Biochemical and Biophysical Research Communications 425 (2012) 150–156

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Synergistic killing effect of chloroquine and androgen deprivation in LNCaP cells

Ramesh R. Kaini, Chien-An A. Hu *

Department of Biochemistry and Molecular Biology and UNM Cancer and Treatment Center, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

ARTICLE INFO

Article history:
Received 10 July 2012
Available online 20 July 2012

Keywords:
Prostate cancer
Androgen deprivation
Chloroquine
Autophagy
Apoptosis
Bioenergetics

ABSTRACT

Modulation of autophagy is a new paradigm in cancer therapeutics. Recently a novel function of chloroquine (CLQ) in inhibiting degradation of autophagic vesicles has been revealed, which raises the question whether CLQ can be used as an adjuvant in targeting autophagic pro-survival mechanism in prostate cancer (PCa). We previously showed that autophagy played a protective role during hormone ablation therapy, in part, by consuming lipid droplets in PCa cells. In addition, blocking autophagy by genetic and pharmacological means in the presence of androgen deprivation caused cell death in PCa cells. To further investigate the importance of autophagy in PCa survival and dissect the role of CLQ in PCa death, we treated hormone responsive LNCaP cells with CLQ in combination with androgen deprivation. We observed that CLQ synergistically killed LNCaP cells during androgen deprivation in a dose- and time-dependent manner. We further confirmed that CLQ inhibited the maturation of autophagic vesicles and decreased the cytosolic ATP. Moreover, CLQ induced nuclear condensation and DNA fragmentation, a hallmark of apoptosis, in androgen deprived LNCaP cells. Taken together, our finding suggests that CLQ may be an useful adjuvant in hormone ablation therapy to improve the therapeutic efficacy.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Prostate cancer (PCa) is the most common cancer among men of all races in the United States [1,2]. About 1 in 6 men will be diagnosed of PCa in his lifetime. There are three standard types of treatment in prostate cancer: surgical resection, radiation therapy and hormone ablation therapy. In general, surgical resection and radiotherapy are conducted when the tumor is localized [3]. However, once metastasized, prostate tumors are hard to cure and bear poor prognosis [1–3]. It is well known that androgens are essential for survival, proliferation, and progression of PCa cells [4,5]. Androgen ablation induces apoptosis and regression of hormone responsive PCa cells [6,7], and thus is a treatment option when PCa cells metastasize. However, unfortunately, some PCa cells can escape and survive the treatment and subsequently develop hormone/castration resistant phenotype [8,9]. Developing combinational therapy that would enhance the therapeutic effectiveness of hormone ablation is crucial for PCa treatment.

We previously reported that during hormone ablation, autophagy was induced in two different hormone sensitive PCa cell lines, LNCaP and LAPC4 cells [1]. Autophagy is a cytosolic mechanism in which a portion of the cytosol constituents including protein aggregates, aberrant organelles and lipid droplets (LDs) is enwrapped by newly formed organelles, known as autophagic vesicles (AVs), and targeted to lysosomes for hydrolysis [10–12]. These AVs can be induced during androgen deprivation and can sequester LDs, a mechanism known as lipophagy [1,13–15]. As PCa cells are rich in LDs and rely predominantly on β-oxidation for their bioenergetic needs, this alternate pathway of lipolysis is crucial for their survival during hormone ablation therapy [1,16,17]. We have demonstrated that inhibition of autophagy by 3-Methyladenine (3-MA) or si-ATG5 significantly killed more cells growing in the absence of androgen than cells growing in regular medium, suggesting that autophagy protects LNCaP cells during androgen deprivation [1]. Similar results were reported in two other studies where genetic knockdown of autophagy caused more cell death in the absence of androgen [18,19]. In addition, modulation of autophagy in cancer therapeutics is promising and inhibition of autophagy by either 3-MA or chloroquine (CLQ) has been shown to sensitizes LNCaP cells to Src kinase inhibitors, such as saracatinib [20]. 3-MA is a PI3-kinase inhibitor which blocks the early stage of autophagosome formation, whereas CLQ, a lysosomotropic drug which inhibits the function of autolysosomes [21], causes accumulation of AVs, and prevents the breakdown of enwrapped materials in AVs. Furthermore, CLQ has been used widely as an effective anti-malarial and anti-rheumatoid drug [24]. Interestingly, CLQ has been shown to potentiate the cytotoxic effects of 5-fluorouracil, Akt inhibitors, and other drugs in cancer cells [21–24]. Taken together, these reports suggest that CLQ is a potential candidate to enhance the anti-cancer effect of androgen deprivation in PCa. As autophagy induction during androgen ablation helps protect LNCaP cells, we hypothesized that CLQ would augment the apoptotic effect of androgen deprivation in LNCaP cells. In this study, we treated LNCaP cells with CLQ in the absence

* Corresponding author. Address: Department of Biochemistry and Molecular Biology University of New Mexico Health Sciences Center, MSC08 4670, Albuquerque, NM 87131-001, USA. Fax: +1 505 272 6587.
E-mail address: AHu@salud.unm.edu (C.-A.A. Hu).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.07.054

of androgen and characterized the cell death pathway that was activated.

2. Materials and methods

2.1. Chemicals and reagents

Charcoal-filtered fetal bovine serum (CFM) was purchased from Hyclone (Thermo Fisher, MA, USA), RPMI 1640, and regular FBS was from Gibco/Invitrogen (Eugene, OR, USA). Chloroquine diphosphate salt was purchased from Sigma Aldrich (St Louis, MO, USA) and dissolved in H_2O. Trihydrochloride trihydrate was purchased from Molecular Probe (Eugene, OR, USA). Si-ATG5 duplexes (SR306286) were purchased from Origene (Rockville, MD, USA) as previously described [1].

