ORIGINAL RESEARCH

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Curcumin synergistically augments the chemotherapeutic activity of doxorubicin in prostate cancer cells

Yalçın Erzurumlu[1], Deniz Çataklı[2], Hatice Kübra Doğan[3]

[1] Suleyman Demirel University Faculty of Pharmacy, Department of Biochemistry, Isparta, Türkiye
[2] Suleyman Demirel University Faculty of Medicine, Department of Pharmacology, Isparta, Türkiye
[3] Suleyman Demirel University Institute of Science, Department of Bioengineering, Isparta, Türkiye

Abstract

Objective: Prostate cancer is one of the most commonly diagnosed cancer types in men and many people die every year due to recurring or acquiring aggressive forms of prostate cancer. Numerous chemotherapeutics, such as paclitaxel and doxorubicin are commonly used in the treatment of prostate cancer. However, acquired resistance to chemotherapeutics and broad systemic side effects substantially limit their usage. Curcumin is one of the most examined phytochemicals of the herbal remedy turmeric. Herein, we aimed to investigate the synergistic capability of curcumin on doxorubicin in prostate cancer cells.

Method: The human adenocarcinoma cell line LNCaP was used in cell culture studies. Cell viability was examined by WST-1 assay. The protein expression levels of Beclin1, p62/SQSTM1, LC3-I/II, Hrd1, gp78, polyubiquitin, PERK, eIF2a, phospho-(Ser51) eIF2a, IRE1a, XBP-1s, PARP-1, caspase-3, AR, PSA, c-Myc, E-cadherin, N-cadherin and VEGF-A were investigated by immunoblotting assay.

Results: Our data indicated that co-administration of curcumin with doxorubicin significantly improved the cytotoxic effect of doxorubicin in LNCaP cells. Also, the combination of curcumin and doxorubicin reduced the autophagic flux and remarkably induced endoplasmic reticulum-associated-degradation (ERAD) and unfolded protein response (UPR) signaling. Also, activation of apoptotic proteins PARP-1 and caspase-3 were strongly enhanced by combined treatment in a dose-dependent manner. Moreover, combined treatment markedly decreased levels of AR, PSA, c-Myc and VEGF-A proteins. Additionally, the epithelial-mesenchymal transition (EMT) was reduced by decreasing N-cadherin and increasing E-cadherin protein levels.

Conclusion: Present data strongly suggest that curcumin synergistically improves the anti-cancer features of doxorubicin in prostate cancer cells. This study will be an important guide for testing the effects of the combined treatment of curcumin and doxorubicin in xenograft animal models with prostate tumors.

Keywords: Autophagy, Curcumin, Doxorubicin, ER-associated degradation, Prostate cancer, Unfolded Protein Response

INTRODUCTION

Cancer is one of the most important health problems and many people die every year due to prostate cancer, which is one of the most diagnosed cancer types in men (1). Family history, age, ethnicity and metabolic disorders like obesity are significant risk factors for prostate cancer. Despite surgery, radiation therapy, cryotherapy, chemotherapy and hormonal therapy being the main treatment options for prostate cancer androgen deprivation therapy is one of the most commonly used treatment approaches against aggressive prostate cancer because of the overactivation of androgen receptor signaling (2-4). Moreover, numerous chemotherapeutics, such as docetaxel, paclitaxel and doxorubicin (DOXO) are commonly used in the treatment of prostate cancer either alone or in combination. However, acquired resistance to chemotherapeutics and also their broad spectrum of systemic side effects substantially limit their therapeutic usage. Accumulated evidence has shown that combined therapies may improve the therapeutic benefits of chemotherapeutics by reducing drug resistance and minimizing side effects compared to monotherapy (5).

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Corresponding Author: Assoc. Prof. Yalçın Erzurumlu, Süleyman Demirel University Faculty of Pharmacy, Department of Biochemistry, Isparta, Türkiye
Email: yalcin.erzurumlu@gmail.com
ORCID iD: 0000-0001-6835-4436

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Numerous bioactive phytochemicals purified from natural products are widely used as therapeutic and chemo-preventive agents for chronic diseases and various types of cancers. These agents are potentially able to suppress carcinogenesis (6,7). Curcumin (CRC) is one of the most examined phytochemicals of the herbal remedy turmeric (*Curcuma longa)*. It has a variety of biochemical activities, including antiseptic, anti-viral anti-inflammatory, antioxidant and antitumor properties (8). Moreover, there are many clinical trials associated with the therapeutic utilization of CRC, including multiple myeloma, pancreatic cancer, myelodysplastic syndromes, psoriasis, Alzheimer's disease, colon cancer and prostate cancer as well (9). The anticancer effects of CRC on androgen-sensitive prostate cancer cell lines have been shown in several studies. It has been reported that CRC dose-dependently suppressed cell growth, survival and proliferation and induced autophagy in LNCaP cells (10,11). Additionally, there are many studies investigating the synergistic effect of combining CRC with many traditional chemotherapeutics and agents in numerous cancers (12). Herein, we efforted the mechanistically investigate the mode of action of the possible booster effect of CRC on DOXO in prostate cancer.

