was resolved on a 4-20% SDS-polyacrylamide gel (Bio Rad, 4568095) and transferred onto PVDF membranes (Bio Rad, 1704272). Membranes were blocked in 2.5% nonfat dry milk in tris-buffered saline/Tween 80 (TBST) (10 mM Tris pH 7.5, 100 mM NaCl, and 0.1% Tween 80 [Fisher Chemicals, BP338-500]) for 1 hour at room temperature. Blots were probed with polyclonal anti-LC3B antibody (Novus Biologicals, NB100-2220) at 1:1,000, monoclonal anti-α-tubulin antibody (Sigma-Aldrich, T5168) at 1:5,000, monoclonal anti-p62/SQSTM1 antibody (Novus Biologicals, H00008878-M01) at 1:1,000, or monoclonal anti-GAPDH antibody (Novus Biologicals, NB300-221) at 1:1,000 in blocking solution overnight at 4°C. Blots were washed three times in TBST then incubated at room temperature for 1 hour with HRP conjugated secondary antibodies anti-rabbit-HRP (Pierce, 31460) at 1:2000 or anti-mouse-HRP (Pierce, 31430) at 1:2,000 for p62, 1:5,000 for α-tubulin, or 1:10,000 for GAPDH in blocking solution. Alpha-tubulin or total protein were used as housekeeping controls. Blots were washed three times with TBST. Immunodetection was carried out using SuperSignal West Dura (Thermo Scientific, 34075) and imaged in a ChemiDoc.
XRS+ (Bio Rad, Hercules, CA) using Image Labs version 3.0 software. Densitometry quantification of LC3 and p62 levels was performed by NIH ImageJ software (http://rsb.info.nih.gov/nih-image/) or by Image Labs total protein normalization.

**Immunofluorescence analysis**

Tumor samples were put into 5% PLP solution for 24 hours at 4°C then placed in 30% sucrose solution for 24 hours at 4°C. Samples were fixed in 4% paraformaldehyde then placed in OCT and cryosectioned at 5 μm. Sample slides were rehydrated in PBS plus 0.05% Tween 20 (PBST) then washed with 0.1M glycine/PBS to reduce tissue autofluorescence. Non-specific binding was blocked with 5% donkey serum in immunofluorescence (IF) buffer (0.2% Triton X-100, 0.1% BSA, 0.05% Tween 20, PBS) for 30 min at room temperature. Sections were probed for primary anti-Ki67 (Cell Signaling Technology, 9027) diluted 1:400, primary anti-cleaved caspase-3 (Cell Signaling Technology, 9664) diluted 1:200, or rabbit IgG isotype control (Cell Signaling Technology, 3900) diluted to same the concentration as other primary antibodies in IF buffer for 1 hour at room temperature. Slides were washed 3 times in PBST then incubated with Cy3 secondary antibody (Jackson ImmunoResearch, 711-165-152 lot #142318) diluted 1:200 in IF buffer for 30 min at room temperature. Three washes were performed then slides were counterstained with filtered DAPI working solution (Thermo Scientific, 62248) diluted 1:500 in PBS for 15 min at room temperature. Slides were coverslipped with ProLong Diamond Antifade Mountant (Invitrogen, P36961) and imaged using an Olympus IX83 confocal microscope and Hamamatsu digital camera.
**Cell culture**

Cell lines were validated mycoplasma free and maintained at 37°C and 5% CO₂ in DMEM (Corning, 10-017-CV) buffered with 10 mM HEPES (Fisher Scientific, BP-299-100) and supplemented with 10% FBS (Peak Serum, PS-FB3), 1% penicillin-streptomycin (Corning, 30-002-CI), and 1% sodium pyruvate (Corning, 25-000-CI). MCF7 media was also supplemented with 10 μg/mL insulin (Fisher Scientific, 12585014). Cells were used for no more than 20 passages.

**Organoid culture**

To form organoids, cells were grown on Cultrex 3D Culture Matrix RGF Basement Membrane Extract (BME) (R&D Systems, 344501001). Briefly, 100 μL/cm² BME was plated and allowed to solidify at 37°C for a minimum of 30 min before plating cells. Cells were grown for 2 days on BME prior to addition of drug.

**Drug sensitivity, cell death, and autophagic flux live cell imaging assays**

For 2D HCQ drug sensitivity, cell death, and autophagic flux assays, 3,000 cells were plated per well in a 96-well plate a few hours before drugging. NucLight red-labeled cells were used for drug sensitivity and cell death assays and cells transduced with LC3-mCherry-GFP were used for autophagic flux assays. For HCQ sensitivity assays, cells were treated with HCQ concentrations ranging from 0 to 40 μM HCQ. For the apoptosis assays, cells were plated in buffered MEM (Corning, 10-010-CV) 5 hours before drugging with 10 μM HCQ (equal dosing) and either 8 μM (MDA-MB-231 and MDA-MB-468) or 15 μM (MCF7) HCQ (equal toxicity) combined with 5 μM caspase 3/7 green fluorescent dye.
For cytotoxicity assays, cells were plated in buffered MEM 5 hours before drugging with 10 μM HCQ and either 8 μM (MDA-MB-231 and MDA-MB-468) or 15 μM (MCF7) HCQ combined with 100 nM YOYO green fluorescent dye (Invitrogen, Y3601). For autophagic flux assays, cells were plated in buffered DMEM 5 hours before drugging with 10 μM HCQ and either 8 μM (MDA-MB-231 and MDA-MB-468) or 15 μM (MCF7) HCQ. Plates were imaged once every 24 hr on an IncuCyte Zoom (Essen BioScience). All organoid cultures were performed the same way except 5,000 cells/well were plated on 100 μL/cm² BME two days prior to drugging.