2.2. Cell culture and transfection with pEGFP-LC3

Human LNCaP cell line was grown in control medium (CM, RPMI medium with 10% fetal bovine serum) at 37 °C and 5% CO_2. LNCaP.EGFP-LC3 cells, LNCaP stably transfected with pEGFP-LC3, were generated as previously described [1]. To investigate the effect of androgen deprivation, cells were grown in CFM (RPMI medium with 10% charcoal-filtered fetal bovine serum). CLQ was added at the indicated concentration.

2.3. Cell viability assay and ATP measurement

LNCaP cells were seeded in equal number (10,000 cells/well) in 96-well plates and cultured in CM or CFM overnight. CLQ was added at indicated concentration and incubated for 24 h or other time points where specified. The cell viability was then examined by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to manufacturer's instructions (Vybrant, Invitrogen, Eugene, OR, USA). Intracellular ATP levels were measured using a colorimetric assay kit (Abcam, San Francisco, CA, USA) followed the manufacturer's instruction.

2.4. LC3-II translocation assay

LNCaP.EGFP-LC3 cells were grown on cover slips, washed in 1X PBS and then incubated in CM or CFM in the presence or absence of CLQ for 24 h, and then observed under a LSM 510 confocal microscope. Number of cells and distinct puncta were counted from 10 random visual fields for each slide. The number of puncta per cell was used as a measure of autophagic activity as previously described [1,25,26].

2.5. Annexin-v/propidium iodide (PI) assay

Cells were treated with CLQ for 36 h in CM or CFM. Both, the floating cells and the attached cells were harvested. Cell pellets were washed twice with 1X DPBS and then resuspended in 1X Binding buffer at a concentration of 1×10^6 cells/ml. One hundred microliter of the suspension was used to label with Annexin V-FITC and PI (BD Biosciences, San Diego, CA, USA) and analyzed by flow cytometry as previously described [25].

2.6. Hoechst nuclear staining

Cells were inoculated on coverslips in 6 well plates, cultured, and treated with CLQ for 36 h, or transfected with 10 nM Si-ATG5 duplexes for for 36 h as previously described [1]. Attached cells were washed and stained with Hoechst 33342 and observed under a Zeiss Axioskop microscope as previously described [25].

2.7. DNA fragmentation assay

Cells were seeded in equal number and cultured in CM or CFM and then treated with CLQ for another 36 h. The floating cells were collected, washed with 1X PBS, lysed with lysis buffer [10 mM Tris (pH7.4), 5 mM EDTA, and 1% Triton X-100] for 20 min on ice, and centrifuged at $11,000 \times g$ for 20 min. The supernatant was collected and treated with RNase A and Proteinase K for 1 h at 37 °C. DNA was extracted using phenol–chloroform method, resuspended in equal volume of $1 \times$ TE, and then electrophoresed in 0.8% agarose gel. DNA was visualized using ethidium bromide staining.

2.8. Statistical analysis

Each experiment was performed in triplicate and the data are presented as the mean ± standard deviation (SD). Data were analyzed by Student's t test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. CLQ and androgen deprivation synergistically kills LNCaP cells

To study the effect of CLQ on the survival of LNCaP cells, 10,000 cells were seeded in 96-well plates in regular medium (CM) or androgen deprived medium (CFM). Both groups were treated with increasing concentrations of CLQ (0–1,000 μM) for 24 h. Viable cells were quantified using MTT assay. The cytotoxicity of CLQ was observed that was correlated with concentrations of CLQ in both CM and CFM (Fig. 1). When cells were treated with 200 μM of CLQ for 24 h, there was little effect on cells grown in CM, whereas there was 20% reduction in cell grown in CFM. Interestingly, at 400 μM, CLQ had greater cytotoxicity (~2 folds) in cells treated with CFM than with CM. At 600–1,000 μM, CLQ had increased cytotoxicity effect on cells grown in either CM or CFM, however, CLQ caused more cell death (approximately 30% greater) in CFM, demonstrating that CLQ and androgen deprivation synergistically killed LNCaP cells. Because CLQ has a long half life ($t^1/_2$ = 2–6 days) [22,27] and 200 μM of CLQ showed cytotoxicity on cells grown in CFM, we looked at the time dependent effect of

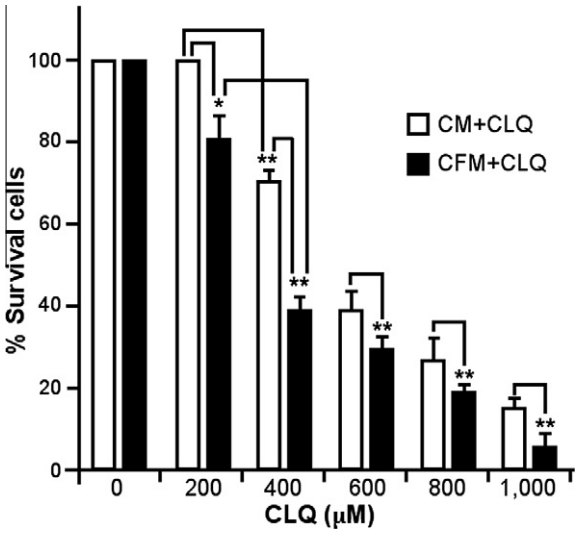

Fig. 1. Survival effect of chloroquine (CLQ) on LNCaP cells. Cells were incubated in the presence (CM) or absence of androgen (CFM). (A) CLQ was added at increasing concentration (0–100 μM) and treated for 24 h. Viable cells were quantified by MTT assay. Bars represent mean ± SEM (n = 4, $^*P < 0.05$, $^{**}P < 0.01$, significant difference comparing to the indicated sample by Student's t-test).

