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CLINICAL TRIAL

A randomized, double-blind, window of opportunity trial evaluating the effects of chloroquine in breast cancer patients

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Abstract

Purpose Chloroquine has demonstrated anti-tumor activities through autophagy inhibition and cell cycle disruption. This study aimed to assess the effect of single-agent chloroquine on breast tumor cellular proliferation in a randomized, phase II, double-blind, placebo-controlled, pre-surgical window of opportunity trial.

Methods Patients with newly diagnosed breast cancer were randomized 2:1 to chloroquine 500 mg daily or placebo for 2- to 6-weeks prior to their breast surgery. The primary outcome was the relative change in measures of proliferation (Ki67) in primary breast cancer cells pre- and post-treatment. Adverse events and toxicity profiles were also evaluated.

Results From September 2015 to December 2016, 70 patients were randomized [46 (66%) chloroquine and 24 (34%) placebo]. Ten patients who were randomized to chloroquine withdrew from study due to adverse events. Mean duration of drug intake was 15 days (range 14–29 days). There were no significant differences between the chloroquine or placebo arms with respect to either the percentage change (−0.4 vs. −1.2, $p=0.088$) or absolute change (−2.0% vs. −5.2%, $p=0.066$) in Ki67 index pre- and post-drug treatment. Although adverse effects were minimal and all classified as grade 1, the effects were significant enough to cause nearly 15% of patients to discontinue therapy.

Conclusions Treatment with single-agent chloroquine 500 mg daily in the preoperative setting was not associated with any significant effects on breast cancer cellular proliferation. It was, however, associated with toxicity that may affect its broader use in oncology.

Keywords Window of opportunity clinical trial · Chloroquine · Breast cancer

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Background

Autophagy permits cells to recycle intracellular organelles and macromolecules, providing the cell with the basic building blocks for growth and survival during periods of stress [1–3]. Pre-clinical evidence has demonstrated improved anti-tumor effects when various types of anti-cancer drug are combined with either genetic or pharmacological autophagy inhibition [1–4].

Chloroquine (CQ) and hydroxychloroquine (HCQ) are commonly used anti-malarial, anti-inflammatory, anti-rheumatoid, and anti-lupus agents. They are also the only clinically available drugs used to inhibit autophagy [5, 6]. CQ arrests the latter step of autophagy, namely degradation of the autolysosome, which results in failure to provide energy through the autophagy pathway [4–9]. In addition to inhibition of autophagy, recent studies have identified modulation of cellular metabolism and cell cycle arrest in certain cell lines as a basis for the anti-tumor activities of CQ, independent of autophagy [10–13]. In vitro studies using CQ have shown inhibition of cell growth and/or induction of cell death in human lung cancer A549 cells, glioma cells, mouse colon cancer CT26 cells [5, 9], and demonstrated efficacy and selectivity against breast cancer cells when used alone and in conjunction with chemotherapy [14–17].

A number of trials have evaluated 150 mg of daily CQ in conjunction with radiation demonstrating prolonged survival in patients with glioblastoma [3, 18–20] and improved control of brain metastasis [21]. A review of clinicaltrials.gov identified 13 studies evaluating CQ as the main intervention against various cancers (Online Resource 1). Only two studies are addressing treatment in breast cancer, one single-agent study in DCIS [22] and one in combination with chemotherapy for metastatic breast cancer [23]; therefore, additional studies with CQ as a single agent against breast cancer in vivo are clearly required.

Window of opportunity trials can provide insight into biological effects and potential therapeutic efficacy of novel therapeutic strategies [24, 25]. In these studies, newly diagnosed patients awaiting curative resection receive a study agent between the diagnostic breast biopsy and planned surgery. This allows the evaluation of effect of agents in tissue samples obtained before and after drug exposure. Here, we describe the results from a window of opportunity trial evaluating the intra-tumoral effects of 500 mg/day administration of CQ on breast cancer cellular proliferation.