DOXO, an anthracycline antibiotic, is extensively used in the treatment protocols of numerous cancers, including breast cancer, leukemia, soft tissue sarcoma and prostate cancer. It prevents the growth of cancer cells by inhibiting nucleic acid synthesis and blocking topoisomerase II enzyme activity (13). While it is highly effective on cancer cells, it targets not only cancer cells but also many other organs like the heart, brain, liver and kidney as well. Therefore, their usage is limited due to their potential cytotoxic effects and the possibility of developing acquired drug resistance. Combined applications of phytochemicals with chemotherapeutic agents offer an excellent alternative option for minimizing the dose ranges used and reducing the systemic cytotoxic effects (14). In 2016, Klippstein et al. reported that the combined treatment of CRC and DOXO has a synergistic effect on metastatic androgen-independent prostate cancer cells through the induction of apoptotic cell death (15). However, detailed studies on the molecular modeling of coadministration of DOXO and CRC on prostate cancer cells were not included in this report.

Herein, we aimed to investigate the possible synergistic effect of CRC on DOXO in non-metastatic prostate cancer cells using a human androgen-sensitive prostate adenocarcinoma cell line LNCaP, which is well-mimic prostate cancer *in vitro*. Firstly, we tested the effect of co-treatment of CRC and DOXO on the viability of LNCaP cells. To understand the molecular mechanism of action of co-administration, we examined its effects on autophagy and endoplasmic reticulum (ER)-associated degradation (ERAD), two primary protein quality control mechanisms in mammalian cells, on the unfolded protein response (UPR) signal, which is involved in the regulation of ER capacity and coordination of ER stress responses, by immunoblotting. Additionally, we examined the cell-death-associated proteins caspase-3 and Poly [ADP-ribose] polymerase 1 (PARP-1), proto-oncogene protein c-Myc, angiogenic factor vascular endothelial factor A (VEGF-A), E-cadherin and N-cadherin which are epithelial-mesenchymal transition markers (EMT) and also prostate tumorigenesis associated proteins, androgen receptor (AR) and prostate-specific antigen (PSA). Present data suggest that co-administration of CRC with DOXO strongly enhanced the anticancer properties of DOXO in prostate cancer cells. The combination of chemotherapeutic agents with CRC could be a promising and powerful strategy to treat prostate cancer.

METHOD

Materials

Fetal bovine serum (FBS), tissue culture media and other cell culture supplements were obtained from Capricorn Scientific (Ebsdorfergrund, Germany). Cell culture plastic materials were obtained from Sarsdeth. Monoclonal rabbit anti-Hrd1 (#14773)(1:3000) and polyclonal rabbit anti-eIF2α (#9722)(1:2500), anti-phospho-eIF2α (Ser51) (#9721) (1:2500), anti-p62/SQSTM1 (#5114)(1:2000), anti-Beclin-1 (#3495)(1:1500), anti-LC3-I/II (#12741)(1:3000) (#2895) and anti-caspase-3 (#9692)(1:1000) were purchased from Cell Signaling Technology (Beverly, MA, USA). Polyclonal rabbit anti-PARP-1 (13371-1-AP)(1:2000), anti-gp78 (16675-1-AP) (1:3000), anti-XBP-1s (#24868-1-AP)(1:2000), anti-ubiquitin (#10201-2-AP)(1:1000), PERK (#24390-1-AP)(1:3500), anti-IRE1α (#27528-1-AP)(1:3000), anti-E-cadherin (20874-1-AP), anti-N-cadherin (22018-1-AP), anti-AR (22089-1-AP)(1:2500), anti-c-Myc (10828-1-AP) and mouse monoclonal anti-PSA (60338-1-Ig) were obtained from Proteintech (Wuhan, China). Polyclonal rabbit anti-VEGF-A (E-AB-53277) was purchased from Elabscience. Mouse monoclonal anti-beta-actin antibody (#A5316)(1:10000) was obtained from Sigma Aldrich. HRP-conjugated goat anti-mouse (#31430)(1:5000) or goat anti-rabbit (#31460)(1:5000) IgG (H+L) was purchased from Pierce (Thermo Fisher Scientific, DE). Curcumin was provided from Sigma-Aldrich (USA) (#C1386)

Cell culture

Human androgen-sensitive prostate adenocarcinoma cell line, LNCaP (CRL-1740TM) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) (Capricorn Scientific, Ebsdorfergrund, Germany) media enriched with 10% FBS, 5 mg ml−1 penicillin/streptomycin and 2 mM L-glutamine (Capricorn Scientific, Ebsdorfergrund, Germany) and were kept in a humidified atmosphere of 5% CO2 and 95% air at a constant temperature of 37 °C. All

compounds were prepared in 1000-fold (1000X) concentration and applied to the cells.

Cell viability assay

Cells were seeded into a 96-well plate (7500 cells/well) and 24 hours later treated with agents for 48 hours. Following the WST-1 assay (Takara Bio Inc., Kusatsu, Shiga, Japan) was performed according to the manufacturer's instructions. The absorbance was determined at 450nm with 600nm set as the reference wavelength by microplate spectrophotometer (BioTek, Epoch 2, USA). Cell viability rates were presented in the graph as a % fold change. IC50 was calculated by GraphPad Prism 7 software (GraphPad Software, La Jolla CA, USA, www.graphpad.com).