200 μM of CLQ during androgen deprivation. LNCaP cells treated with CFM in the presence of 200 μM CLQ reduced the number of viable cells by 30%, whereas treatment for 3 days reduced the number by more than 50% compared to cells grown in CFM (Fig. 2 A and B).

3.2. CLQ blocks autophagic degradation and reduces cytosolic ATP in LNCaP cells

To investigate if CLQ affected the maturation of autophagic vesicles (AVs), we incubated LNCaP.EGFP-LC3 cells with CLQ (200 μM) in the presence or absence of androgen. We previously showed that incubation of LNCaP cells in CFM induced translocation of cytosolic EGFP-LC3 to puncta like AVs. We also showed that blocking of autophagy by genetic (si-ATG5) of pharmacological (3-MA) means induced cell death in PCa cells grown in CFM [1]. As shown in Fig. 3A and B, CLQ also induced increase of puncta in cells cultured in CM (>2 folds); however the accumulation of puncta was even greater (>7 folds) in cells cultured in CFM, suggesting that CLQ blocked degradation of AVs (Fig. 3A, panels c and d; B, columns 3 and 4).

To study the effect of CLQ on bioenergetics, the cytosolic ATP was measured in LNCaP cells treated with CLQ in CM or CFM. Interestingly, without CLQ, there was no change in the amount of ATP in cells cultured in CM and CFM (Fig. 3C, column c and a). However, CLQ alone decreased the cytosolic ATP by 20% in cells grown in CM (Fig. 3C, column b), whereas combinational treatment of CLQ and CFM decreased the cytosolic ATP level by 38% (Fig. 3C, column d), suggesting that autophagy dependent metabolism is more prominent in androgen-deprived LNCaP cells.

3.3. CLQ induces DNA fragmentation and nuclear condensation, hallmarks of apoptosis, in LNCaP cells

To characterize the death pathway during CLQ treatment, cells were incubated in CM or CFM in the presence or absence of 200 μM CLQ. Cells were harvested at 36 h time point and

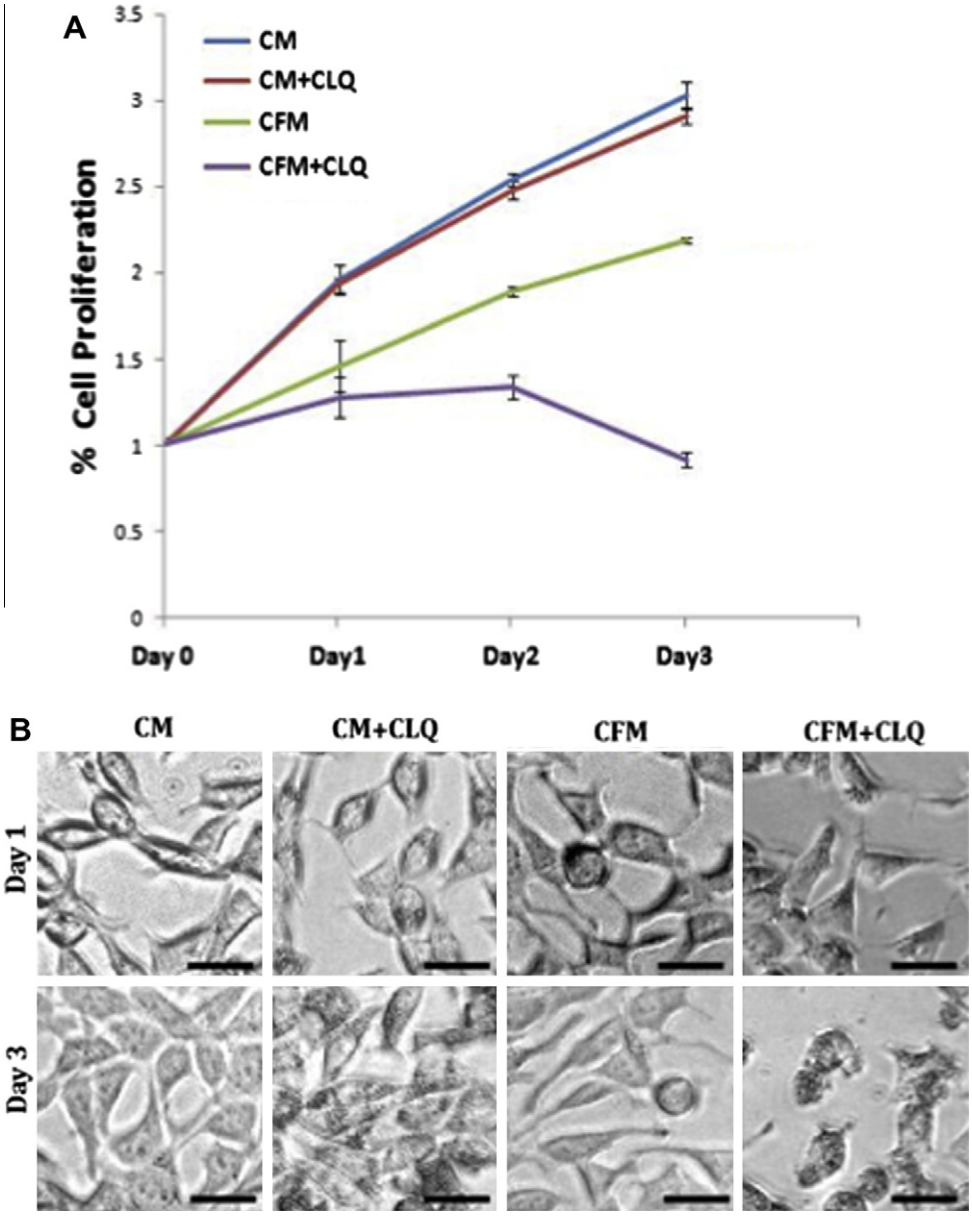

Fig. 2. Time dependent effect of 200 μM CLQ on the proliferation of LNCaP cells. (A). Equal number of cells were seeded and incubated in CM or CFM. CLQ (200 μM) was added to the medium and cells were treated for 1–3 days. Viable cells were quantified by MTT assay at each time point. Bars represent mean ± SEM ($n = 4$). (B). Light microscopic view of cells taken from day 1 and day 3. Scale bar = 30 μm.