**Cell cycle analysis**

300,000 cells/well were plated in 6-well plates the day before drugging with HCQ. Cells grew for 48 hr following drugging then were trypsinized, spun down, washed once with PBS, then fixed with 70% ethanol and stored at -20°C for up to two weeks. Cells were stained with FxCycle PI/RNase (Life Technologies, F10797) at a concentration of 2 million cells/mL then analyzed on a Gallios flow cytometer (Beckman Coulter). Flow results were analyzed via FlowJo.

**Cell proliferation via 5-ethynyl-2′-deoxyuridine (EdU) staining**

For 2D cell culture experiments, 750,000 cells/plate were plated in 60 mm dishes the day before drugging with HCQ. Once drugged with HCQ, cells grew for 48 hr. One hr (MCF7 and MDA-MB-231) or two hr (MDA-MB-468) before 48 hr time point, cells were treated with 10 μM EdU. Cells were trypsinized, spun down, washed once with cold PBS, then fixed in ice cold 70% ethanol. Fixed cells were stored at 4°C for a minimum of two days.
Cells were re-hydrated in PBS for 1 hr then stained using the Click-iT EdU flow cytometry Alexa Fluor 647 kit (Invitrogen, C10424) by permeabilizing and staining cells. Cells were washed an extra time with PBS just before putting at final concentration of 1 million cells/mL in PBS. 100 μg/mL RNase A (Thermo Scientific, FEREN0531) and 1 μL of a 1:4 dilution from stock Sytox Blue (Thermo Scientific, S34857) was added per 1 million cells. Cells incubated overnight before flow on a Gallios flow cytometer.

For tumor organoid experiments, 100 μL/cm² BME was plated then 30 min later, 750,000 cells were plated. Tumor organoids were allowed to establish for two days before treatment with HCQ. Once drugged with HCQ, cells grew for 48 hr. Four hr before 48 hr time point, cells were treated with 10 μM EdU. Cells were isolated from BME using cell harvesting buffer (R&D Systems, 3448020CH) protocol. Briefly, cells were washed 3 times with PBS, cell harvesting buffer was added to the plate on ice, pipetted up and down, moved to a 15 mL conical, topped with cell harvesting buffer, and shaken on a plate shaker at 650 RPM for 30 min at 4°C. Conical tubes were centrifuged, supernatant was removed, and cells were washed once more with cell harvesting buffer. To break up organoids, cells were incubated with trypsin at room temp for 10 min then fixed with 4% PFA for 15 min. Cells were permeabilized and stained following the Click-iT EdU flow cytometry Alexa Fluor 647. Cells were washed an extra time with PBS then incubated with 100 μg/mL RNase A and 1 μL of a 1:4 dilution from stock Sytox Blue per 1 million cells for a minimum of 30 min before analyzing on a Gallios flow cytometer. All results were analyzed via FlowJo.
**Autophagic flux flow cytometry**

Cells transduced with a LC3-mCherry-GFP reporter were used. Cells were plated at 325,000 cells/well in a 6-well plate for 24 hr, 200,000 cells/dish in a 60 mm dish for 96 hr, or 50,000 (MDA-MB-231) or 100,000 (MCF7, MDA-MB-468) cells/plate for 144 hr in a 60 mm dish, allowed a day to attach and grow, then drugged with HCQ. For use in flow cytometry analysis, control cells were treated with 10 nM bafilomycin A1 (Fisher Scientific, AAJ67193XF) 24 hr prior to the endpoint to completely inhibit autophagy. Cells were trypsinized, washed once with cold PBS, and resuspended in PBS, keeping cold and on ice. Samples were flowed on an Aurora 4 laser (Cytek Biosciences) flow cytometer then data analyzed via FlowJo. To determine the percent of cells using autophagy, the bafilomycin-treated control cells were gated so that 5-10% of cells were positive in an angled, diagonal area to the upper left of the events on a mCherry versus GFP plot. This gate was pasted directly onto all other samples for the same cell line from the same replicate.

**Live/Dead staining**

700,000 cells/plate for 48 hr, 200,000 cells/plate for 96 hr, or 50,000 cells/plate for 144 hr were plated in 60 mm dishes the day before drugging with HCQ. Cells were grown for given time frame then trypsinized, spun down, and washed once with cold PBS. Cells were re-suspended in PBS at a concentration of 1 million cells/mL then 1 μL of a 1:4 dilution from stock Sytox Blue was added. Cells were incubated for 15 min at room temperature then kept on ice until ready to flow and analyzed on a Gallios flow cytometer as soon as possible. Results were analyzed via FlowJo.
**Statistical analysis**

Statistical analysis was performed using Graphpad Prism. Two-way ANOVA using multiple comparisons with Dunnett correction between controls and drug-treated cells at each time point or unpaired two-tailed t-tests between controls and HCQ-treated mice were used to determine statistical significance with *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, or ****p ≤ 0.0001. All error bars are standard deviation.