Protein isolation and immunoblotting

Cells were lysed within RIPA buffer and then centrifugated at 14.000 rpm for 20 min at 4°C. The supernatant was collected and total protein ingredients were defined by bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, DE). 20-30 µg protein was used in immunoblotting studies. Samples were denatured in 4x Laemmli buffer (Bio-Rad Laboratories, Hercules, California, USA) at 70°C for 15 min and proteins were electrophoretically separated on hand-cast polyacrylamide gels and transferred to Immobilon®-P polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, California, USA). The membrane was blocked with 5% nonfat dry milk in Phosphate-buffered saline (PBS) containing 0.1% Tween (PBS–Tween) for 1 h at room temperature and then incubated with primary antibody and secondary antibodies, respectively. Target proteins were visualized by clarity enhanced chemiluminescence western blotting substrate (Bio-Rad Laboratories, Hercules, California, USA) in ChemiDoc XRS+ (Bio-Rad Laboratories, Hercules, California, USA). Densitometric analysis of protein bands was carried out by ImageJ software (National Institutes of Health, USA).

Statistical analysis

Data were presented as means ± standard deviation. The statistical significance of differences between groups was determined by a two-tailed equal variance Student's t-test or One-way ANOVA with a minimum of 95% confidence interval by GraphPad Prism 7. The significant level was set at 5% ($p<0.05$) for all tests.

RESULTS

Curcumin enhances the anti-cancer activity of doxorubicin on LNCaP cells

Firstly, we evaluated the effect of DOXO and CRC on cell viability of LNCaP cells carried out the WST-1 based cell viability assay. LNCaP cells were treated with 1, 2.5, 5, 7.5, 10, 12.5 and 15mM DOXO or 2.5, 5, 10, 25, 50, 100, 250, 500, 1000 and 2000nM CRC for 48 hours. Our results revealed that DOXO and CRC treatment significantly decreased the cell viability of LNCaP cells in a dose-dependent manner (Figure 1a, b). We calculated IC50 values for DOXO and CRC, 10.652mM and 0.369nM, respectively. According to these findings, we decided to study with doses of 5, 10 and 20nM of CRC, where 10mM DOXO and CRC had low effects on the viability of LNCaP cells.

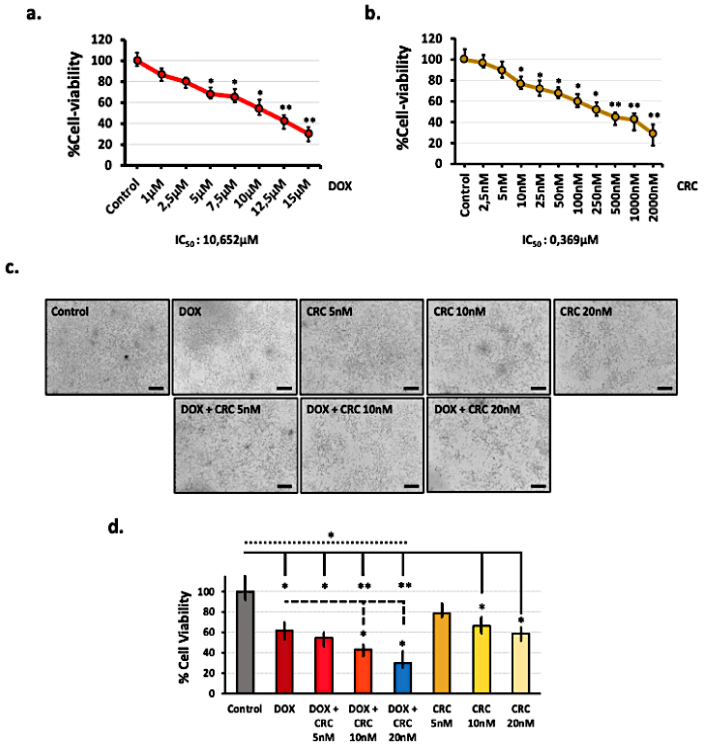

Figure 1. The testing of the effect of curcumin and doxorubicin on cell viability LNCaP cells were treated with (a) 1, 2.5, 5, 7.5, 10, 12.5 and 15mM DOXO or (b) 2.5, 5, 10, 25, 50, 100, 250, 500, 1000 and 2000nM CRC for 48 hours and then cell viability examined by WST-1 assay. IC50 values were calculated by GraphPad Prism 7 software. (c) Cells were treated with vehicle or 10µM DOXO, 5, 10 and 20nM CRC or their combination for 48 hours. The following cells were photographed by an inverted microscope. 4x Scale bar: 5 µm, 20x Scale bar: 10 µm. (d) Cell viability was analyzed by WST-1 assay. Three independent biological and three technical repeats per experiment were used. Statistical significance among the groups was analyzed by Student's t-test or one-way ANOVA and Tukey's tests. (*$p<0.05$, **$p<0.001$).

To examine the improvement effect of CRC on DOXO-induced cell death, LNCaP cells were treated with 10mM DOXO, 5, 10 and 20nM CRC and the combined administration of CRC and DOXO as indicated doses for 48 hours. Microscopic examination results indicated that alone DOXO and CRC decreased the viability of LNCaP cells compared to the control group. Moreover, we observed that the co-administration of CRC and DOXO more efficiently reduced the viability of LNCaP cells in a dose-dependent manner (Figure 1c). Next, to quantify the %cell viability, we carried out the WST -1-based cell viability assay. Our data indicated that 10mM DOXO reduced the viability of LNCaP cells by 60%. Also, 5, 10 and

20nM CRC treatment decreased the cell viability by 22, 35 and 40%, respectively. Co-administration of 10mM DOXO and 5, 10 and 20nM CRC more strongly reduced the % cell viability by 62, 46 and 35%, respectively (Figure 1d).