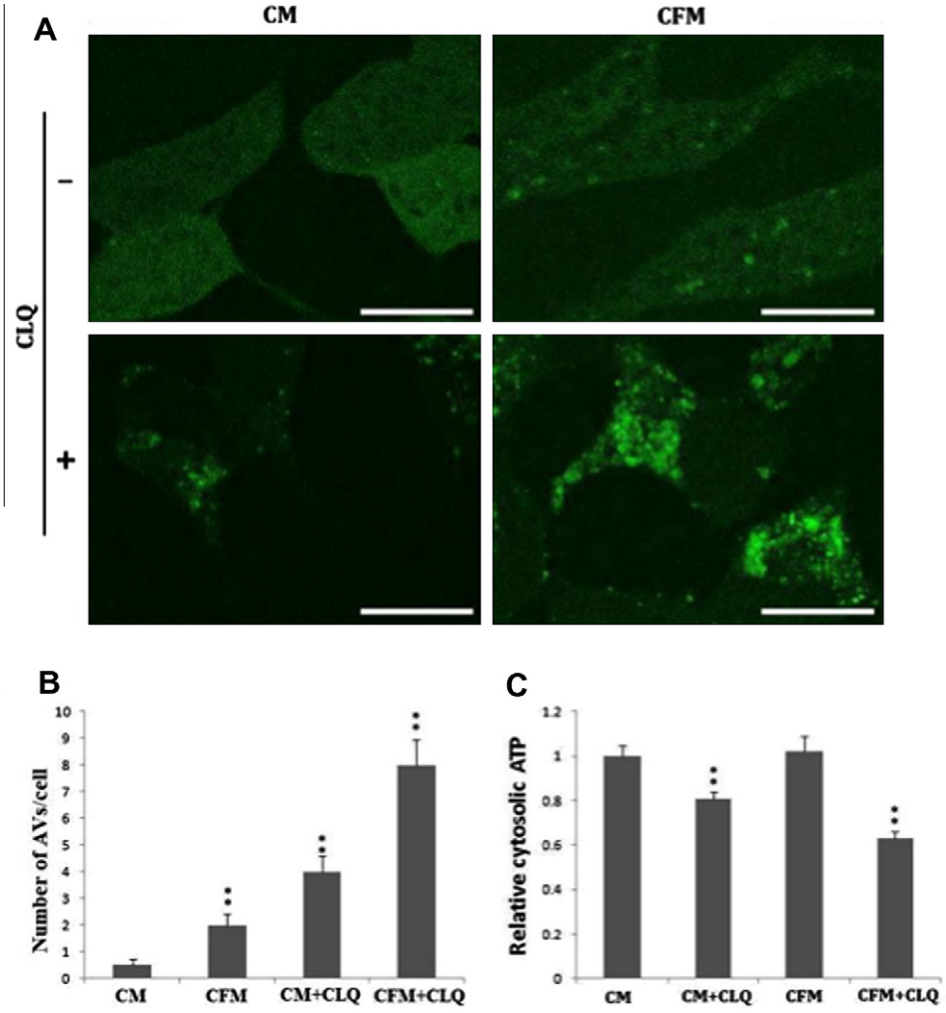

Fig. 3. Effect of CLQ on autophagic vesicles. LNCaP.EGFP-LC3 cells were incubated in CM or CFM as indicated. CLQ (200 μM) was added in the medium and incubated for 24 h. (A). Cells were fixed and observed under a confocal microscope. Numbers of cells and intracellular puncta were counted from 10 random visual fields for each group. Average numbers of puncta per cell were plotted and shown in (B. C). Intracellular ATP level was measured. Scale bar = 30 μm. B. Bars represent mean ± SEM ($n = 4$, $^{**}P < 0.01$, significant difference comparing to the indicated sample by Student's *t*-test).

co-stained with Annexin V-FITC and Propidium iodide (PI), and analyzed by flow cytometry. Data displayed in Fig. 4A were 2-color dot plots with FITC-Annexin V (green fluorescence, X axis) vs. PI (red fluorescence, Y axis). Previously, we and others observed that ~3% of LNCaP cell population underwent spontaneous apoptosis in regular medium [1,5,28]. Thus, we first set the line thresholds to reflect 3% cell population undergone apoptosis in the lower right quadrant of CM (panel a, Fig. 4A). We then applied the same line thresholds to calculate apoptosis in the other treatments. We observed that CFM or CM ± CLQ had little effect on apoptosis as compared to the control (7.04, 13.5 and 3.1% in CFM, CM + CLQ and CM, respectively; Fig. 4A panels a, b and d). CLQ, however, caused more synergistically with androgen deprivation (CFM + CLQ; 35.9%, ~12-fold increase; Fig. 4A, panel c). Notably, CLQ also caused increased necrosis in both medium as shown in the upper right quadrants of panels c and d (panel c vs. panel b; panel d vs. panel a).