Methods

Study design and participants

A randomized, double-blinded, placebo-controlled window of opportunity trial was performed. Study eligibility criteria included: biopsy proven operable invasive cancer clinically and/or radiologically ≥ 1.5 cm in size; surgery planned for 2–6 weeks after initial consultation; and ECOG performance status 0–2. Patients were excluded if they had known recurrent or metastatic breast cancer, were currently on or have been exposed to CQ or HCQ within the past 3 months, were pregnant or actively nursing, or had known history of pre-existing auditory, retinal or ocular pathology, psoriasis, epilepsy or seizures, or G6PD deficiency. They were also excluded if their baseline electrocardiogram showed QT prolongation based on QTc interval > 450 ms or if their baseline bloodwork showed abnormal hepatic function (serum AST or ALT $> 3 \times$ upper limit of normal) or creatinine > 100 umol/L.

Randomization and intervention

Eligible patients were randomly allocated 2:1 to 500 mg CQ (Novo-Chloroquine, 2×250 mg tabs) or lactose placebo with identical appearance. Study preparations were taken once a day, for 2- to 6-weeks ending the day before their surgery. The randomization sequence was generated using computer software to produce randomly permutated blocks of size 6, developed by the Ottawa Methods Centre. Each arm was stratified based on baseline Ki67 index of the initial diagnostic core biopsy into three levels: Ki67 low group (0–14%), Ki67 intermediate group (14.1–30%), and Ki67 high group ($> 30\%$). The study protocol was approved by the Ottawa Hospital Research Ethics Board and registered at Clinicaltrials.gov (NCT02333890).

Ki67

Ki67 is a proliferation-associated nuclear antigen that is considered an important prognostic and predictive biomarker associated with patient outcomes [26, 27]. All diagnostic core biopsies (14 g needles) were immediately fixed in 10% neutral buffered formalin. If surgical specimen was used, excisional breast specimens were sliced and exposed to formalin within 1 h. The fixation time for biopsies and excisional specimens were in compliance with ASCO/CAP guidelines for hormone receptor testing [28]. After standard tissue processing and embedding in paraffin wax, sections were cut and stained with Hematoxylin and Eosin or left unstained for immunohistochemistry.

Immunohistochemistry was performed within 5 days after sectioning, using Ki67 antibody diluted 1:7 (clone MIB-1; Dako, Denmark) with 20-min retrieval with BOND Epitope Retrieval Solution 1 (citrate based pH 6.0) on BOND-Max platform, and visualized with a BOND Polymer Refine Detection Kit. The ''Ki67 index'' (percentage of nuclei showing nuclear immunoreactivity of any intensity) was determined by computer image assisted count by a single blinded pathologist (SR). In each case, after a low-power scan of the entire tissue section, hot spot regions of highest activity were selected and from these 1000 tumor nuclei were counted at ×400 to ×600 magnification.

Toxicity

As prolonged administration of high dose CQ can cause visual disturbances and retinal toxicity with decreased vision [29], patients had ophthalmologic exams at baseline, 1 month post-surgery, and 4–6 months after CQ intake has completed. During the study, patients were interviewed weekly via telephone for toxicity assessments following the Common Terminology Criteria for Adverse Events (CTCAE) [30]. Compliance was monitored by measuring the remaining capsules returned on the day of the surgery.

Statistical analyses

Descriptive statistics were used to summarize patient, tumor, treatment, and outcome characteristics. Analysis was intention-to-treat and involved all patients randomized to the two study arms. All data were analyzed with SPSS software (version 20). The absolute change in Ki67 was calculated as: post-surgery measure—pre-surgery measure, while the percent change was calculated as: absolute change/pre-surgery measure*100%. Comparisons between CQ vs placebo patients were performed using Wilcoxon rank sum tests. Linear regression was performed to investigate possible prognostic factors of the absolute change in average Ki67. The baseline Ki67 score was used as a covariate in the linear regression analysis. Univariate models were initially constructed for selected potentially prognostic factors. A multivariable model was constructed using forward selection. All tests were two-sided and a p value of 0.05 or less was considered statistically significant.

