Chloroquine Inhibits Autophagy to Potentiate Antiestrogen Responsiveness in ER\textsuperscript{+} Breast Cancer

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Abstract

\textbf{Purpose:} Estrogen receptor-\(\alpha\) (ER\(\alpha\))-targeted therapies including tamoxifen (TAM) or Faslodex (ICI) are used to treat ER\textsuperscript{+} breast cancers. Up to 50\% of tumors will acquire resistance to these interventions. Autophagy has been implicated as a major driver of antiestrogen resistance. We have explored the ability of chloroquine (CQ), which inhibits autophagy, to affect antiestrogen responsiveness.

\textbf{Experimental Design:} TAM-resistant MCF7-RR and ICI-resistant/TAM cross-resistant LCC9 ER\textsuperscript{+} breast cancer cells were injected into mammary fat pads of female athymic mice and treated with TAM and/or ICI in combination with oral low-dose CQ.

\textbf{Results:} We show that CQ can increase antiestrogen responsiveness in MCF7-RR and LCC9 cells and tumors, likely through the inhibition of autophagy. However, the combination of ICI+CQ was less effective than CQ alone \textit{in vivo}, unlike the TAM+CQ combination. Antiestrogen treatment stimulated angiogenesis in tumors but did not prevent CQ effectiveness. The lower efficacy of ICI+CQ was associated with ICI effects on cell-mediated immunity within the tumor microenvironment. The mouse chemokine KC (CXCL1) and IFN\(\gamma\) were differentially regulated by both TAM and ICI treatments, suggesting a possible effect on macrophage development/activity. Consistent with these observations, TAM+CQ treatment increased tumor CD68\textsuperscript{+} cells infiltration, whereas ICI and ICI+CQ reduced peripheral tumor macrophage content. Moreover, macrophage elimination of breast cancer target cells \textit{in vitro} was reduced following exposure to ICI.

\textbf{Conclusion:} CQ restores antiestrogen sensitivity to resistant tumors. Moreover, the beneficial combination of TAM+CQ suggests a positive outcome for ongoing neoadjuvant clinical trials using this combination for the treatment of ER\textsuperscript{+} ductal carcinoma \textit{in situ} lesions. \textit{Clin Cancer Res}; 20(12); 3222–32. ©2014 AACR.

Introduction

One out of every eight American women will develop invasive breast cancer over the course of her lifetime. An estimated 230,000 new cases of breast cancer are diagnosed annually in the United States (1). Of these cases, 70\% will express estrogen receptor-\(\alpha\) (ER\(\alpha\)). ER\(\alpha\)-targeted therapies include the selective ER modulator tamoxifen (TAM), the selective ER downregulator Faslodex (fulvestrant, ICI), or aromatase inhibitors that block the conversion of androgens to estrogens. Even with the success of these drugs, some tumors fail to respond (\textit{de novo} resistance) or acquire resistance over time (2–4).

Autophagy is a process by which a double membrane vesicle surrounds cellular contents, such as damaged organelles and misfolded or protein aggregates, and recycles the material through lysosomal degradation (5). Studies in breast cancer cells show that the induction of autophagy by various therapeutics is usually prosurvival (6–8). Furthermore, TAM and ICI both induce autophagy in ER\textsuperscript{+} breast cancer cells (6, 9–13). Antiestrogen-resistant cell lines exhibit increased basal autophagy when compared with their antiestrogen-sensitive parental cells (10). Inhibiting autophagy through autophagy-related gene 5 (ATG5) silencing potentiated antiestrogen-mediated cell death, indicating that antiestrogen-stimulated autophagy is pro-survival and a critical mechanism of therapy resistance (10). Analysis of publically available human datasets indicates that autophagy-related genes, ATG5, ATG7, and p62 SQSTM1, are elevated in early recurring breast cancer when compared with breast cancer that never recurs. Moreover, elevated p62 is significantly correlated with poor survival and a critical mechanism of therapy resistance (10). Analysis of publically available human datasets indicates that autophagy-related genes, ATG5, ATG7, and p62 SQSTM1, are elevated in early recurring breast cancer when compared with breast cancer that never recurs. Moreover, elevated p62 is significantly correlated with poor survival and a critical mechanism of therapy resistance (10).
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Translational Relevance
Breast cancer affects more than 230,000 American women each year. An estimated 70% of these cases have tumors that express the estrogen receptor and are eligible to be treated with antiestrogens such as tamoxifen (TAM) or, for the treatment of advanced postmenopausal disease, Faslodex. However, 50% of these breast cancers will either fail to respond (de novo resistance) or lose therapy responsiveness over time (acquired resistance). We found that inhibiting autophagy through low-dose oral chloroquine (CQ) administration increases the responsiveness of resistant mammary tumors to antiestrogens. Moreover, we show that the combination of TAM and CQ is more effective than that of Faslodex and CQ due to activities within the tumor microenvironment. These preclinical data highlight the relevance of a clinical trial combining chloroquine and TAM for the treatment of ER+ in situ breast lesions (Preventing Invasive Neoplasia with Chloroquine (PINC)) and suggest a clinical benefit of the addition of CQ to antiestrogen therapy for the treatment of ER+ breast cancer. Furthermore, this study predicts a more modest result for combining Faslodex and chloroquine-based therapies for prevention and/or treatment of breast cancer.