**Results**

**HCQ exposure is dose dependent in vivo**

HCQ and DHCQ levels were measured in whole blood, liver, gut, kidney, and brain following a single IP dose of 20, 40, or 80 mg/kg HCQ (Figure 1A and Figure S1). Drug levels were dose dependent and concentrations of HCQ and DHCQ decreased over time. Highest concentrations were observed in the liver. In whole blood, there were still detectable levels of HCQ and DHCQ at 72 hours following all doses. HCQ levels were undetectable in liver after 24 hours, but DHCQ levels were detectable at relevant concentrations up to 72 hours. Concentrations of HCQ and DHCQ in the gut were similar over the entire 72 hours, with slightly more DHCQ than HCQ present at all doses 24 hr and later. Kidney drug levels were similar to gut and showed the same trends with higher DHCQ levels following 12 hr. HCQ can cross the blood brain barrier (Amaravadi et al., 2011) and therefore can be detected in the brain. The concentrations of HCQ and DHCQ in the brain were approximately ten-fold less than whole blood, liver, kidney, and gut,
suggesting that HCQ and DHCQ enter the central nervous system to a lesser degree than other tissues.

HCQ PK parameters (Table 1 and Table S1) show that maximal concentration ($C_{\text{max}}$) in whole blood, liver, gut, kidney, and brain increased in a dose dependent manner. Time to maximal concentration ($T_{\text{max}}$) occurred at 3 hours in whole blood, liver, gut, kidney, and brain at all doses except for brain at 80 mg/kg where $T_{\text{max}}$ occurred at 6 hours. Half-lives for whole blood, liver, gut, kidney, and brain were approximately 14.5 hours, 3.5 hours, 17.5 hours, 15 hours, and 48 hours, respectively, regardless of dose. The area under the drug concentration versus time curve (AUC$_{0-\text{inf}}$) increased as dose increased in all tissues, showing exposure of HCQ is dose dependent. DHCQ PK parameters in whole blood (Table S2) and tissues (Table S3) had similar trends as HCQ. $C_{\text{max}}$ and AUC$_{0-\text{inf}}$ increased in a dose-dependent manner. DHCQ $T_{\text{max}}$ occurred mostly between 5 and 9 hours in all tissue. DHCQ half-lives are all slightly longer than HCQ for all tissues except brain where DHCQ is more rapidly eliminated from the brain. Both HCQ and DHCQ clearance is not constant between 20 and 80 mg/kg. Given that AUC$_{0-\text{inf}}$ are not dose proportional from 40 to 80 mg/kg and clearance is concurrently increasing, this signifies non-linear PK with less drug in the blood at higher HCQ concentrations. A potential explanation is an increase in cellular lysosomal volume associated with lysosomal biogenesis triggered by HCQ (Collins et al., 2021) leading to an increase in tissue uptake and subsequent lower blood levels. This explanation is supported by the relationship between dose and AUC for blood vs. tissues where whole blood shows a 1.80 and 3.07-fold increase in AUC when doses are increased 2 and 4-fold whereas tissues (brain, gut, kidney and liver) all show fold increases greater than 2 and 4 with averages of 2.47±0.28
and 4.78 ± 0.71. Thus, although dose and exposure (AUC) are related for all tissues analyzed, tissue accumulation increases greater than the fold dose increase whereas blood increases at a rate lower than the fold increase.

To determine equivalent dose exposure for HCQ in mice compared to humans, area under drug concentration versus time curves from both mice and humans were directly compared since these AUC’s represent exposure in each species. The human equivalent dose (HED) of steady state HCQ concentration was calculated by using the average human patient AUC over 24-hour dosing time period estimated at steady state in humans given an oral 600 mg dose (Rosenfeld et al., 2014) and comparing this to the AUC$_{0-24\text{ hr}}$ corrected with an accumulation factor in mice found in this study. A comparison of the predicted trough level (24hr) at 60 mg/kg in the Balb/c non-tumor bearing mice at steady-state versus the measured steady-state levels in tumor bearing mice at 24 hours showed a predicted value of 227 ng/ml and measured values at 288 ± 75 ng/ml. The HED in mice is 60 mg/kg in whole blood with a standard deviation of 20 mg/kg (Figure 1B), suggesting most doses used for in vivo mouse studies are within the limits of exposure achievable in humans. The AUC$_{0-24\text{ hr}}$ that was measured in the tumor-bearing mice at 60 mg/kg (Table 1) corrected for accumulation at steady-state (1.47-fold) gives a calculated exposure of 32.8 µg•hr/ml, consistent with the exposure range shown in Figure 1B.

**Autophagy inhibition is variable in tissues**

Pharmacodynamic response was assessed by western blot analysis of LC3 and p62 in the liver, kidney, gut, and brain at various time points (Figure 1C and Figure S2).
Autophagy inhibition in the liver was observed at later time points, most noticeably at 48 hr based on the LC3-II/tubulin ratio. Since liver autophagy was inhibited most at later time points, only 24 hr, 48 hr, and 72 hr time points were assessed in the gut, kidney, and brain. The gut and the kidney showed no autophagy inhibition by either LC3 or p62 levels. Brain autophagy inhibition was evident in a few mice at 24 and 48 hr but was not dose related. There was no difference in p62 expression in controls compared to HCQ-treated mice in liver or kidney. Effects of HCQ and/or DHCQ on LC3-II expression over time measured by western blot in the liver demonstrate counterclockwise hysteresis, showing it takes time for enough HCQ and DHCQ levels to build up before an effect is observed (Figure 1D and Figure S3). There is not much difference between the curves when comparing LC3-II levels to either whole blood or liver HCQ concentrations, indicating that HCQ whole blood concentrations can be used as a surrogate for autophagy effects. Further, DHCQ has a similar effect as HCQ on LC3-II levels. When HCQ and DHCQ concentrations are added together to determine total active drug, the hysteresis curves are still counterclockwise and similar to HCQ or DHCQ alone, indicating that although DHCQ is active, it does not significantly change how quickly autophagy is affected and also requires a buildup to achieve an effect.