Sample size

The primary outcome was the absolute change in Ki67 in primary breast cancer cells from pre- to post-treatment (post-treatment Ki67 score—pre-treatment Ki67 score). Based on a previously reported mean percentage decrease in Ki67 of 10% to 40% in patients receiving metformin [31, 32], it was conservatively assumed that CQ will result in a 20% mean decrease in Ki67, and 0% mean decrease for patients receiving placebo, with standard deviation of the change of 25%. Thus, with these hypotheses, a two-sided, alpha = 0.05, two-sample t-test would require 40 patients in the CQ group and 20 patients in the placebo group for a total of 60 evaluable patients to achieve at least 80% statistical power.

Results

Study population and characteristics

Between September 2015 and December 2016, 508 patients newly diagnosed with invasive breast cancer underwent initial surgical consultation. 100 patients (20%) were potentially eligible and approached for the study (Fig. 1). Out of the 100 patients approached, 15 patients declined the study; of these, 6 specified a reason for declining including refusing repeat eye exam ($n = 5$) and size of CQ capsules ($n = 1$). There were 15 screen failures due to: abnormal baseline ophthalmologic exam ($n = 3$); pre-existing ocular or auditory symptoms ($n = 3$); abnormal blood work ($n = 3$), abnormal ECG ($n = 1$); change in the decision or date for surgery ($n = 3$); and neoadjuvant therapy ($n = 2$).

70 patients consented to participate. 46 were allocated to the CQ group (66%) and 24 to placebo (34%). Table 1 summarizes baseline patient, pathologic, and treatment characteristics of these 70 patients. Ten patients who received CQ withdrew after developing symptoms and did not complete their allocated intervention (Table 2). As these patients, who received CQ between 3 and 8 days, did not have on-treatment Ki67 values they were excluded from the analysis of Ki67. Overall, 60 patients completed the study: 36 (60%) in the CQ group and 24 (40%) in the placebo control group.

Duration of treatment

The median duration of drug intake for those who completed treatment was 15 days (range 14 to 29) for both the CQ and placebo group.

Primary outcome

Changes in tumor Ki67

Baseline and post-treatment Ki67 indices are listed in Table 3. No statistically significant difference was observed in the absolute change in Ki67 from pre- to post-treatment between the CQ group (median change −0.4, range −13.2 to 15.3) and the placebo group (−1.2, −19.4 to 4.2) ($p = 0.088$). The difference in percent change in Ki67 index was also not statistically significant between the CQ group (−2.0%, −75% to 390%)