Co-administration of curcumin with doxorubicin diminishes the autophagic flux in LNCaP cells

CRC and chemotherapeutic drugs are known to have potent effects on autophagic flow. Therefore, we evaluated the effects of co-administration on autophagy. Firstly, we evaluated the morphological alterations of LNCaP cells depending on the CRC, DOXO or co-administration of CRC and DOXO by microscopic examination. We observed that DOXO treatment generated the vacuole-like cellular formation on LNCaP cells. CRC also induced the vacuole-like structures in a dose-dependent manner (Figure 2a). Co-administration more strongly increased the vacuole formation and caused the shrinking cell form. Also, it formed the gathering around the nucleus in the cells (Figure 2a).

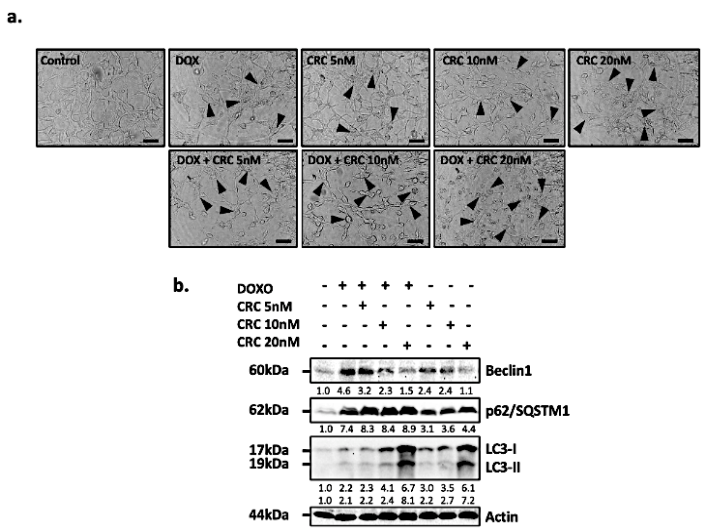

Figure 2. Evaluation of the effect of curcumin and doxorubicin on autophagy proteins in LNCaP cells. Cells were treated with vehicle or 10μM DOXO, 5, 10 and 20nM CRC or their combination for 24 hours. (a) Cells were photographed by an inverted microscope. Vacuolar structures are indicated by arrows. Scale bar: 25 μm. (b) The expression level of Beclin1, p62/SQSTM1 and LC3-I/II levels were analyzed by immunoblotting. Beta-actin was used as a loading control

We examined the impacts of the co-administration of CRC and DOXO on autophagy. To this aim, we treated the cells with 10μM DOXO, 5, 10 and 20nM CRC or their combinations for 24 hours and then the levels of critical autophagy proteins, including Beclin1, p62/sequestosome-1 (SQSTM1) and microtubule-associated protein 1A/1B-light chain 3 (LC3-I/II) were analyzed by immunoblotting studies. Our data showed that DOXO treatment increased the Beclin1, p62/SQSTM1 and LC3-I/II levels compared to the control group. Alone CRC application also elevated the levels of the p62/SQSTM1 and LC3-I/II, whereas Beclin1 was downregulated in a dose-dependent manner (Figure 2b). Combined treatment of CRC with DOXO remarkably increased the p62/SQSTM1 and LC3-I/II levels in a dose-dependent manner. Also, Beclin1 levels were decreased by co-treatment (Figure 2b).

Combined treatment of curcumin with doxorubicin induced the ERAD and UPR signaling in LNCaP cells

To test the possible booster effect of co-administration of CRC with DOXO on ERAD and UPR signaling, we evaluated the state of polyubiquitination, hydroxymethyl glutaryl-coenzyme A reductase degradation protein 1 (Hrd1) and glycoprotein 78 (gp78) which are ERAD E3 ligase enzymes and Inositol-requiring enzyme 1α (IRE1a) and Protein Kinase RNA-like ER Kinase (PERK) branches of UPR proteins, including eukaryotic initiation factor 2α (eIF2a), phosphorylated at serine 51 position eIF2a, PERK, X-box Binding Protein-1 (XBP-1s) and IRE1a by immunoblotting. Our results indicated that DOXO treatment increased the polyubiquitination levels whereas gp78 and Hrd1 levels did not affect compared to the control (Figure 3). CRC treatment markedly elevated the polyubiquitination levels at 10 and 20nM doses. 5nM CRC administration did not affect the polyubiquitination. Similar to these results, Hrd1 and gp78 levels were increased by 10 and 20nM CRC in a dose-dependent manner. However, 5nM CRC treatment did not affect the Hrd1 and gp78 levels and also similar results were obtained by 10mM DOXO administration (Figure 3). Co-administration of CRC with DOXO more strongly increased the steady-state level of polyubiquitination compared to alone CRC and DOXO treatment in a dose-dependent manner. Moreover, Hrd1 and gp78 levels were remarkably increased by the combined treatment of CRC and DOXO (Figure 3).

Additionally, we evaluated the effects of co-treatment of CRC with DOXO on PERK and IRE1a branches of UPR signaling. Alone CRC or DOXO treatment increased the PERK and phosphorylated eIF2a levels whereas total eIF2a levels were not affected compared to the control group. Co-administration more strongly increased the phosphorylated eIF2a and PERK levels and also total eIF2a levels were decreased in a dose-dependent manner (Figure 3b). IRE1a level was not affected by DOXO whereas the level of downstream effector protein of IRE1a, XBP-1s was increased. CRC treatment gradually increased the IRE1a and XBP-1s levels in a dose-dependent manner (Figure 3b). Co-administration of CRC with DOXO more strongly increased the PERK, phosphorylated eIF2a, IRE1a and XBP-1s levels (Figure 3b). Overall, these results indicated that co-administration of CRC with DOXO more efficiently induced the IRE1a and PERK branches of UPR signaling in LNCaP cells.