**HCQ exhibits anti-proliferative effects and decreases autophagic flux but does not induce significant cell death in in vitro 2D culture**

*In vitro* two-dimensional cell culture experiments were performed to validate standard cell culture methods as a sufficient pharmacodynamic model comparable to *in vivo* results and to assess how HCQ is affecting cell growth, death, and long-term autophagy in breast
cancer. To investigate differences in HCQ uptake and response between breast cancers with different sensitivities to autophagy inhibition determined via shRNA knockdowns and responses to chloroquine in vitro and when grown as tumor xenografts (Maycotte et al., 2014), MDA-MB-468 (triple negative basal), MDA-MB-231 (triple negative claudin-low), and MCF7 (luminal) cells, listed from most autophagy sensitive to least autophagy sensitive, were treated with increasing doses of HCQ in buffered DMEM for up to 120 hr. Cells are more affected by lower doses of HCQ at later time points (Figure 2A). The median dose at which half of cells were affected (Dm) at 96 hr was calculated. As expected, autophagy independent MCF7 had the highest Dm of $14.6 \pm 2.9$ μM, while autophagy dependent MDA-MB-231 and MDA-MB-468 Dm’s were lower at $7.8 \pm 3.9$ μM and $7.9 \pm 2.6$ μM, respectively (Figure 2A).

Since cancer patients are generally treated with 200 mg to 400 mg HCQ, the maximal concentration of HCQ achievable in patients is equivalent to 10 μM and 20 μM in vitro based on dosing an average size patient. Therefore, cells were treated with either 10 μM (equal dosing) or their Dm value (equal toxicity, 8 μM for MDA-MB-231 and MDA-MB-468 and 15 μM for MCF7) for the following experiments. Equal dosing means cells were treated with the same concentration of HCQ while equal toxicity means cells were treated at the HCQ concentration in which half of the cells were affected. To determine if the cells were dying via apoptosis, cells were treated with HCQ for 144 hr with a Caspase 3/7 fluorescent dye and monitored in a live cell imaging system. There were no differences in Caspase 3/7 signal between control and HCQ-treated cells in any cell line (Figure 2B and Supplemental Figure 6A), indicating that either cell growth is inhibited or that cells are dying by another cell death pathway. To assess whether HCQ causes death in a
caspase 3/7 independent manner, cell death was measured in a live cell imaging system using the cytotoxicity agent YOYO, a fluorescent dye that is cell membrane impermeable and binds free DNA in solution as an indicator of cell death (Figure 2C and Supplemental Figure 6B). No difference between control and HCQ-treated cell death was detected. However, when cells were stained with a live/dead stain and analyzed via flow cytometry after 48 hr, 96 hr, or 144 hr of HCQ treatment, modest cell death that was time and dose dependent was observed in the autophagy dependent cells (Supplemental Figure 4A). Overall, this indicates that cell death is not the major cellular pharmacodynamic response to HCQ in 2D culture.

To discern whether cells were growth inhibited, cell cycle assays were performed following HCQ dosing for 48 hr. Cell cycle analysis showed that when HCQ concentration is high enough, an increase in G1 and a decrease in G2/M and S is observed (Supplemental Figure 4B). MCF7 cells treated with 10 μM (less than the Dm) have similar percentages of cells to control in these phases compared to 15 μM. In contrast, MDA-MB-231 and MDA-MB-468 cells experienced a decrease in G2/M and an increase in G0/G1 at all HCQ doses used (their Dm and higher). These results were further supported by cell cycle analysis via EdU incorporation. After HCQ treatment for 48 hr, there were more MDA-MB-231 and MDA-MB-468 cells in G1 and less cells in S and G2/M phases of the cell cycle when treated with both HCQ concentrations but MCF7 cells treated with 10 μM were not different from their control counterparts as the MCF7 cells treated with 15 μM were (Figure 2D). Changes in cell cycle were most enhanced in MDA-MB-468, indicating that HCQ more greatly affects cells that are inherently autophagy dependent.
Autophagic flux following HCQ treatment was assessed by flow cytometry using cells transduced with a LC3-mCherry-GFP reporter. This reporter works by tagging autophagosomes. GFP gets quenched in acidic environments. Therefore, when an autophagosome fuses with a lysosome, GFP signal decreases and indicates autophagic flux is occurring. Autophagy was inhibited significantly in the MCF7 cells following as little as 24 hr but autophagy inhibition was not significant in the MDA-MB-231 and MDA-MB-468 until 144 hr or 96 hr, respectively (Figure 2E and Supplemental Figure 6C). Results were similar using a live cell imaging system (Supplemental Figure 4C and Supplemental Figure 6D). This indicates that HCQ has prolonged PD affects that may not be observed at short time points.