To further confirm CLQ-induced cell death during androgen deprivation was apoptosis, we investigated nuclear/chromatin condensation and DNA laddering, two other hallmarks of apoptosis. Fig. 4B showed that combined treatment of LNCaP cells with CLQ and androgen deprivation (CFM + CLQ) or si-ATG5 and androgen deprivation (CFM + siATG5) caused significant alterations in nuclear morphology, known as nuclear condensation/fragmentation (Fig. 4B, panels c and e, respectively). Approximately 30% of the nuclei in cells treated with CFM + CLQ or CFM + siATG5 showed nuclear condensation. In contrast, nuclei in cells cultured in other media (CM (a), CFM (b), CM + CLQ (d), and CM + siATG5 (f)) were healthy. Moreover, Fig. 4C demonstrated that genomic DNA isolated from cells treated with CLQ + CFM was systematically fragmented in laddered pattern (lane 3), but not in samples from cells treated with androgen deprivation or CLQ alone (lane 2 and 4, respectively), demonstrating that blocking autophagy by CLQ or si-ATG5 in the presence of androgen deprivation resulted in apoptosis in LNCaP cells.

4. Discussion

In this study, we showed that combinational treatment of CLQ and androgen deprivation had better efficacy in killing LNCaP cells: CLQ synergistically killed LNCaP cells that were cultured in androgen-deprived medium in a dose- and time-dependent manner. We also observed that CLQ blocked the degradation of autophagic vesicles and therefore the completion of autophagy. Furthermore, we confirmed that autophagy is important for the generation of bioenergetic precursors as CLQ treatment lowered the cytosolic ATP. Moreover, CLQ treatment resulted in chromatin condensation and DNA degradation in androgen deprived LNCaP cells, as