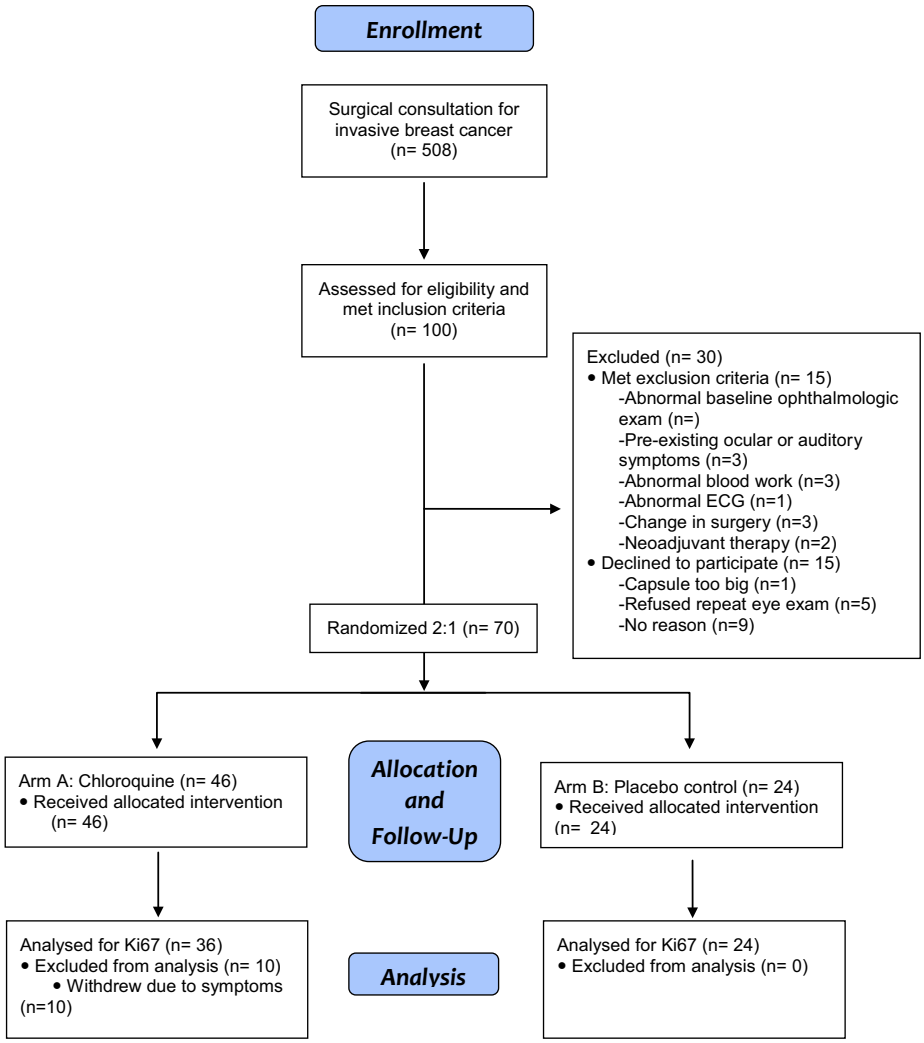

Fig. 1 Consort diagram

and the placebo group (−5.2%, −65% to 18.4%) ($p=0.066$). Figure 2 demonstrates the Ki67 results before and after drug treatment.

In order to identify factors potentially prognostic for absolute change in Ki67, regression model analysis was used. In the univariate analysis, higher T stage (estimate = −6.86, standard error, SE, 2.52, $p=0.009$; T3 versus T1-2), higher baseline Ki-67 (−0.10, SE 0.04, $p=0.019$), and receiving CQ (3.71, SE 1.70, $p=0.0034$) were statistically significant prognostic factors for a decrease in Ki-67. After adjusting for T stage, baseline Ki-67, and age in the multivariable model, treatment with CQ was not statistically significant (2.46, SE 1.63, $p=0.14$). A linear regression analysis was also performed for the percent change in Ki67, with similar results (data not shown).

Secondary outcomes

Toxicity

All reported adverse events were grade 1 (Table 2). Visual (blurriness, light sensitivity) (4/46, 8.7%), auditory symptoms (tinnitus) (1/46, 2.2%), muscle weakness (4/46, 8.7%), and dry mouth (2/46, 4.3%) occurred only in the CQ group. Among the 10 patients on the CQ arm who withdrew early due to symptoms, 7 (70%) had nausea, 3 (30%) had diarrhea, 3 (30%) had dizziness, 2 (20%) had visual symptoms, 3 (30%) had muscle weakness, and 1 (10%) had dry mouth. All these symptoms are recognized

Table 1 Baseline patient, pathologic, and treatment characteristics ($n=70$)