Materials and Methods

Materials
The following materials were obtained as indicated: 4-hydroxytamoxifen and CQ diphosphate salt (Sigma-Aldrich); ICI 182,780 (Tocris Bioscience); Improved Minimal Essential Medium (IMEM; Gibco Invitrogen BRL); FBS and bovine calf charcoal-stripped serum (CCS; Equitech-Bio Inc); crystal violet (Fisher Scientific). Growth factor reduced Matrigel (BD Biosciences). Antibodies were obtained from the following sources: LC3A/B, VEGFR2, phospho-VEGFR2 (Cell Signaling Technology); p62 [Western, BD Biosciences; immunohistochemistry (IHC), MBL International]; CD68 (AbD Serotec); CD31 (Abcam); ERα, β-actin, and polyclonal and horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology).

Cell culture
MCF7-RR and LCC9 human breast carcinoma cells were grown in phenol-red free IMEM media containing 5% CCS. MCF7 and ZR-75-1/ICI-R cells were grown in phenol-red IMEM media containing 5% FBS. MCF7 cells were obtained from Dr. Marvin Rich at the Michigan Cancer Foundation. MCF7-RR cells were obtained from Indiana University of Pennsylvania (Indiana, PA; ref. 21). LCC9 and ZR-75-1/ICI-R cells were generated by our group at Georgetown University (Washington, D.C.; ref. 22). Cells were grown at 37°C in a humidified, 5% CO2:95% air atmosphere.

Crystal violet assay
Human breast cancer cells (3 x 10^5 cells/mL) in IMEM containing 5% CCS were plated in 24-well tissue culture plates. On day 1 after plating, and every 3 days, thereafter cells were treated with varying doses (10–1,000 nmol/L) of either TAM or ICI and 1 µmol/L CQ. On day 6, media were aspirated and cells were stained with crystal violet. Cells were permeabilized using citrate buffer and absorbance was read at 480 nm on a plate reader.

Western blot hybridization
MCF7-RR and LCC9 cells were solubilized by sonication in radioimmunoprecipitation assay buffer lysis buffer.
Proteins were size fractionated by PAGE and then transferred to a nitrocellulose membrane. Nonspecific binding was blocked by incubation for one hour at room temperature with Tris-buffered saline containing 5% powdered milk and 1% Triton X-100. Membranes were incubated overnight at 4°C with primary antibodies, followed by incubation with polyclonal HRP-conjugated secondary antibodies (1:2000) for 1 hour at room temperature. Immunoreactive products were visualized by chemiluminescence (SuperSignal Femto West, Pierce Biotechnology) and quantified by densitometry using the ImageJ digital densitometry software (http://rsbweb.nih.gov/ij/).

**Orthotopic human breast cancer xenografts**

Five-week-old, intact, athymic nude mice (Harlan Laboratories) were injected with $1 \times 10^6$ LCC9 or MCF7-RR cells in Matrigel:IMEM (50:50%). Cells were injected orthotopically through a 3-mm skin cut into 4 different mammary fat pads per mouse (one abdominal and one thoracic mammary gland on each side). For the MCF7-RR xenografts, mice also received a low-dose 17β-estradiol pellet (0.36 mg, 60-day release; Innovative Research of America) and Biomathematics at Georgetown University Medical Center (Washington, D.C.). Tumor volumes were obtained by measuring the longest perpendicular axes. We estimated the number of vessels/microvessels in each tumor section by counting the number of vessels/microvessels that intersected the longest diameter of the tumor section. The number of vessels/microvessels was counted at 200 magnification, and a final score was expressed as the mean vessel density (24, 25). The effect of antiestrogen treatment was confirmed by staining of the estrus cycle; mice on TAM or ICI therapy did not cycle.