**HCQ induces cell death and decreases autophagic flux in tumor organoids**

Cells were grown on a basement membrane matrix to obtain three-dimensional tumor organoids to better recapitulate *in vivo* tumors. Caspase 3/7-dependent cell death was assessed by live cell imaging in the IncuCyte. There was significant caspase 3/7 cell death in MCF7 at 96 hr but by 144 hr, the difference between control and HCQ-treated organoids was no longer significant. No significant caspase 3/7 cell death was observed in MDA-MB-468 organoids but there was caspase 3/7 dependent cell death in MDA-MB-231 organoids treated with HCQ 96 hr and later (Figure 3A and Supplemental Figure 7A). Cytotoxicity measured by YOYO staining showed significant cell death in MDA-MB-231 and MDA-MB-468 organoids at time points as early as 72 hr following HCQ treatment but not significantly in the MCF7 organoids at equal dosing until 120 hr (Figure 3B and
Supplemental Figure 7B), indicating that HCQ causes more cell death in autophagy dependent tumors than autophagy independent tumors.

Cell cycle analysis was assessed in tumor organoids via EdU incorporation (Figure 3C). Results were similar to 2D culture; there was no difference in HCQ-treated MCF7 cells compared to controls but MDA-MB-231 and MDA-MB-468 cells both had significantly less cells in S phase at both HCQ concentrations used. Consistent with the 2D results, MDA-MB-468 cells also had a significant increase in G1 cells following HCQ treatment.

To assess autophagic flux, organoids transduced with a LC3-mCherry-GFP construct were imaged in the IncuCyte over 6 days. Similar to the 2D cell culture results, autophagy was inhibited significantly between control and HCQ-treated organoids at later time points (Figure 3D and Supplemental Figure 7C). MCF7 and MDA-MB-468 organoid autophagic flux inhibition was sustained and enhanced by 144 hr. MDA-MB-231 organoid autophagic flux inhibition following HCQ was less marked compared to control which was consistent with the 2D cell culture results.

**Autophagy dependent tumors take up more HCQ in vivo**

To assess how HCQ affects autophagy dependent and independent tumors in vivo, MCF7, MDA-MB-231, or MDA-MB-468 cells were implanted into mice and once the tumors were at least 100 mm$^3$, the mice were treated with either 60 mg/kg HCQ once or daily for one week to analyze steady state levels.

Whole blood HCQ and DHCQ amounts were similar in both MDA-MB-231 and MCF7 cohorts while both tumor HCQ and DHCQ amounts were higher in MDA-MB-231
compared to MCF7 following single HCQ doses. MDA-MB-468 HCQ and DHCQ amounts were also similar to MDA-MB-231 (Figure 4A). Tumor levels of HCQ and DHCQ were higher in MDA-MB-231 and MDA-MB-468 compared to MCF7 at steady state (Figure 4B), indicating that more HCQ and DHCQ is distributed into autophagy sensitive tumors. In the steady state cohort, whole blood levels were similar for HCQ and DHCQ in MDA-MB-231 and MCF7 but the MDA-MB-231 cohort had higher tumor HCQ and DHCQ levels compared to the MCF7 cohort (Figure 4C). When the steady state tumor group was compared to the 24 hr single dose tumors in each tumor type, HCQ and DHCQ levels were significantly higher in tumors in the steady state group compared to the single dose groups (Figure 4C), indicating that HCQ and DHCQ move into the tumor after 24 hr and tumor and blood saturation is not achieved by 24 hr. Whole blood of tumor-bearing mice treated with a single dose of HCQ for 24 hr was compared to the non-tumor bearing mice treated with HCQ for 24 hr from Figure 1. HCQ levels from the tumor-bearing mice fell between the 40 and 80 mg/kg non-tumor bearing levels as expected (Figure 4C) and PK parameters fell within the expected range (Table 1). DHCQ levels were not as high in whole blood in tumor-bearing mice compared to levels that would be predicted based on the non-tumor bearing mice but this could be due to DHCQ sequestering in tumors in the tumor-bearing mice. When analyzing DHCQ:HCQ ratios found in steady state treated tumors, MDA-MB-231 and MDA-MB-468 had higher ratios compared to MCF7 (Figure 4D), indicating that more DHCQ was formed compared to HCQ in the autophagy dependent tumors.
**Autophagic responses were not different but proliferation was less in autophagy dependent tumors in vivo**

Pharmacodynamic response was evaluated via western blot analysis of LC3 and p62 in the MDA-MB-231, MCF7, and MDA-MB-468 cohorts. There were no major differences between control and treated mice at 24 hr, 48 hr, or steady state doses although p62 levels trended higher in treated MDA-MB-468 tumors at 24 hr (Figure 4E and Figure S5). LC3-II densitometry area normalized to total protein were highest at 12 hr, 24 hr, and following steady state dosing in MDA-MB-231 tumors but these results were variable depending on the mouse (Figure 4F). Tumor cell proliferation was measured via immunofluorescence staining of Ki67 in the MDA-MB-231 and MCF7 control and steady state cohorts. MDA-MB-231 tumors had significantly less Ki67 staining following HCQ treatment while there was no difference in cell proliferation in the MCF7 tumors after HCQ treatment (Figure 4G). These results are consistent with the cell cycle results in 2D culture (Figure 2D) and tumor organoids (Figure 3C) because the cells that are more sensitive to autophagy inhibition (MDA-MB-231 and MDA-MB-468) have less proliferative cells at lower HCQ concentrations compared to those that are not (MCF7). Apoptotic cell death via immunofluorescence staining of cleaved caspase-3 was also performed but no tumors expressed cleaved caspase-3 (results not shown).

**Discussion**

Autophagy, a lysosomal degradation process that recycles cellular components, has been linked to enhanced cancer cell survival and chemotherapy resistance. HCQ is repurposed as an anticancer agent that inhibits autophagy. Although it is currently being used in over
90 cancer clinical trials alone or in combination treatments, pharmacodynamic responses associated with drug dosages used clinically is unclear. Further, pre-clinical studies in mice use varying doses of HCQ but there is no rationale behind those doses or the affect the associated drug exposures have on autophagy inhibition in vivo.