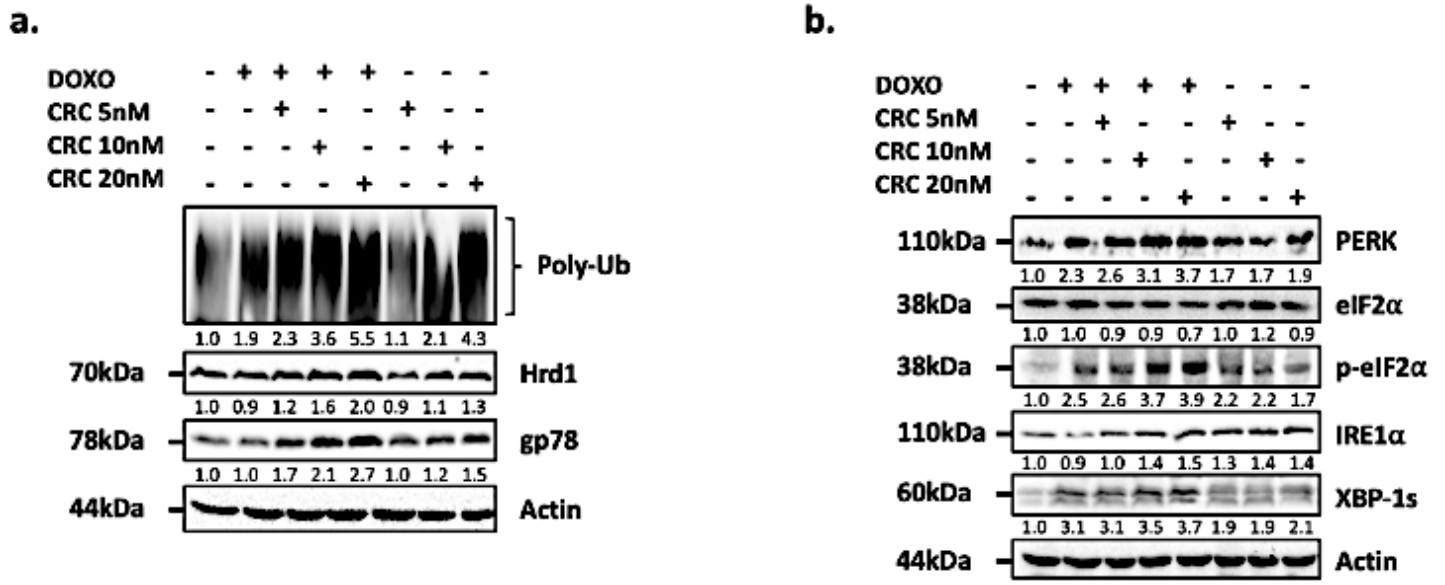

Figure 3. Assessment of the effect of curcumin and doxorubicin on ERAD and UPR proteins in LNCaP cells . Cells were treated with vehicle or 10μM DOXO, 5, 10 and 20nM CRC or their combination for 24 hours. The expression level of (a) ERAD components, including poly-Ub, Hrd1 and gp78 levels and (b) UPR signaling proteins, eIF2α, p-eIF2α, PERK, IRE1α and XBP-1s were analyzed by immunoblotting. Beta-actin was used as a loading control.

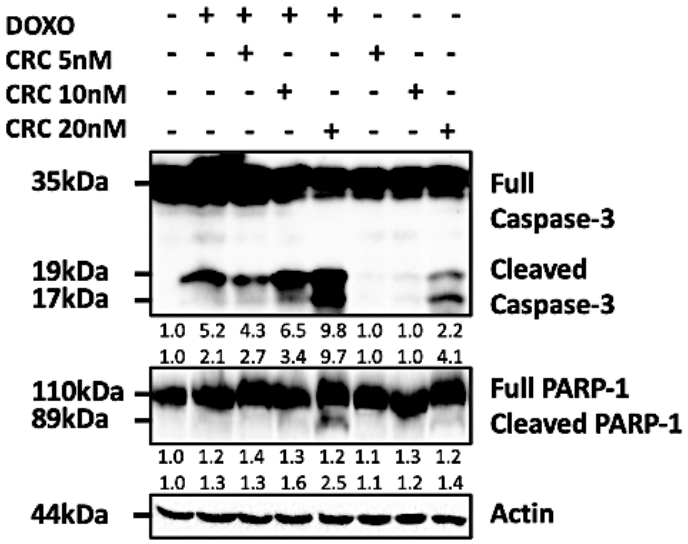

Figure 4. Evaluation of the effect of curcumin and doxorubicin on cell-death-related proteins in LNCaP cells . Cells were treated with vehicle or 10μM DOXO, 5, 10 and 20nM CRC or their combination for 24 hours. The expression levels of full and cleaved caspase-3 and PARP-1 were analyzed by immunoblotting. Beta-actin was used as a loading control.