**Tissue staining and immunohistochemistry**

Mammary tumors were fixed in 10% phosphate-buffered formalin for 24 hours before embedding in paraffin. Embedded tissues were cut into 5 μm thick sections and stained with hematoxylin and eosin (H&E) to determine morphology. Immunostaining was performed with an antibody to CD31 (1:100), pVEGFR2 (1:100), p62 (1:1000), LC3 (1:100), CD68 (1:100) using the streptavidin-biotin method. Stained sections were visualized and photographed. Because CD31 and pVEGFR2 staining was heterogeneous, data were quantified using a common method of angiogenic marker quantification defined as "hotspot" quantification. In summary, three "hotspots" in the tumor were selected, the number of vessels/microvessels in each "hotspot" was counted at ×200 magnification, and a final score was expressed as the mean vessel density (24, 25). The longest diameter of each CD31+ blood vessel was measured using ImageJ and shown as average vessel diameter (Supplementary Fig. S3B).

**Cytokine analysis**

Plasma from treated mice was collected at necropsy and immediately frozen. Quansys Biosciences Q-Plex Array kits were used to measure the following mouse cytokines and chemokines: interleukin (IL) 1β, IL2, IL3, IL4, IL5, IL6, IL12p70, IL17, TNFa, IFNγ, MCP1, RANTES, Eotaxin, KC, MDC, TARC, TCA3.

**Macrophage differentiation and cytotoxicity assay**

U937 human monocyte cells were treated in the presence of IFNγ (to differentiate monocytes into activated macrophages) and either 100 nmol/L TAM, 1 μmol/L CQ, 100 nmol/L ICI, or the combination of an antiestrogen and CQ for 72 hours. Macrophages were counted and plated with 70% confluent MDA-MB-231 (triple-negative, antiestrogen non-responsive breast cancer cells) in a 1:5 ratio of macrophages to cancer cells for 72 hours. Macrophage killing was then assessed by RTCA-ACEA xCELLigence® system through electrical impedance (26).

**Statistical analysis**

Data are presented as the mean ± SEM. For most studies, Student t test (pairwise) or ANOVA followed by Bonferroni posthoc tests (group wise) was used (Prism software). Statistical differences for in vivo tumor area were evaluated by Dr. Fang in the Department of Biostatistics, Bioinformatics and Biomathematics at Georgetown University Medical Center (Washington, D.C.). Tumor volumes were obtained from measurements of the longest perpendicular axes. We use the weighted F test for comparison between two groups with two tumor volumes because the behavior of two tumors growing in a single mouse are not independent events (27). All computation was performed in the R-environment. There were 26 mice for LCC9 xenografts, and 19 mice for MCF7RR xenografts. Criterion for statistical significance was set at $P \leq 0.05$.

**Results**

Antiestrogen-sensitive MCF7 cells and antiestrogen-resistant MCF7-RR (TAM resistant), ZR-75-1 ICI-R (ICI resistant), and LCC9 (ICI resistant/TAM cross-resistant) breast cancer cells were plated in 24-well dishes and treated with vehicle, 1 μmol/L CQ, and/or various concentrations of TAM or ICI (vehicle, 10, 100, 1,000 nmol/L) for 6 days. The effect on cell density was determined by crystal violet assays (28). The combination of antiestrogen and CQ potentiated TAM and ICI-mediated cell death in endocrine-sensitive MCF7 cells (Fig. 1A). Furthermore, CQ and antiestrogen therapy inhibited LCC9 cells (Fig. 1B). MCF7-RR (Fig. 1C), and ZR-75-1 ICI-R (Fig. 1D). Moreover, the addition of CQ resulted in increased LC3-II formation (lipidated form of LC3 that is a marker of autophagosome formation) and accumulation of p62 (marker of autophagosomes) in both MCF7-RR (Fig. 1E) and LCC9 (Fig. 1F) as determined by Western blot hybridization. Increased LC3-II and p62 accumulation of p62 (marker of autophagosomal flux) in LC3 was a marker of autophagosome formation and accumulation of p62 (marker of autophagosomes) in both MCF7-RR (Fig. 1E) and LCC9 (Fig. 1F) as determined by Western blot hybridization.
expression is indicative of inhibited autophagic flux resulting in the cellular accumulation of autophagosomes. Treatment of MCF7-RR cells with TAM or LCC9 cells with TAM or ICI resulted in increased LC3-II with a corresponding decrease in p62 levels confirming previous studies that antiestrogens stimulate autophagic flux (10).