	Chloroquine $N=46$	Placebo $N=24$	All patients $N=70$
Age, mean (SD), years	57.4 (9.7)	55.7 (8.4)	56.8 (9.2)
BMI, median (range), kg/m^2	26.7 (18.8, 46.1)	28.3 (19.0, 40.8)	27.5 (18.8, 46.1)
Weight, median (range), kg	69.4 (45.8, 114.3)	71.2 (46.7, 117.9)	70.3 (45.8, 117.9)
Primary pathologic tumor type, no. (%)			
Ductal	36 (78.3)	21 (87.5)	57 (81.4)
Lobular	9 (19.6)	3 (12.5)	12 (17.1)
NE	1 (2.2)	0	1 (1.4)
Pathologic grade, no. (%) (on core biopsy)			
1	4 (8.7)	2 (8.3)	6 (8.6)
2	14 (30.4)	10 (41.7)	24 (34.3)
3	28 (60.9)	12 (50.0)	40 (57.1)
ER positive, no. (%)	42 (91.3)	22 (91.7)	64 (91.4)
PR positive, no. (%)	33 (71.7)	18 (75.0)	51 (72.9)
HER2, no. (%)			
Positive	7 (15.2)	4 (16.7)	11 (15.7)
Negative	38 (82.6)	20 (83.3)	58 (82.9)
Equivocal	1 (2.2)	0	1 (1.4)
Pathological tumor size, median (range), cm (post-surgical)	2.5 (1.1, 7.5)	2.9 (1.7, 8.7)	2.5 (1.1, 8.7)
Pathologic T stage, No. (%) (post-surgical)			
T1c	7 (15.2)	2 (8.3)	9 (12.9)
T2	38 (82.6)	16 (66.7)	54 (77.1)
T3	1 (2.8)	6 (25.0)	7 (10.0)
Pathologic N stage, no. (%) (post-surgical)			
N0	30 (65.2)	10 (41.7)	40 (57.1)
N1	11 (23.9)	8 (33.3)	19 (27.1)
N2	3 (6.5)	4 (16.7)	7 (10.0)
N3	0	2 (8.3)	2 (2.9)
Breast surgery type: mastectomy, no. (%)	13 (28.3)	5 (20.8)	18 (25.7)
Axillary lymph node surgery type: sentinel, no. (%)	41 (89.1)	16 (66.7)	57 (81.4)
Baseline stratum, $n=60$			
Group 1 (Ki-67 $\leq$ 14%)	13 (36.1)	8 (33.3)	21 (35.0)
Group 2 (14.1–30%)	12 (33.3)	8 (33.3)	20 (33.3)
Group 3 (> 30%)	11 (30.6)	8 (33.3)	19 (31.7)

side effects of CQ. Both the CQ and placebo groups experienced nausea and/or abdominal cramps (CQ: 11/46, 23.9% vs. placebo: 3/24, 12.5%), diarrhea (CQ: 8/46, 17.4% vs. placebo: 1/24, 4.2%), dizziness (CQ: 4/46, 8.7% vs. placebo 3/24, 12.5%), and fatigue (CQ: 1/46, 2.2% vs. placebo: 1/24, 4.2%). All the above symptoms subsided once the medication ceased. No patients experienced any documented visual changes.

Discussion

Chloroquine with its well established side effect profile and demonstrated effects on autophagy inhibitory activity and cell cycle arrest makes it an interesting agent to evaluate in breast cancer. To date, results on the therapeutic efficacy of CQ in cancer have been published for 4 clinical

Table 2 All reported adverse events[a]

	Chloroquine			Placebo $N=24$
	Completed $N=36$	Withdrawn $N=10$	Total $N=46$	
Nausea and/or abdominal cramps	4 (11.1)	7 (70.0)	11 (23.9)	3 (12.5)
Diarrhea	5 (13.9)	3 (30.0)	8 (17.4)	1 (4.2)
Dizziness	1 (2.8)	3 (30.0)	4 (8.7)	3 (12.5)
Fatigue	1 (2.8)	0 (0)	1 (2.2)	1 (4.2)
Visual symptoms	2 (5.6)	2 (20.0)	4 (8.7)	0 (0)
Auditory symptoms	1 (2.8)	0 (0)	1 (2.2)	0 (0)
Muscle weakness	1 (2.8)	3 (30.0)	4 (8.7)	0 (0)
Dry mouth	1 (2.8)	1 (10.0)	2 (4.3)	0 (0)

Values are number (percentage) ($n=70$)