This study showed that HCQ and its major active metabolite DHCQ levels are dose dependent in whole blood and multiple tissues in vivo (Figure 1A and Supplemental Figure S1). Autophagy inhibition was achieved at all doses in liver and gut at multiple time points (Figure 1C). Although some autophagy inhibition was observed at various doses in multiple tissues, there was high variability between different mice. Variability and non-significant differences in autophagy inhibition is also evident in the clinic (Wolpin et al., 2014, Mahalingam et al., 2014, Barnard et al., 2014) and the results here show that this is difficult to control from patient to patient based on dose alone, suggesting HCQ doses may need to be tailored based on their individual PD response. It further implies that autophagy inhibition may not be reliably achievable using HCQ and that more potent autophagy inhibitors such as DC661 should be considered. This work clarified that 60 mg/kg ± 20 mg/kg HCQ is the HED to give mice in pre-clinical studies and validates the clinical relevance for studies that choose HCQ doses within this range (Figure 1B). Calculating the HED in this way highlights the importance of normalizing pre-clinical and clinical drug exposure because mouse model efficacy is predictive of clinical response when drug concentrations in mice are appropriately corrected for therapeutic exposure (Wong et al., 2012, Kerbel 2003).

Mice with breast tumors treated with the HED of 60 mg/kg HCQ had similar whole blood HCQ and DHCQ levels but varying tumor levels with more detected in the
autophagy dependent MDA-MB-231 and MDA-MB-468 tumors compared to autophagy independent MCF7 tumors (Maycotte et al., 2014) (Figure 4A), suggesting that autophagy dependent tumors sequester more HCQ over time. This could be due to an increase in lysosomes in autophagy dependent tumors compared to autophagy independent tumors (Collins et al., 2021). There are important implications of this when considering dual treatment with HCQ and other drugs, especially chemotherapies that sequester in lysosomes. It could be advantageous to treat patients with HCQ prior to these drugs since HCQ also sequesters in lysosome and could make these drugs more potent, as seen in a phase I clinical trial in dogs with lymphoma (Barnard et al., 2014). Although much information exists on HCQ effects in cancer, it is also important to consider DHCQ since it is present at relevant concentrations and correlates to liver autophagy inhibition (Figure 1D and Supplemental Figure S3). Further, data here suggests that DHCQ:HCQ ratios may be predictive of patient efficacy based on autophagy dependency of the tumor; this ratio has been implicated in patient response to HCQ treatment in other studies (Munster et al., 2002, Lee et al., 2016). Similar to HCQ, DHCQ has a long half-life of approximately 160 hr in people (Munster et al., 2002). However, DHCQ is not produced in cell culture experiments because there are no cytochrome P450 enzymes. Since in vitro studies do not take into account DHCQ but it has the same action as HCQ, drug concentrations used in vitro likely do not reflect the full efficacy HCQ and subsequent DHCQ will have on PD and this should be taken into consideration when choosing HCQ doses in vitro. This is evidenced by comparing HCQ exposure in vitro to in vivo exposure, where 20 µM HCQ dosing in cell culture correlates best with the autophagy dependent tumors while there is no correlation between MCF7 and in vitro exposure (Figure 5). Further, adding in relevant
DHCQ exposure means that a higher concentration of HCQ can be used in cell culture to achieve the same exposure observed in vivo (Figure 5). This also highlights the importance of calculating maximum achievable clinically relevant doses for cell culture experiments.

Autophagy inhibition measured by western blot in vivo was less conclusive in all tumor types which is consistent with the non-tumor bearing data and autophagy inhibition measured clinically. However, autophagy inhibition was observed in vitro at clinically relevant concentrations. Inhibition was most enhanced at later time points indicating that HCQ does not decrease autophagic flux rapidly (Figure 2E and 3D) even though HCQ uptake is observed at early time points in the tumors. This is consistent with observing decreased cell growth compared to control following HCQ treatment at later time points (Figure 2A). Further, MDA-MB-231 showed high basal autophagy even when treated with HCQ, implying that HCQ is inhibiting autophagy but not as well as other autophagy inhibitors since this assay was based on autophagy inhibition via bafilomycin A1. Both autophagy dependent and independent cell lines had significant decreases in autophagic flux indicating autophagy is still inhibited by HCQ irrespective of autophagy dependency. In contrast, one study found the basal breast cancer line SUM190 to be the most sensitive to HCQ induced autophagy inhibition in vitro but that could be because it only analyzed short time points (Wang et al., 2019). Overall, autophagy inhibition was variable in vivo but observed in vitro, suggesting that other autophagy PD measures may be necessary to better understand how HCQ is causing autophagy inhibition in vivo and clinically.

Although autophagy inhibition was inconsistent across tumor types and between controlled and treated mice, cell proliferation was consistently affected in autophagy
sensitive MDA-MB-231 and MDA-MB-468 tumors treated with HCQ but not in autophagy independent MCF7 tumors (Figures 2D, 3C, and 4G) indicating that autophagy dependent tumors are more affected by single agent HCQ. Further, cell proliferation was more affected in autophagy dependent tumors at lower doses of HCQ compared to MCF7 (Figures 2D and 3C), suggesting that the MCF7 tumors in vivo did not have high enough HCQ levels to cause decreased cell proliferation that was observed in MDA-MB-231. The ability of HCQ alone to inhibit cell proliferation varies in tumor types (Xie et al., 2013, Arnaout et al., 2019), suggesting that CQ and HCQ alone may not provide effective anti-proliferative effects in many cancer patients but that HCQ alone is anti-proliferative if tumors are inherently dependent on autophagy. Even though HCQ did decrease cell proliferation in autophagy dependent tumors, more potent autophagy inhibitors may produce more robust results and have less variability in achieving autophagy inhibition as a single agent compared to HCQ.