LCC9 and MCF7-RR cells were orthotopically injected into the mammary fat pads of female athymic mice. Tumors were grown to 20 to 35 mm$^2$ before treatment with CQ and/or TAM (MCF7-RR), or with CQ and/or TAM or ICI (LCC9). In LCC9 xenografts, TAM+CQ was most effective at reducing initial tumor growth (Fig. 2A and B), but after 3 weeks of treatment, CQ alone was just as effective at inhibiting LCC9 as the TAM+CQ combination. Unexpectedly, the combination of ICI+CQ was less effective than either TAM+CQ or CQ treatment alone. TAM or ICI treatment alone had no significant difference in tumor area when compared with control tumors. When tumor growth was measured as wet weight at necropsy, LCC9 tumors treated with CQ alone, TAM+CQ, or ICI+CQ had significantly reduced tumor wet-weight when compared with control tumors (Fig. 2C). However, CQ alone was significantly more effective than ICI+CQ, suggesting a potentially antagonist interaction between ICI and CQ. In
MCF7-RR orthotopic xenografts, control tumors continued to grow, whereas TAM+ or TAM significantly reduced tumor size (Fig. 2D and E). CQ or TAM alone had no significant effect on MCF7-RR tumor growth when compared with controls. The combination of TAM+CQ significantly reduced MCF7-RR tumor wet weight when compared with control-treated tumor weight measured at necropsy (Fig. 2F).

Formalin-fixed LCC9 tumors were embedded in parafin and cut into 5 μm sections. Tumor sections were stained with specific antibodies against either LC3 (Fig. 3A) or p62 (Fig. 3B) using an avidin-biotin technique that reacts with peroxidase-conjugated streptavidin substrate to determine the effects of treatments on autophagy markers. LCC9 tumors from mice treated with ICI, TAM, or CQ show elevated LC3A/B staining (Fig. 3C). Measuring autophagic activity using immunohistochemistry can be challenging often because it is difficult to differentiate between LC3 and LC3-II by IHC in tissue sections. However, the high level of magnification (∗1,000) shows positive LC3 staining forming puncta-like structures broadly consistent with autophagy induction. Tumors from animals treated with ICI or TAM have reduced p62 expression, consistent with increased autophagic flux (Fig. 3C). In contrast, tumors from mice treated with CQ have elevated p62 expression, suggesting a block in the later stages of autophagy (29, 30). To corroborate the systemic effect of CQ on autophagy, uterine tissue from control mice or mice treated with CQ were isolated and Western blot hybridization was performed on protein lysates for LC3-II and p62 levels. As shown in Fig. 3D, CQ dosing increases LC3-II formation and results in the accumulation of p62, suggesting that CQ systemically inhibits autophagy.

To confirm antiestrogen drug effectiveness, LCC9 parafin-embedded tumor sections were stained for ERα or progesterone receptor (PR; Supplementary Fig. S2). ICI reduces ERα staining and PR staining in LCC9 tumors, consistent with the known effects of this drug. TAM
treatment resulted in a cytoplasmic diffuse distribution of ERα and reduced PR staining, consistent with observed drug activity.