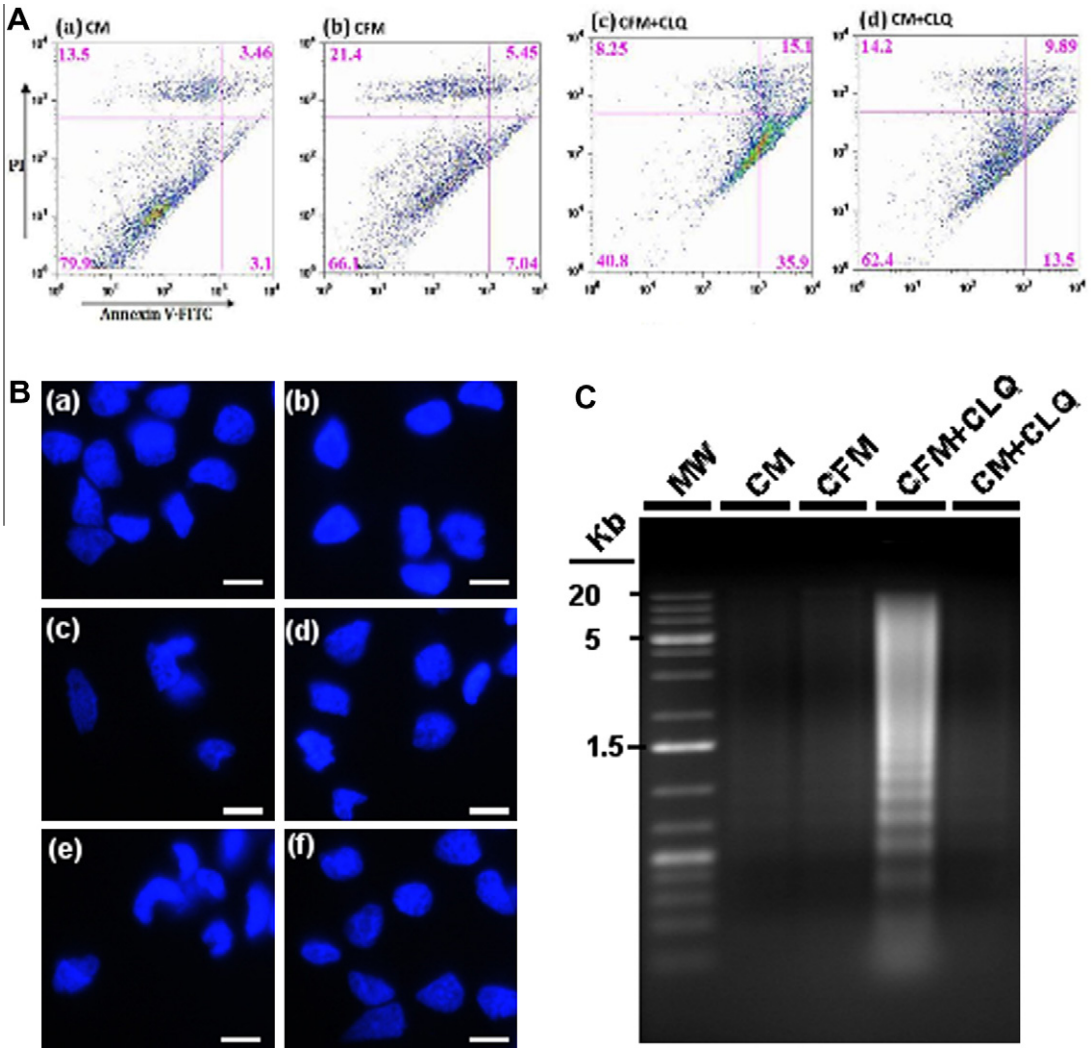

Fig. 4. CLQ treatment induces apoptosis in LNCaP cells during androgen deprivation. (A). Cells were incubated in CM or CFM and treated with CLQ (200 μM) for 36 h. Cells were harvested, double-labeled with Annexin-V-FITC (X axis) and PI (Y axis), and analyzed by flow cytometer. The number in the lower right quadrant of each panel represented the percentage of apoptotic cells, i.e., 3.1, 7.04, 35.9, and 13.5% in CM (a), CFM (b), CFM + CLQ (c), and CM + CLQ (d), respectively. (B). Cells grown on coverslips were treated with CLQ (200 μM) or si-ATG5 (10 nM) in CFM for 36 h. The attached cells were stained with Hoechst nuclear dye and observed under a fluorescent microscope. Panels were CM (a), CFM (b), CFM + CLQ (c), CM + CLQ (d), CFM + si-ATG5 (e), and CM + si-ATG5 (f). Scale bar = 10 μm. (C). DNA gel electrophoresis. DNA was extracted from the floating cells treated with CM (a), CFM (b), CFM + CLQ (c), and CM + CLQ (d) for 36 h and separated in 0.8% agarose gel. Lane c, representing the DNA isolated from (c), showed DNA laddering, a hallmark of apoptotic cell death. Sizes of DNA molecular weight (MW) makers were also indicated.

evidenced by the increase of Annexin V-positive and PI-negative, DNA laddering and nuclear abnormality in dying/dead cells. Our results are consistent with other reports that autophagy inhibition by CLQ or other autophagy inhibitors (e.g., si-ATG5) induces cell death in cancer cell types [1,29–38]. However, to our best knowledge, this is the first report that demonstrates a synergistic killing effect of CLQ and androgen deprivation in hormone-responsive PCa cells.

CLQ has been used to treat various diseases (e.g., malaria, rheumatoid arthritis and lupus) for more than 6 decades [22,39,40]. The application of CLQ as an adjuvant in cancer therapeutics has just emerged because it works as a weak intercalating agent of DNA and thus can halt the DNA repair process [41]. This function has been found useful in potentiating the killing effect of radiated cancer cells [22]. In addition, CLQ is a weak base that would be widely distributed in human body if administrated. At the cellular level, CLQ in its unprotonated form can diffuse through the plasma membrane. When CLQ gets into the acidic organelles (late endosomes and lysosomes), it is protonated and trapped inside the lumen of these organelles [22,27,28], causing increase in the pH, inactivating the lysosomal hydrolases, and thus lysosomal function. Dysfunctional lysosomes inhibit the maturation of autophagic vesicles (amphisomes and autolysosomes), and increase the accumulation of autolysosomes in human cells [27,29–33].

Effects of CLQ on autophagic vesicles are potentially important in cancer therapies where cells induce autophagy as a survival mechanism. It has been shown that in HT-29 colorectal cancer cells, treatment with 5-fluorouracil induces autophagy, however, in the presence of CLQ, autophagosomal degradation is inhibited and therefore potentiates the anti-cancer effect of 5-FU [21]. In another study, CLQ was shown to induce dose-dependent cell death in 5 different glioblastoma cell lines independent of p53 status suggesting that CLQ also affects cell death beyond its effect in DNA repair process. In addition, CLQ elevated accumulation of autophagic vesicles in glioblastoma cells, similar to that in colorectal cancer cells, and affected the levels and subcellular distribution of Cathepsin D [33]. These findings suggest that disrupted lysosomal function may play a role in CLQ-induced cell death. In another study, Wu and colleagues [20] showed that inhibition of autophagy by CLQ sensitized PCa cells to the treatment of Src

family kinase (SFK) inhibitors. Interestingly, SFK inhibitors have been shown to induce autophagic survival in PCa cells. Blocking autophagic survival by CLQ redirected SFK inhibitor-treated cells to apoptosis [42]. Importantly, similar to SFK inhibitors, many cancer therapeutics induce both apoptosis and autophagy in treated cells, and in most cases autophagy, functioning as a survival mechanism, counteract apoptotic cell death, resulting in drug treatment failure. Thus, a new paradigm in cancer therapeutics would be to develop drug modalities that can simultaneously induce apoptosis and inhibit autophagic survival.

We and others demonstrated that autophagy is induced in hormone-sensitive prostate cancer cells either by incubation in androgen deficient medium or treatment of an androgen inhibitor, Casodex [1,18,19]. Chemical inhibition of autophagy by 3-MA or genetic ablation of autophagy machinery (si-BECLIN1, si-ATG5) causes more cell death, suggesting that autophagy protects PCa cells during androgen deprivation. We also observed that one survival mechanism of autophagy is to sequester lipid droplets and target to lysosomes for the generation of precursors for cellular bioenergetic needs [1]. Lysosomes are cellular digestive organelles critically regulate the pH-dependent catabolism of extracellular and intracellular macromolecules delivered from the endocytic/heterophagy and autophagy pathways, respectively. The unique ability of lysosomes to compartmentalize degradation within their lumen protects the rest of the cell from the induction of oxidative stress, the accumulation of aggregated macromolecules and organelles, and the random degradation of cytosolic constituents. Under conditions of cell stress, for example, change of intracellular pH, however, lysosome function and integrity may become compromised and can trigger apoptosis [13–15].

Androgen ablation therapy is one of the major treatment modalities in PCa. However, it relapses as hormone/castration resistant phenotype in many patients by some poorly defined mechanisms [1,4,8,9]. CLQ is a drug already in clinical usage, repurposing it and its analogs as a new adjuvant in hormone ablation therapy is practical and cost effective. Further study, for example, in pre-clinical trial using PCa animal models of hormone sensitive PCa is required to test the efficacy and efficiency of CLQ *in vivo*.