Curcumin enhances the doxorubicin-induced caspase-3 and PARP-1 activation in LNCaP cells

It is known that caspase enzymes and PARP-1 activation play a key role in programmed cell death (16). Also, chemotherapeutics strongly stimulate the activation of these enzymes (17). Thus, we examined the booster effect of CRC on DOXO-induced caspase-3 and PARP-1 activation. 10μM DOXO treatment slightly generated the 89kDa cleavage fragment of PARP-1 and it strongly induced the 19kDa cleavage fragment of caspase-3 compared to the control group (Figure 4). Moreover, alone CRC treatment weakly induced the PARP-1 activation at 10 and 20nM doses. 5nM CRC did not activate caspase-3 and PARP-1 compared to the control group. Despite 10nM CRC treatment slightly induced the cleavage of caspase-3, 20nM CRC markedly increased the caspase-3 activation (Figure 4). Co-administration of CRC and DOXO remarkedly induced cleavage of caspase-3 and PARP-1 in a dose-dependent manner compared to alone DOXO or CRC as indicated doses (Figure 4). These results indicated that co-administration of CRC with DOXO more strongly induced cleavage of caspase-3 and PARP-1 activation in LNCaP cells.

Co-administration of curcumin with doxorubicin strongly reduced the tumorigenic protein levels in LNCaP cells

We tested the effect of co-administration of CRC and DOXO on tumorigenic protein levels, including PSA, AR, c-Myc, VEGF-A and the EMT-related proteins E-cadherin and N-cadherin by immunoblotting. We found that only CRC or DOXO administration reduced AR, PSA, c-Myc, N-cadherin and VEGF-A levels compared to the control group. E-cadherin levels were increased by CRC or DOXO treatment (Figure 5). Co-treatment of CRC with DOXO more strongly decreased the PSA, AR, c-Myc, N-cadherin and VEGF-A levels, whereas E-cadherin was increased in a dose-dependent manner (Figure 5). These results suggested that CRC and DOXO alone treatment reduced the tumorigenic protein levels and co-treatment more effectively reduced the PSA, AR, c-Myc and VEGF-A levels. Moreover, EMT-related proteins N-cadherin more strongly decreased with co-administration whereas E-cadherin was induced by the co-administration of CRC and DOXO.

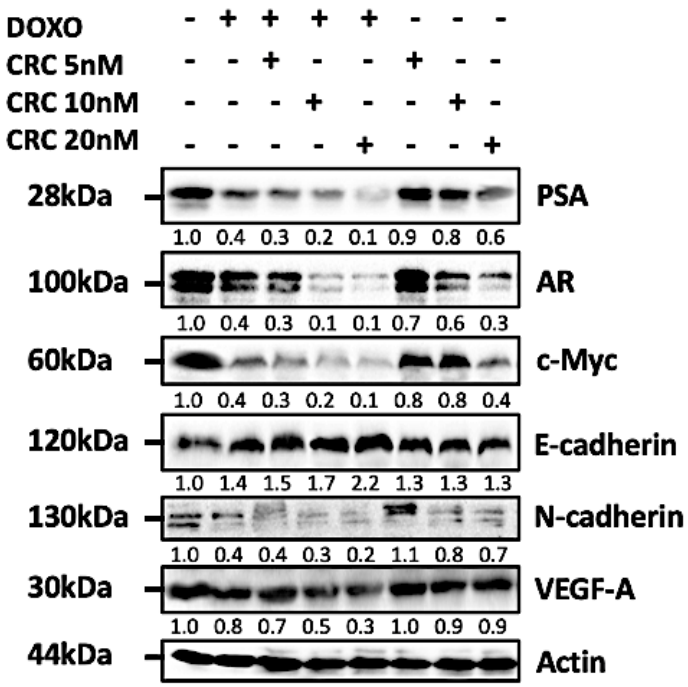

Figure 5. Evaluation of the effect of curcumin and doxorubicin on prostate cancer tumorigenesis-related proteins in LNCaP cells . Cells were treated with vehicle or 10µM DOXO, 5, 10 and 20nM CRC or their combination for 24 hours. The expression levels of PSA, AR, c-Myc, E-cadherin, N-cadherin and VEGF-A were analyzed by immunoblotting. Beta-actin was used as a loading control.

DISCUSSION

Since ancient times, natural compounds have been extensively used as medicinal remedies in different cultures owing to their antioxidant, anti-inflammatory and antimicrobial properties (18). Most of the chemotherapeutics with strong anti-cancer properties were discovered after further characterization of the active ingredients in natural products. Today, studies focusing on plant-derived agents are continuing intensively (19).

CRC, a polyphenol, is the active ingredient of turmeric (*Curcuma longa Linn*) and has diverse biochemical activities, including neuroprotective, anti-inflammatory, anti-proliferative, anti-angiogenic, antioxidant, antiviral and anti-tumorigenic effects (20). Studies have shown that caspase-related apoptosis is induced by CRC administration in androgen-dependent and castration-resistant prostate cancer cells (21,22). CRC and its analogues have been extensively studied for their anticancer properties, including prostate cancer (20). In addition, there have been many ongoing and finalized phase studies related to the effectiveness of CRC on various types of cancers for the last 20 years (23). Furthermore, *in vitro* studies demonstrated that CRC can potentiate the cytotoxic effects of chemotherapeutic drugs, including tamoxifen, cisplatin, vincristine, daunorubicin and DOXO (24-26). Also, multi-drug administrations clinically have been used such as combined administration of DOX with other chemotherapeutics, including cyclophosphamide, 5-fluorouracil, docetaxel, vinblastine and bleomycin (27-29). Moreover, Klippstein et al. reported that the combinatory administration of CRC nanocapsule and DOXO has a synergistic effect on metastatic androgen independent prostate cancer cells, PC3 and DU145 through the induction of apoptotic cell death (15). Moreover, recent studies have reported that CRC administration may reduce the adverse effects of DOX (30). Based on these studies, we aimed to mechanistically investigate the possible booster effect of CRC on DOXO in human androgen-dependent prostatic adenocarcinoma LNCaP cells.