Treatment of LCC9 cells with CQ, ICI, or TAM in vitro increased pVEGFR2 expression determined by Western blot hybridization (Supplementary Fig. S3A), suggesting that antiestrogen therapy stimulates proangiogenic signaling in drug-resistant breast cancer cells. Thus, LCC9 tumor sections were stained with antibodies against either CD31 (an endothelial cell marker) or phosphorylated pVEGFR2. TAM and ICI treatment increased CD31-positive vessel staining (Fig. 4A and B) and elevated levels of pVEGFR2 (Fig. 4C and D), implying increased angiogenesis. To confirm the effect of antiestrogen therapy on angiogenesis, mice were injected with 500 μL of Matrigel between the abdominal wall and the skin to form a Matrigel plug. Mice were either untreated (as control) or treated with TAM, ICI, CQ, ICI+CQ, or TAM+CQ. After 5 days, the plug was removed, fixed in formalin, and embedded in paraffin. Sections of Matrigel plug were stained with H&E, as shown in Fig. 5A. Mice treated with ICI showed increased number of infiltrating cells into the Matrigel plug (Fig. 5B), which was not observed in Matrigel plugs from animals treated with TAM. However, treatment with CQ in combination with either antiestrogen had no effect on CD31 staining or pVEGFR2 staining, suggesting that the stimulation of angiogenesis likely does not explain the differential effects observed between ICI+CQ and TAM+CQ treatment.

Cytokine production and reticuloendothelial cell infiltrates are common in some breast cancers (31). Thus, blood was collected at the time of euthanasia to measure various cytokine concentrations. We measured the mouse cytokines and chemokines IL1β, IL2, IL3, IL4, IL5, IL6, IL12p70, IL17, TNFα, IFNγ, MCP1, RANTES, Eotaxin, KC, MDC, TARC, and TCA3 using Q-Plex Array kits from Quansys Biosciences (Supplementary Figs. S4–S6). Although variability in plasma cytokines is high, several cytokine trends are evident between treatment groups. Of particular interest, the mouse chemokine KC (human analog: chemokine C-X-C motif ligand 1; CXCL1) is elevated in the plasma of TAM and TAM+CQ mice and decreased in ICI and ICI+CQ-treated mice. Also, IFNγ is reduced in ICI and ICI+CQ-treated animals.

Because CXCL1 is expressed by macrophages and IFNγ stimulates macrophages, we investigated whether there were differential effects of antiestrogens and CQ on macrophages. LCC9 tumor sections were stained with antibodies against ERα and/or GPR30. The precursor U937 monocyte cells do not express GPR30 but express...
ERα, albeit at a lower level than the breast cancer cells. Conversely, differentiated U937 macrophage cells do not express detectable ERα but express higher levels of GPR30 than MCF7-RR, LCC9, or their parental U937 monocyte cells.

To determine whether antiestrogens or CQ affect macrophages’ tumor cell killing capacity, U937 cells were differentiated in the presence of IFNγ and vehicle control, 1 μmol/L CQ, 100 nmol/L ICI, 1 μmol/L CQ + 100 nmol/L ICI, 100 nmol/L TAM, or 1 μmol/L CQ + 100 nmol/L TAM for 72 hours. Macrophages were then collected, resuspended in fresh media to remove drug, and added to preplated MDA-MB-231 breast cancer cells. Use of these ER-negative cells prevented any remaining antiestrogen from inhibiting the target cells. Monocytes, differentiated into macrophages in the presence of ICI, ICI + CQ, and TAM, had a decreased breast cancer cell killing capacity at 24 and 48 hours after plating when compared with their vehicle-treated controls (Fig. 6D). However, in the presence of CQ or TAM + CQ, macrophages had no significant difference in their cell killing capacity when compared with the vehicle-treated control macrophages. Seventy-two hours after the addition of macrophages, only macrophages in the presence of ICI and ICI + CQ had significantly reduced breast cancer cell killing capacity as measured by cell index.

Discussion
Breast cancer remains the most prevalent cancer in women, with the majority of these tumors expressing ERα. Resistance to endocrine therapies remains a critical limitation in the ability of these agents to cure some patients. Autophagy is a key pathway in the development of endocrine resistance in breast cancer, and targeting autophagy can reverse antiestrogen resistance (6). Chloroquine, an antimalarial drug, inhibits autophagy by preventing degradation of autolysosomes. Moreover, chloroquine derivatives, such as hydroxychloroquine (HQC), in combination with antineoplastic chemotherapeutic drugs or radiotherapy treatments inhibit multiple cancer cell types (32, 33). We now show that using chloroquine therapy in combination with antiestrogens increased the sensitivity of resistant breast cancer cells to endocrine therapies (Fig. 1A–C).