Acknowledgments

This work was supported, in part, by NIH/NCI Grants 5R01CA106644 (to CAAH), and a grant from the National Center for Research Resources (5P20RR016480-12), and DOD PCRP Predoctoral Award W81XWH-08-1-0183 (to RRK).

References

[1] Kaini RR, Sillerud L, Zhaorigetu S, Hu CA. Autophagy regulates lipolysis and cell survival through lipid droplet degradation in androgen-sensitive prostate cancer cells. Prostate. 2012 Jan 31. doi: 10.1002/pros.22489. [Epub ahead of print].
[2] A.R. Patel, E.A. Klein, Risk factors for prostate cancer, Nat. Clin. Pract. Urol. 6 (2) (2009) 87–95.
[3] G.W. Chodak, K.S. Warren, Watchful waiting for prostate cancer: a review article, Prostate Cancer Prostatic Dis. 9 (1) (2006) 25–29.
[4] S.M. Dehm, D.J. Tindall, Molecular regulation of androgen action in prostate cancer, J. Cell. Biochem. 99 (2006) 333–344.
[5] M. Bisoffi, I. Klima, E. Gresko, P.N. Durfee, W.C. Hines, J.K. Griffith, U.E. Studer, G.N. Thalmann, Expression profiles of androgen independent bone metastatic prostate cancer cells indicate up-regulation of the putative serine-threonine kinase GS3955, J. Urol. 172 (3) (2004) 1145–1150.
[6] B. Szende, I. Romics, L. Vass, Apoptosis in prostate cancer after hormonal treatment, Lancet 342 (1993) 1422.
[7] S.R. Denmeade, X.S. Lin, J.T. Isaacs, Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer, Prostate 28 (1996) 251–265.
[8] B.J. Feldman, D. Feldman, The development of androgen-independent prostate cancer, Nat. Rev. Cancer 1 (1) (2001) 34–45.
[9] S.S. Dutt, A.C. Gao, Molecular mechanisms of castration-resistant prostate cancer progression, Future Oncol. 5 (9) (2009) 1403–1413.
[10] G. Kroemer, G. Mariño, B. Levine, Autophagy and the integrated stress response, Mol. Cell 40 (2) (2010) 280–293.
[11] G. Wan, S. Zhaorigetu, Z. Liu, R. Kaini, Z. Jiang, C.A. Hu, Apolipoprotein L1, a novel Bcl-2 homology domain 3-only lipid-binding protein, induces autophagic cell death, J. Biol. Chem. 283 (31) (2008) 21540–21549.
[12] S. Zhaorigetu, G. Wan, R. Kaini, Z. Jiang, C.A. Hu, ApoL1, a BH3-only lipid-binding protein, induces autophagic cell death, Autophagy 4 (8) (2008) 1079–1082.
[13] R. Singh, S. Kaushik, Y. Wang, Y. Xiang, I. Novak, M. Komatsu, K. Tanaka, A.M. Cuervo, M.J. Czaja, Autophagy regulates lipid metabolism, Nature 458 (7242) (2009) 1131–1135.
[14] R. Singh, A.M. Cuervo, Autophagy in the cellular energetic balance, Cell Metab. 13 (5) (2011) 495–504.
[15] J.D. Rabinowitz, E. White, Autophagy and metabolism, Science 330 (6009) (2010) 1344–1348.
[16] Y. Liu, Fatty acid oxidation is a dominant bioenergetic pathway in prostate cancer, Prostate Cancer Prostatic Dis. 9 (2006) 230–234.
[17] S. Zha, S. Ferdinandusse, J.L. Hicks, S. Denis, T.A. Dunn, R.J. Wanders, J. Luo, A.M. De Marzo, W.B. Isaacs, Peroxisomal branched chain fatty acid beta-oxidation pathway is upregulated in prostate cancer, Prostate 63 (4) (2005) 316–323.
[18] M. Li, X. Jiang, D. Liu, Y. Na, G.F. Gao, Z. Xi, Autophagy protects LNCaP cells under androgen deprivation conditions, Autophagy 4 (1) (2008) 54–60.
[19] H.L. Bennett, J.T. Fleming, J. O'Prey, K.M. Ryan, H.Y. Leung, Androgens modulate autophagy and cell death via regulation of the endoplasmic reticulum chaperone glucose-regulated protein 78/BiP in prostate cancer cells, Cell. Death Dis 1 (2010) e72.
[20] Z. Wu, P.C. Chang, J.C. Yang, C.Y. Chu, L.Y. Wang, N.T. Chen, A.H. Ma, S.J. Desai, S.H. Lo, C.P. Evans, K.S. Lam, H.J. Kung, Autophagy blockade sensitizes prostate cancer cells towards src family kinase inhibitors, Genes Cancer 1 (1) (2010) 40–49.
[21] K. Sasaki, N.H. Tsuno, E. Sunami, G. Tsurita, K. Kawai, Y. Okaji, T. Nishikawa, Y. Shuno, K. Hongo, M. Hiyoshi, M. Kaneko, J. Kitayama, K. Takahashi, H. Nagawa, Chloroquine potentiates the anti-cancer effect of 5-fluorouracil on colon cancer cells, BMC Cancer 10 (2010) 370.
[22] V.R. Solomon, H. Lee, Chloroquine and its analogs: a new promise of an old drug for effective and safe cancer therapies, Eur. J. Pharmacol. 625 (1–3) (2009) 220–233.
[23] V. Espina, B.D. Mariani, R.I. Gallagher, K. Tran, S. Banks, J. Wiedemann, H. Huryk, C. Mueller, L. Adamo, J. Deng, E.F. Petricoin, L. Pastore, S. Zaman, G. Menezes, J. Mize, J. Johal, K. Edmiston, L.A. Liotta, Malignant precursor cells pre-exist in human breast DCIS and require autophagy for survival, PLoS One 5 (4) (2010) e10240.
[24] J. Sotelo, E. Briceño, M.A. López-González, Adding chloroquine to conventional treatment for glioblastoma multiforme: a randomized, double-blind, placebo-controlled trial, Ann. Intern. Med. 144 (5) (2006) 337–343.
[25] Z. Liu, H. Lu, Z. Jiang, A. Pastuszyn, C.-A.A. Hu, Apolipoprotein L6, a novel pro-apoptotic BH3-only protein, induces mitochondria-mediated apoptosis in cancer cells, Mol. Can. Res. 3 (1) (2005) 21–31.
[26] S. Zhaorigetu, Z. Yang, T.A. McCaffrey, C.A. Hu, Apolipoprotein L6, an interferon-gamma-inducible gene, promotes ROS generation and apoptosis in atherosclerotic lesion-derived cells, J. Biol. Chem. 286 (2011) 27389–27398.
[27] P.M. Chen, Z.J. Gombart, J.W. Chen, Chloroquine treatment of ARPE-19 cells leads to lysosome dilation and intracellular lipid accumulation: possible implications of lysosomal dysfunction in macular degeneration, Cell. Biosci. 1 (1) (2011) 10.
[28] M. Ricote, I. García-Tuñón, B. Fraile, C. Fernández, P. Aller, R. Paniagua, M. Royuela, P38 MAPK protects against TNF-alpha-provoked apoptosis in LNCaP prostatic cancer cells, Apoptosis 11 (11) (2006) 1969–1975.
[29] Y.H. Yoon, K.S. Cho, J.J. Hwang, S.J. Lee, J.A. Choi, J.Y. Koh, Induction of lysosomal dilatation, arrested autophagy, and cell death by chloroquine in cultured ARPE-19 cells, Invest Ophthalmol. Vis. Sci. 51 (11) (2010) 6030–6037.
[30] B. Ramser, A. Kokot, D. Metze, N. Weiss, T.A. Luger, M. Böhm, Hydroxychloroquine modulates metabolic activity and proliferation and induces autophagic cell death of human dermal fibroblasts, J. Invest. Dermatol. 129 (10) (2009) 2419–2426.
[31] C.J. Jones, M.I. Jayson, Chloroquine: its effect on leucocyte auto- and heterophagocytosis, Ann. Rheum. Dis. 43 (2) (1984) 205–212.
[32] A. Oikarinen, Hydroxychloroquine induces autophagic cell death of human dermal fibroblasts: implications for treating fibrotic skin diseases, J. Invest. Dermatol. 129 (10) (2009) 2333–2335.
[33] Y. Wang, R.Q. Peng, D.D. Li, Y. Ding, X.Q. Wu, Y.X. Zeng, X.F. Zhu, X.S. Zhang, Chloroquine enhances the cytotoxicity of topotecan by inhibiting autophagy in lung cancer cells, Chin. J. Cancer 30 (10) (2011) 690–700.
[34] Y. Geng, L. Kohli, B.J. Klocke, K.A. Roth, Chloroquine-induced autophagic vacuole accumulation and cell death in glioma cells is p53 independent, Neuro Oncol. 12 (5) (2010) 473–481.
[35] P. Singh, M. Godbole, G. Rao, S. Annarao, K. Mitra, R. Roy, A. Ingle, G. Agarwal, S. Tiwari, Inhibition of autophagy stimulate molecular iodine-induced apoptosis in hormone independent breast tumors, Biochem. Biophys. Res. Commun. 415 (1) (2011) 181–186.
[36] W. Han, J. Sun, L. Feng, K. Wang, D. Li, Q. Pan, Y. Chen, W. Jin, X. Wang, H. Pan, H. Jin, Autophagy inhibition enhances daunorubicin-induced apoptosis in K562 cells, PLoS One 6 (12) (2011) e28491.
[37] A.M. Godbole, P. Purushottamachar, M.S. Martin, C. Daskalakis, V.C. Njar, Autophagy inhibition synergistically enhances anti-cancer efficacy of RAMBA,