Firstly, we evaluated the impacts of co-administration of CRC with DOXO on cell viability, we found that combined treatment more strongly reduced the viability of LNCaP cells (Figure 1a, b). It was also determined that co-administration increased the cytotoxic effect and decreased the IC50 value. These results suggest that CRC raised the anti-tumorigenic ability of DOXO by elevating the susceptibility of LNCaP cells to DOXO treatment (Figure 1a, b). Our results supported the results of Klippstein et al., on androgen-independent metastatic prostate cancer cells (15).

Autophagy is an evolutionarily conserved mechanism that delicately regulates the cell content, including long-lived and unfolded proteins and damaged organelles. Today, the role of autophagy in cancer is enigmatic and it may work as a tumor support or suppressor depending on the status of the cancer cells (31). Autophagy involves the formation of the double-membrane vesicle degradation through lysosomes, which consists of sequential steps, including membrane nucleation, phagophore expansion, formation of autophagosome and fusion with lysosomes. The formation of the double-membrane vesicle is a complex process in which a large number of autophagy-related proteins (Atg) work in tandem (32). Therefore, many proteins need to be studied simultaneously in the cellular-level monitoring of autophagy, which has multiple steps (33). For this aim, we examined the effect of co-administration on Beclin1 protein levels, p62/SQSTM1 turnover and LC3-I to LC3-II conversion by immunoblotting. Beclin1 is a key regulator of autophagy which plays a role in the initiation of autophagy through interaction with lipid kinase complex and coordinates the membrane trafficking (34). The ubiquitin receptor protein, p62/SQSTM1 directly binds to the LC3 proteins and is degraded by autophagy. The steady-state level of p62/SQSTM1 increases or decreases depending on the autophagic activity in the cells. Therefore, it is often used to monitor the autophagic flux (35). During autophagy, the cytosolic form of LC3, LC3-I, is modified with phosphatidylethanolamine and it covers to the LC3-II, which is recruited to autophagosomal membranes

and degraded during the fusion of the autophagosome with the lysosome (36). Our data indicated that either alone DOXO or CRC treatment increased the steady-state level of p62/SQSTM1 compared to the control group. Also, LC3-I and LC3-II levels were increased by CRC and DOXO treatment. Combination of DOXO and 5 nM CRC enhanced the Beclin1 levels whereas 10nM and 20nM CRC administration reduced the Beclin1 levels in a dose-dependent manner (Figure 2b). Considering that autophagy is a physiological mechanism, it can be thought that LNCaP cells respond through the reorganization of autophagy against 5nM CRC-mediated reduced autophagic activity by increasing Beclin1 levels. Co-administration of CRC and DOXO increased the accumulation of p62/SQSTM1 and increment of LC3-I and LC3-II levels stronger than either alone CRC or DOXO treatment whereas Beclin1 was decreased in a dose-dependent manner (Figure 2b). Collectively, these results suggested that CRC and DOXO alone reduced the autophagic flux in LNCaP cells while co-administration more strongly reduced autophagic activity. Additionally, microscopic examination results also support these findings (Figure 2a).

ERAD is another important protein quality control mechanism in mammalian cells. The ER is an important centre for the synthesis of one-third of the cellular proteome. Therefore, it hosts advanced protein quality control mechanisms. ERAD is a sophisticated mechanism that selectively recognizes misfolded, unfolded and incorrectly oligomerized proteins and directs them to proteosome-mediated degradation. Moreover, ERAD also controls the endogenous levels of physiologically important proteins (37). Besides, ubiquitination, a posttranslational modification, is the molecular marking required for directing proteins to degradation (38). We found that DOXO treatment did not affect the levels of E3 ubiquitin ligase enzymes (Hrd1 and gp78), whereas poly-ubiquitination slightly increased by DOXO. CRC treatment increased the Hrd1, gp78 and poly-ubiquitination levels in a dose-dependent manner. Co-administration remarkably increased the levels of E3 ligase enzymes and poly-ubiquitination state (Figure 3a). These data indicated that co-administration strongly induced the ERAD-mediated protein turnover in LNCaP cells.

It is known that the unfolded protein response (UPR) signaling plays a pivotal role in improving the capacity of the ER and re-establishing impaired ER homeostasis (39,40). The UPR signaling is regulated through ER membrane-localized three transmembrane proteins, IRE1α, PERK and ATF6 (41). We found that either DOXO or CRC treatment induced the IRE1α and PERK branches of UPR. Our data indicated that CRC markedly increased the IRE1α and its effector protein XBP-1s levels in a dose-dependent manner. PERK protein levels and phosphorylation of eIF2 were increased by CRC treatment. Similar to these results, DOXO administration induced phosphorylated eIF2α, PERK and XBP-1s levels. Co-administration of DOXO with CRC more strongly increased the levels of PERK and IRE1α signaling proteins in a dose-dependent manner (Figure 3b). The severe ER stress signaling can induce programmed cell death in cells by increasing the levels of pro-apoptotic proteins, such as C/EBP-homologous protein (CHOP) by causing overstimulation of the UPR (42,43). Our results suggest that the potentiating effect of CRC on DOXO is through potentiation of DOXO-induced cell death due to overstimulation of the UPR in prostate cancer cells.