Resensitization to antiestrogens is characterized by an increase in LC3-II levels, the lipidated form of LC3 that is found in the autophagosomal membrane, implying an increase in the rate of autophagy initiation. In contrast, increased levels of p62 indicate autophagosome accumulation (Fig. 1E–F) leading to undegraded autophagosome accumulation in the cytoplasm (34), suggesting that the later steps in autophagy are not completed. When measured with changes in LC3, decreased p62 generally indicates that the autolysosome and its cargo have been degraded and that autophagic flux is intact. LCC9 or MCF7-RR cells treated with either TAM or ICI showed increased LC3-II and reduced p62 expression, consistent with prior reports that endocrine-targeting therapy induces autophagic flux (6, 10, 35).

A synthetic quinine analog often used for chloroquine-resistant malarial cases, mefloquine (Lariam), was also shown to inhibit autophagy and induce cell death in MCF7 antiestrogen-sensitive breast cancer cells when given as a single agent (36). Chloroquine prevented DCIS xenografts’ outgrowth in athymic mice (37, 38) and...
inhibited N-methyl-N-nitrosurea–induced mammary carcinogenesis, suggesting chloroquine-based therapy as a possible agent in the prevention of initial premalignant lesions from progressing to breast cancer (39).

We show that oral low-dose CQ given to tumor-bearing mice in combination with an antiestrogen resulted in the resensitization of resistant MCF7-RR (Fig. 2A and C) and the potentiation of antiestrogen-mediated cell death in LCC9 breast tumors (Fig. 2B and D). Overall, CQ is likely to be an effective chemotherapeutic agent in combination with specific antiestrogen therapy for the treatment of some ER$^+$ breast cancers. Nonetheless, because established tumors were not eliminated completely, that is, “cured,” during the 5-week treatment, improvements in overall response rates to CQ in adjuvant settings may be meaningful but modest. The efficacy of CQ in prevention remains to be evaluated with the results of the ongoing PINC clinical trial. Importantly, because increased LC3A/B and p62 were observed in LCC9 tumors from mice treated with CQ (Fig. 3), consistent with an inhibition of autophagy, changes in expression of these markers in tumors may be worth evaluating as possible biomarkers for predicting treatment responses in patients.

Although a combination of both CQ+TAM and CQ+ICI resensitized resistant breast cancers to therapy, the combination of CQ and ICI was less effective at reducing tumor burden than the combination of TAM+CQ at the doses tested. We hypothesized that systemic ICI therapy negatively affects the tumor microenvironment to reduce effectiveness of the CQ+ICI combination. LCC9 tumors from mice treated with ICI, ICI+CQ, TAM, or TAM+CQ, each displayed increased CD31 (Fig. 4A–B) and phospho-VEGFR2 (Fig. 4C–D) immunoreactivity. Thus, antiestrogens can stimulate tumor angiogenesis in endocrine-resistant tumors and CQ has no effect on this response. Moreover, LCC9 cells treated with antiestrogens in vitro displayed increased expression of phospho-VEGFR2 (Supplementary Fig. S3), suggesting that the effect of TAM and ICI on angiogenesis is mediated directly by the tumor cells. Because both TAM and ICI stimulated angiogenesis, increased blood vessel formation is unlikely to explain the differential antitumor effects observed between TAM+CQ and ICI+CQ treatments. Previous reports showed that TAM but not ICI increased VEGFR2 expression in antiestrogen-sensitive MCF7 cells, further suggesting that the increase in angiogenesis observed in both TAM and ICI-treated tumors is due to a direct effect mediated by the tumor epithelial cells (40). To determine the effect of antiestrogen therapy on cell invasion, standard Matrigel plug assays were performed (Fig. 5). Although both TAM and ICI stimulated tumor angiogenesis (Fig. 4), only ICI and ICI+CQ-treated mice exhibited increased cell invasion into their Matrigel plugs. These data suggest that ICI and TAM have different effects on the tumor microenvironment.