[a]All reported adverse events were classified as grade 1

trials for the treatment of glioblastoma multiforme (GBM) or brain metastases [18–21]. All of these studies evaluated CQ in combination with either systemic therapy and/or radiation therapy. In a trial involving 18 patients with glioblastoma, treatment with 150 mg/day of CQ in conjunction with radiation and the alkylating agent carmustine experienced a statistically significant prolonged median survival compared to controls (33 months compared with 11 months) [20]. Follow up randomized placebo clinical trials, and retrospective data supported the findings of the initial study [3, 18, 19]. Additional early phase clinical trial combining radiation with 150 mg/day of CQ for 4 weeks also found improved control of brain metastasis [21]. Despite their small size these studies all provide evidence that CQ when combined with conventional cancer therapies may have anti-tumor activity.

Table 3 Ki67 labeling index (%)

	Chloroquine $N=36$	Placebo $N=24$	p value
Baseline	25.9 (1.0, 97.0)	20.9 (3.8, 75.0)	0.83
Post-surgery	28.4 (1.0, 83.8)	18.0 (3.5, 75.7)	0.64
Absolute change in Ki-67[a]	−0.4 (−13.2, 15.3)	−1.2 (−19.4, 4.2)	0.088
% Change in Ki67[b]	−2.0 (−75, 390)	−5.2 (−65, 18.4)	0.066

Values are median (range) ($n=60$)

[a]The absolute change was calculated as: post-surgery measure—pre-surgery measure

[b]The percent change was calculated as: absolute change/pre-surgery*100%

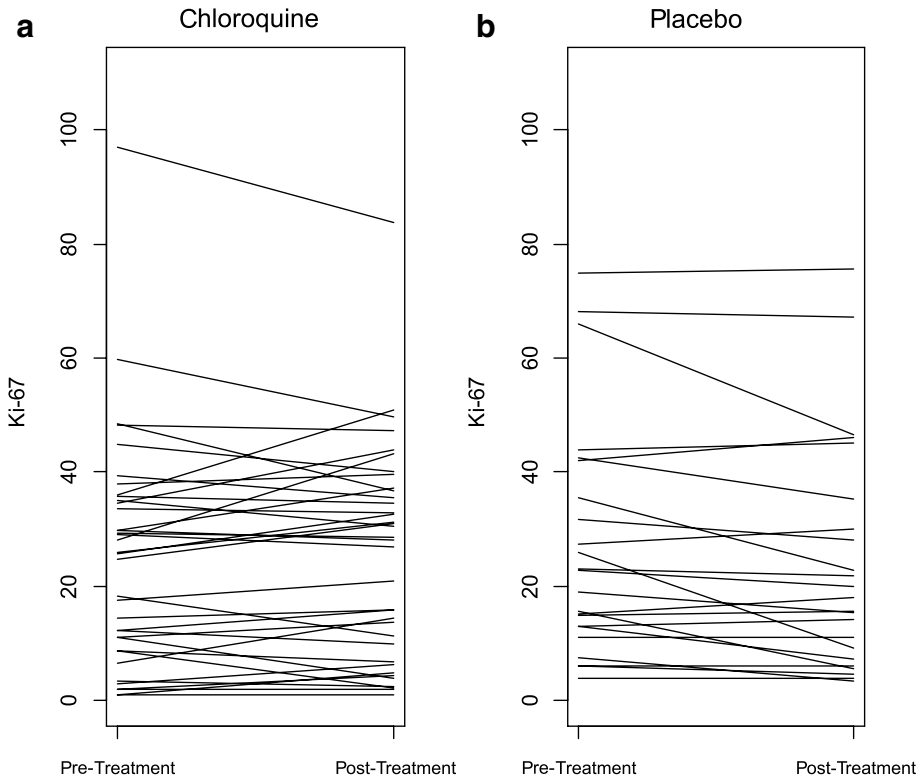

Fig. 2 Change in Ki67 index pre- and post-treatment with chloroquine (**a**) and placebo (**b**)

As proof of principle for evaluating efficacy of a potential therapeutic agent, window of opportunity trials provides a unique opportunity to evaluate potential the biological effects of agents in vivo [33, 34]. The current study evaluated the effects of daily CQ supplementation (500 mg) on the tumor Ki67 index of breast cancer patients. The proliferate maker Ki67 was chosen as it has been widely used in window trials and is associated with response to a range of active therapies [26]. Despite a minimum of 2 weeks of study medication, we were unable to demonstrate any significant effect on cancer cell proliferation.