Next, we evaluated the programmed cell death-related caspase-3 and PARP-1 protein levels. Effector caspases enzymes such as caspase-3, -6, -7 regulate the programmed cell death apoptosis through fragmentation of DNA, blebbing and shrinkage of cells (44). Cysteine–aspartic acid protease, caspase-3 is one of the main executioner proteins of apoptosis and leads to the cleavage of numerous key proteins, including PARP-1 (44,45). Activation of caspase-3 leads to proteolytic cleavage of the full version of caspase-3 and produces 17/19 kDa cleavage caspase-3 form (44,45). Our results indicated that either alone DOXO or CRC administration induced the activation of caspase-3 whereas co-administration remarkably increased the caspase-3 activation in a dose-dependent manner (Figure 4). Also, we tested the level of full (116kDa) and cleavage form (89kDa) of PARP-1 protein, which is a 116 kDa nuclear protein and cleavable through the caspase-3 enzyme (46,47). We found that DOXO or CRC treatment weakly produced the 89kDa fragment of PARP-1 protein, whereas the combination of DOXO and 20nM CRC strongly increased the cleavage form of PARP-1 (Figure 4). These data suggest that CRC improves the anti-cancer properties of DOXO on prostate cancer cells through inducing executioner protein levels.

Lastly, we evaluated the tumorigenic protein levels, including PSA, AR, c-Myc and VEGF-A. We also tested the level of N-cadherin and E-cadherin proteins, which are related to the arrangement of the invasion and migration capability process. Androgen receptor signaling has a crucial role in the progression of prostate cancer. It regulates the transcription of AR target genes, including PSA as an active transcription factor after stimulation of AR with its ligand (48). c-Myc is a well-known oncogene and is a major driver in prostate cancer malignancy. Moreover, a positive correlation has been determined between expression levels of c-Myc and AR in human prostate cancer samples (49). VEGF-A is an important regulator of angiogenesis and it promotes the growth of tumors (50). We observed that either DOXO or CRC treatment importantly decreased the AR, PSA and c-Myc levels compared to the control group. Furthermore, co-administration more strongly reduced the levels of these proteins in a dose-dependent manner (Figure 5). Additionally,

VEGF-A levels were slightly decreased by alone CRC and DOXO whereas co-administration remarkably reduced VEGF-A levels in a dose-dependent manner compared to either alone DOXO or CRC (Figure 5). These data suggest that co-administration more effectively decreases the tumorigenic protein levels in prostate cancer cells.

EMT works as a key mechanism in cancer cells for acquiring mobility and increasing invasiveness/migrative features. Reduction of E-cadherin and elevated level of N-cadherin induces epithelial to mesenchymal transition (51). Our data showed that CRC or DOXO administration increased E-cadherin and decreased N-cadherin levels compared to the control group. Co-administration more potently increased the level of E-cadherin and reduced N-cadherin. Collectively, these results indicated that CRC efficiently evolved the anti-tumorigenic properties of DOXO on prostate cancer cells.

Prostate cancer is one of the main health problems among men worldwide and it causes the death of many people (52). Today, numerous therapies depending on the stage and subtype of prostate cancer, including surgery, cryotherapy, androgen-deprivation therapy and chemotherapy, while the efficacy of the treatments may be limited due to the serious side effects caused by the long-term use of high-dose chemotherapeutics or acquired drug resistance (53). The results of numerous *in vitro* and in vivo experiments show that natural products in treatment protocols can offer a promising approach (19).

The anthracycline DOXO is one of the most commonly used anticancer drugs in clinical practice and has a broad spectrum of use, such as in childhood and adult malignancies, including prostate cancer (54,55). However, accumulated evidence has shown that DOXO could affect non-targeted tissues; thereby, acute and chronic toxicity can develop in these tissues as a side effect (54). Herein, we examined the booster effect of CRC on DOXO on prostate cancer cells through investigating the detailed molecular signaling mechanisms, including autophagy, ERAD, UPR, AR signaling, angiogenic signal and EMT.

Limitations of the study

The present study investigates the possible potentiator effect of CRC on DOXO *in vitro*. The more effective anti-cancer responses demonstrated by the co-administration of CRC with DOXO need to be confirmed in vivo studies.

CONCLUSION

Overall, the present data strongly suggest that CRC potently and synergistically improves the anti-cancer features of DOXO by blocking autophagic flux, inducing ERAD and UPR signaling, activating the executioner proteins caspase-3 and PARP-1 and negatively modulating the AR signaling and EMT mechanism and also decreasing angiogenic factor VEGF-A and protooncogene c-Myc levels.

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Peer-Review

Both externally and internally peer reviewed.

Conflict of Interest

The authors declare that they have no conflict of interests regarding content of this article.

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Ethical Declaration

This study does not require any ethical permission, and Helsinki Declaration rules were followed to conduct this study.

Authorship Contributions

Concept: YE, Design: YE, Supervising: YE, Financing and equipment: YE, Data collection and entry: YE, HKD, DC, Analysis and interpretation: YE, Literature search: YE, HKD, DC, Writing: YE, Critical review: YE, HKD, DC.

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