Interestingly, analysis of circulating cytokines and chemokines in mice treated with CQ and antiestrogens indicate that ICI and TAM may have a differential effect on the macrophage population (Supplementary Figs. S4–S6).
Macrophages can be classified as M1-like (cytotoxic) or M2-like (immunosuppressive) and often serve opposing roles, highlighting the plasticity of these cell types (41). Estrogen was previously shown to affect the monocyte-macrophage system (42, 43), suggesting that antiestrogen therapy may also perturb these cells. When LCC9 tumors were stained for the macrophage marker CD68 (Fig. 6A), mice treated with ICI and ICI+CQ showed decreased staining when compared with other treatments (Fig. 6B). Thus, ICI seems to affect negatively macrophage development and/or chemotaxis. Moreover, circulating levels of IFNγ in mice treated with ICI and ICI+CQ were reduced when compared with TAM and TAM+CQ-treated mice. IFNγ can increase development of the M1-like cytotoxic macrophages (41), suggesting that systemic ICI treatment may reduce the cell-killing capacity of macrophages. As shown in Fig. 6C, human monocytes express ERα albeit at a reduced level than the breast cancer cells, whereas ERα expression is lost in the macrophages. Moreover, macrophages have elevated GPR30 when compared with their parental monocyte cells and breast cancer cells. GPR30 is a GPR that may act as an ER and promotes cellular growth and activation. TAM’s partial estrogen agonist activity was shown to also activate GPR30 (44), which may explain, in part, the elevated macrophage number in the tumors from TAM+CQ-treated animals.

To confirm the effect of antiestrogen treatment on macrophage development and cytotoxicity, U937 monocytes...
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were differentiated into macrophages in the presence of IFN and either ICI, TAM, CQ, CQ + TAM, or CQ + ICI. Macrophages that were differentiated in the presence of ICI, ICI + CQ, or TAM had decreased tumor cell cytotoxicity, indicating that systemic antiestrogen treatment reduces macrophage activity (Fig. 6D). Furthermore, only ICI and ICI + CQ-treated macrophages had a significantly higher cell index (reduced cytotoxicity) at 72 hours postmacrophage addition to breast cancer cells, whereas TAM and TAM + CQ produced no significant difference in cell index when compared with vehicle-treated controls. These data suggest that ICI but not TAM had deleterious effects on macrophage distribution, activation, and/or cytotoxicity that may result in the potentially antagonistic interaction seen with the ICI + CQ combination therapy. An ongoing clinical trial is examining the effect of combining TAM and chloroquine for the treatment of ERþ DCIS patients: PINC (see clinicaltrials.gov/show/NCI01023477). Preclinical data showed that chloroquine treatment inhibited DCIS cell tumorigenesis in NOD/SCID mice, indicating the possibility of using chloroquine as a chemopreventive drug for breast cancer (38). In this study, we show that CQ in combination with antiestrogens potentiates endocrine therapy sensitivity in acquired resistant ERþ breast tumors. More importantly, this study highlights a potentially nonbeneficial effect of Faslodex (ICI) on the tumor microenvironment that reduces the value of combining chloroquine-based therapies with Faslodex. Future clinical trial study designs may consider using TAM as the preferred antiestrogen to optimize the effects of an antiestrogen in the tumor microenvironment. In our studies, a combination of CQ + TAM was more effective than either drug alone.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: K.L. Cook, R. Clarke
Development of methodology: K.L. Cook, A. Warri
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.L. Cook, D.R. Soto-Pantoja, P.A.G. Clarke, M.I. Cruz, A. Zwart
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): K.L. Cook, D.R. Soto-Pantoja, R. Clarke
Writing, review, and/or revision of the manuscript: K.L. Cook, A. Warri, R. Clarke
Study supervision: R. Clarke

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References


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Correction: Hydroxychloroquine Inhibits Autophagy to Potentiate Antiestrogen Responsiveness in ER⁺ Breast Cancer

In this article (Clin Cancer Res 2014;20:3222–32), which was published in the June 15, 2014, issue of Clinical Cancer Research (1), the corresponding author informed us of a nomenclature error in the published article. The primary chemical form of the drug used in each of the in vitro and in vivo experiments presented in the study was chloroquine (CQ) diphosphate and not hydroxychloroquine (HCQ) diphosphate as reported in the text. CQ is chloroquine diphosphate (Sigma-Aldrich, catalog number: C6628), i.e., \(N^4\)-(7-chloro-4-quinolinyl)-\(N^1\).\(N^1\)-dimethyl-1,4-pentanediamine diphosphate salt; Sigma does not sell HCQ diphosphate. Hydroxychloroquine is a hydroxyl derivative of chloroquine. The conclusions put forth in this article remain unchanged.

The online version of this article has been changed to reflect the correct nomenclature and no longer matches the print version. The authors regret this error.

Reference

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