Clinically relevant HCQ concentrations were used in this study to determine if HCQ causes cancer cell death. Based on in vivo and in vitro apoptosis and cell death assays, breast cancer treated with HCQ alone at the concentrations used here do not appear to die via caspase 3/7-dependent apoptosis but do undergo some cell death in 2D and organoids (Supplemental Figure S4A and Figure 3B) and this cell death is enhanced in autophagy dependent tumors at later time points. Another study also observed no caspase 3-dependent cell death in head and neck squamous cell carcinoma following 20 μM HCQ alone (Gao et al., 2018). Other studies have shown that HCQ alone can induce caspase 3-dependent cell death in gastric (Wang et al., 2019) and bladder cancer (Lin et al., 2017) at HCQ concentrations of 14 μM and 20 μM respectively, indicating that HCQ
alone will only induce this kind of cell death at clinically achievable concentrations in certain cancer types. However, when clinically relevant HCQ doses are combined with other treatments, HCQ enhances apoptotic cell death in other cancer types such as head and neck squamous cell carcinoma (Gao et al., 2018), melanoma (Xie et al., 2013), and gastric cancer (Wang et al., 2019), demonstrating that although low enough doses of HCQ alone do not cause caspase 3/7-dependent apoptosis in certain cancer types, in combination with other therapies it does induce caspase 3/7-dependent apoptosis. Combination therapies were not tested in this study, but the results here and in other studies suggest that combining HCQ with other treatments may enhance cell death in breast cancer.

Overall, this study shows that 2D cell culture, 3D tumor organoids, and *in vivo* studies produce similar results and *in vitro* studies can be used as surrogates to recapitulate *in vivo* tumor responses. Tumor autophagy dependency is important in the evocation of cellular responses including autophagy inhibition, cell proliferation, and cell death. Further, in certain contexts, HCQ may not be an adequate drug as a single agent depending on the clinical objective. DHCQ is an active metabolite whose effects needs to be considered in *in vitro* experiments since it is not produced but would add to toxicity if it were present. Lastly, better biomarkers to measure autophagy inhibition clinically are necessary to understand how the PD relates to the PK of autophagy inhibitors.

**Acknowledgements**

We would like to thank Drs. Ryan Hansen and Keagan Collins for contributions in the pharmacokinetic studies as well as other staff in the Pharmacology Shared Resource.
Authorship Contributions

Participated in research design: Van Eaton and Gustafson

Conducted experiments: Van Eaton

Performed data analysis: Van Eaton and Gustafson

Wrote or contributed to the writing of the manuscript: Van Eaton and Gustafson

References


Footnotes

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Legends for Figures

**Figure 1.** HCQ PK and associated PD in non-tumor bearing mice. (A) Exposure of HCQ and DHCQ in whole blood, liver, gut, kidney, and brain. (B) Clinical trial human HCQ AUC data (Rosenfeld et al., 2014) was compared to mouse AUC data to find the human equivalent dose in mice. Dotted line represents the mean human HCQ AUC and dashed lines represent human HCQ AUC standard deviation. The red dot represents the AUC\(_{0-24}\) estimated at steady-state from the tumor-bearing mouse PK study dosed at 60 mg/kg. (C) LC3 western blots for liver 24 hr, 48 hr, and 72 hr following HCQ in three mice. LC3 and p62 western blot quantification for liver and kidney from western blots shown in Figure S2. (D) Hysteresis curves showing how LC3-II levels as measured in the liver are affected by HCQ and/or DHCQ concentrations in the liver or whole blood following 40 mg/kg dose
of HCQ. Numbers on the curves correspond to the time points. N=3 mice per group. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

Figure 2. Cell death, cell cycle, and autophagic flux following HCQ treatment in 2D cell culture. (A) HCQ screen in breast cancer lines in buffered media from 0.625 to 40 μM HCQ. The median effective dose (Dm) at which half of the cells were affected is calculated at 96 hr. (B) Caspase 3/7 assays on HCQ-treated cells using the live cell imaging IncuCyte Zoom imaging system. (C) Cell death via YOYO staining using the IncuCyte Zoom. (D) Cell cycle analysis via EdU incorporation of HCQ-treated cells for 48 hr. (E) Autophagic flux was assessed using cells transduced with a LC3-mCherry-GFP reporter and reporting the percent of cells using autophagy. Equal toxicity doses were 8 μM for MDA-MB-231 and MDA-MB-468 and 15 μM for MCF7. Equal dosing was 10 μM HCQ. N=3 or more biological replicates of 3 technical replicates each. Significance indicated is compared to control. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

Figure 3. Cell death, cell cycle, and autophagic flux following HCQ treatment in tumor organoids. (A) Cell death via Caspase 3/7 staining in the IncuCyte. Green object integrated intensity was normalized to red object integrated intensity. (B) Cell death analysis by YOYO staining in the IncuCyte. Green object integrated intensity was normalized to red object integrated intensity. (C) Cell cycle analysis via EdU incorporation. (D) Autophagy inhibition assessed by monitoring LC3-mCherry-GFP labeled cells in the IncuCyte over six days. N=3 or more biological replicates of 3 technical
replicates each. Significance indicated is compared to control. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