The lack of a therapeutic effect of CQ in the current study may be attributed to the mechanism of autophagy. Autophagy is induced by signals of cellular stress, and is activated as a survival mechanism in response to anti-cancer therapies such as chemotherapy or radiation [35]. In this context, autophagy has a cytoprotective function that enables cancer cells to cope with stress. Therefore, the inhibition of autophagy by CQ may only be beneficial during stress and show limited efficacy when used alone. The majority of pre-clinical research, both in vitro and in vivo, has focused on combination therapy with CQ, and many have reported positive therapeutic efficacy when combined with existing anti-cancer therapy as compared with monotherapy.

Other potential explanations for the negative results could be: too low a dose of CQ, short interval of treatment, or lack of statistical power. The dosage of CQ ranges between 100 and 1000 mg/day; with 250 mg/day normally recommended for treatment of rheumatologic conditions and higher doses for treatment of malaria [36]. The dose required for autophagy inhibition and induction of apoptosis in humans has not yet been elucidated. Dosages of CQ used in the aforementioned clinical trials (Online Resource 1) range from 100 to 600 mg/day, with the most commonly used dose being 250 mg/day. The concentrations of CQ required to induce cell death in vitro (~ 20 uM) is considerably higher than the clinically acceptable doses of CQ (~ 5 uM) [12]. A recent review also suggested that autophagy activities were mostly demonstrated at high doses of CQ [37]. We chose to administer 500 mg of CQ per day, a high dose, to allow for maximal possible effect. In regards to drug duration, as it is a window of opportunity trial, delay of planned curative resection for extended treatment was not possible. Finally, although the sample size was small, the median decrease in Ki67 was actually larger amongst placebo patients than CQ patients, indicating that a larger sample size would be unlikely to demonstrate statistically significant effects.

Despite no significant effect on cancer cell proliferation, administration of CQ was associated with toxicity that resulted in 10 patients (21.7%) not completing the study. Retinal toxicity with prolonged administration of high dose CQ has been previously reported [29]. Other known toxicity include extremely low incidences of auditory damage, muscle weakness, and minor side effect such as nausea, dizziness, headache, and pruritus [38].

This clinical trial had limitations including being a relatively small study at a single center. Challenges with the window of opportunity model are well recognized [39]. Variability in Ki67 staining as a result of duration of tissue ischemia, formalin quality, duration of fixation, immunohistochemical technique and assessor differences, is described [40, 41] and these were controlled for in our study. The major remaining variable that is impossible to control is sampling variability using pre- intervention core biopsies. Despite these challenges, Ki67 remains the most validated biomarker for window trials [39]. These changes should be equally present in both the treatment and control arms in a randomized controlled clinical trial. Finally, with over 20% of patients who received CQ withdrawn from the study, this could have substantial effect on the results.

Further insights on the effects of CQ would be obtained by examining autophagy and apoptosis markers. Immunohistochemical detection of proteins such as Beclin 1, LC3, and p62 which are required for autophagosome formation are recognized markers of autophagy [2, 5], and TUNEL or caspase assays have been widely used to quantify apoptosis [31, 32, 42]. Future trials could evaluate longer duration of CQ, or possibly higher doses to assess therapeutic effects. Although the severity was minimal, the number of patients who reported adverse effects from administering CQ was substantial. An alternate choice of treatment may be hydroxychloroquine, which has less side effects than CQ [43–45].

Conclusion

Single-agent CQ administration for a minimum of 2 weeks, in this window of opportunity trial, was not associated with any significant effects on tumor proliferation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Ottawa Hospital Research Ethics Board) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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