VN/12-1 in SKBR-3 cells and tumor xenografts, Mol. Cancer Ther. 11 (4) (2012) 898–908.
[38] X.L. Guo, D. Li, F. Hu, J.R. Song, S.S. Zhang, W.J. Deng, K. Sun, Q.D. Zhao, X.Q. Xie, Y.J. Song, M.C. Wu, L.X. Wei, Targeting autophagy potentiates chemotherapy-induced apoptosis and proliferation inhibition in hepatocarcinoma cells, Cancer Lett. 320 (2) (2012) 171–179.
[39] I.M. Meinão, E.I. Sato, L.E. Andrade, M.B. Ferraz, E. Atra, Controlled trial with chloroquine diphosphate in systemic lupus erythematosus, Lupus 5 (3) (1996) 237–241.
[40] G.G. Haydu, Rheumatoid arthritis therapy; a rationale and the use of chloroquine diphosphate, Am. J. Med. Sci. 225 (1) (1953) 71–75.
[41] S.R. Meshnick, Chloroquine as intercalator: a hypothesis revived, Parasitol. Today 6 (3) (1990) 77–79.
[42] S.I. Park, J. Zhang, K.A. Phillips, J.C. Araujo, A.M. Najjar, A.Y. Volgin, et al., Targeting src family kinases inhibits growth and lymph node metastases of prostate cancer in an orthotopic nude mouse model, Cancer Res. 68 (9) (2008) 3323–3333.