**Figure 4.** HCQ PK and PD in breast tumor-bearing mice. (A) Exposure of HCQ and DHCQ in whole blood and tumor. Solid lines represent HCQ and dotted lines represent DHCQ. (B) Blood and tumor HCQ and DHCQ amounts detected in steady-state dosed mice compared to 24 hr single dose mice (top) and HCQ and DHCQ amounts detected in blood in single dose 24 hr tumor-bearing mice compared to non-tumor bearing mice at 24 hr (bottom). Numbers listed above the bars are the mean amount of HCQ/DHCQ of the respective cohort. (C) Tumor levels of HCQ and DHCQ at steady state. (D) DHCQ:HCQ ratios in tumors. (E) Western blot quantification of LC3-II/tubulin, LC3-II densitometry area normalized by total protein, or p62 densitometry area normalized by total protein in breast tumors following 24 hr, 48 hr, or steady state dosing of HCQ in mice. Western blots used for protein quantification in Figure S5. (F) Western blot and quantification of LC3-II density normalized to total protein over 72 hr or at steady state. (G) Immunofluorescent analysis of Ki67 in MDA-MB-231 and MCF7 control and steady state tumors. Blue=DAPI and red=Ki67. Quantification based on 6 or more separate fields of view on each tumor slice. N=3 mice per group. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

**Figure 5.** HCQ PK exposure in vitro compared to in vivo at 24 hr and 48 hr. For the breast tumors, exposure is based on AUC of concentration versus time curves. The 10 and 20
µM \textit{in vitro} AUC is based on the theoretical exposure cells will experience based on those doses for the given time (e.g., 10 µM * 24 hr = 240 hr* µM).
Tables

Table 1. Pharmacokinetic parameters of HCQ from whole blood in non-tumor bearing and tumor bearing mice following a single intraperitoneal dose.

<table>
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<tr>
<th>PK Parameter</th>
<th>Units</th>
<th>20 mg/kg</th>
<th>40 mg/kg</th>
<th>80 mg/kg</th>
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*CL and V<sub>Dz</sub> calculations represent values that are not dose corrected for the bioavailability (F) and total dose was used in the calculation.
**Table:**

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<th>Cell Line</th>
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<td>MDA-MB-468</td>
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**Figure 2**

**A**

Fraction of control at 96 hr

**B**

Percent dead CASP3/7

**C**

Percent dead YOYO

**D**

Percent of cells

**E**

Percent of autophagic cells
Supplemental Figure S1. DHCQ levels in tissues over time.
**Supplemental Table S1.** Pharmacokinetic parameters of HCQ in tissues in mice.

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Supplemental Table S2. Pharmacokinetic parameters of DHCQ in whole blood in mice.

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**Supplemental Table S3.** Pharmacokinetic parameters of DHCQ in tissues in mice.

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Supplemental Figure S2. Western blots for tissues in non-tumor-bearing mice.
Supplemental Figure S3. Hysteresis plots of LC3-II in the liver and whole blood of mice following (A) 20 mg/kg HCQ or (B) 80 mg/kg HCQ.
Supplemental Figure S4. HCQ in vitro 2D cell culture studies. (A) Cell death via live/dead staining. (B) Cell cycle analysis via PI/RNase staining of HCQ-treated cells for 48 hr. (C) Autophagy inhibition assessed by monitoring LC3-mCherry-GFP labeled cells in the IncuCyte over six days. N=3 or more biological replicates of 3 technical replicates each. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001
MDA-MB-231, MCF7, and MDA-MB-231 LC3 and p62 Western Blots

Supplemental Figure S5. Western blots for tumors from tumor-bearing mice.
**Supplemental Figure S6.** Same data as shown in Figure 2 for *in vitro* 2D experiments but includes positive controls for cell death and autophagy inhibition. For cell death, MCF7 was treated with 1 μM, MDA-MB-231 was treated with 6 μM, and MDA-MB-468
was treated with 2 µM etoposide. For autophagic flux inhibition, all cell lines were treated with 10 nM bafilomycin. (A) Cell death Caspase 3/7 measurement in the IncuCyte. (B) Cell death via YOYO staining measured in the IncuCyte. (C) Autophagy inhibition assessed by flow cytometry of LC3-mCherry-GFP labeled cells. (D) Autophagy inhibition assessed by monitoring LC3-mCherry-GFP labeled cells in the IncuCyte over 6 days. N=3 or more biological replicates of 3 technical replicates each. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001
Supplemental Figure S7. Same data as shown in Figure 3 for in vitro tumor organoid experiments but includes positive controls for cell death and autophagy inhibition. For cell death, MCF7 was treated with 0.2 µM, MDA-MB-231 was treated with 0.01 µM, and MDA-MB-468 was treated with 0.02 µM staurosporine. For autphagic flux inhibition, all cell lines were treated with 5 nM bafilomycin. (A) Cell death Caspase 3/7 measurement in the IncuCyte. (B) Cell death via YOYO staining measured in the IncuCyte. (C) Autophagy inhibition assessed by monitoring LC3-mCherry-GFP labeled cells in the
IncuCyte over 6 days. No significance calculated for CASP3/7 or YOYO data since only one staurosporine experiment was performed. N=3 or more biological replicates of 3 technical replicates each. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001