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Combinatorial effect of curcumin with docetaxel modulates apoptotic and cell survival molecules in prostate cancer

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1. ABSTRACT

Docetaxel is the most commonly used chemotherapeutic agent to target androgen signaling in metastatic prostate cancer (PCa); however, prolonged treatment with docetaxel results in drug-resistant cancer cells. Combination therapies have the potential of increasing the effectiveness of drug treatment as well as decreasing the side effects. Curcumin is a nontoxic organic compound with multifaceted chemopreventive potential. In this study, we evaluated whether curcumin can reinforce the effect of docetaxel on PCa cells. The PCa cell lines DU145 and PC3 were treated with curcumin and docetaxel alone or in combination. After completion of the treatment cell proliferation and the expression of pro-survival and anti-apoptotic markers and the signaling molecules were analyzed. The combined treatment of curcumin and docetaxel inhibited the proliferation and induced apoptosis significantly higher than the curcumin and docetaxel-treated group alone. Interestingly, the combined treatment with curcumin and docetaxel modulates the expression of RTKs, PI3K, phospho-AKT, NF-kappa B, p53, and COX-2. These results suggest that curcumin can be a potential therapeutic contender in enhancing the efficacy of docetaxel in PCa treatment.

2. INTRODUCTION

Prostate cancer (PCa) comprises 21% of all total cancer-related diseases leading to death in 8% of men in the United States in 2016 (1). As PCa is a potential health threat for men due to its inadequate treatment options for advanced-stage PCa. The most common therapeutic strategies attainable for localized androgen-dependent PCa are prostatectomy and radiotherapy followed by androgen deprivation therapy (2). However, androgen deprivation therapy inevitably

loses its effectiveness, results in a metastatic stage referred to as castration-resistant prostate cancer (CRPC) (3). Currently, taxane-based chemotherapeutic drugs are the first line of treatment for CRPC (4).

Docetaxel is a microtubule-stabilizing agent and clinically approved for the treatment of metastatic castration-resistant PCa (mCRPC) when androgen deprivation therapy fails (5). Treatment of PCa with docetaxel inhibits proliferation and activates apoptosis, however, promotes cytotoxicity in PCa (6-8). Thus, patients receive prolonged treatment with docetaxel experience a transient benefit but develop incurable drug resistance and cytotoxicity (9). The clinical use of taxane-based chemotherapy in castration-resistant prostate cancer (CRPC) has limitation. Therefore, recent studies are focused on combining nature-based agents with the conventional chemotherapies to augment the current cure rates in prostate cancer (3, 10).

Curcumin or diferuloylmethane, a dietary nontoxic polyphenol compound has the multifaceted chemopreventive potential (11, 12). Several preclinical and animal studies have demonstrated that the anticancer activity of curcumin is attributed to target the different signaling molecules associated with proliferative, apoptotic, angiogenic and metastatic pathways (13, 14) and proven to be advantageous in sensitizing the cancer cells when exploitation in conjunction with chemotherapeutic agents (15).

The goal of our study is to elucidate whether curcumin could augment the efficacy of docetaxel treatment in PCa cell lines by targeting multiple signaling molecules associated with survival and proliferation. Therefore, two androgen-independent prostate cancer cell lines (DU145 and PC3) were treated with curcumin and docetaxel alone or in combination, followed by the estimation of pro-survival and proliferative status was analyzed along with different signaling molecules.

3. MATERIALS AND METHODS

3.1. Materials

The human prostate cancer cell lines PC3 (CRL-1435), and DU145 (HTB-81) was obtained from ATCC (Manassas, VA). Docetaxel was purchased from Sigma (St. Louis, MO), and curcumin was obtained from Thermofisher Scientific (Grand Island, NY). Both docetaxel and curcumin were dissolved in DMSO (Sigma, St. Louis, MO) and diluted with media prior to the assays. All the primary and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA). The APC Annexin V Apoptosis Detection Kit with PI was procured from Biolegend (San Diego, CA) and the Click-iT® TUNEL Alexa Fluor® 594 Imaging Assay, for microscopy & HCS was obtained from Thermofisher Scientific (Grand Island, NY).

3.2. Cell culture

PC3 cells were grown in F12K supplemented with 10% fetal bovine serum (FBS), L-glutamine, sodium bicarbonate and Penicillin/streptomycin antibiotic solution (Fisher scientific, Pittsburgh, PA). DU145 cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS and Penicillin/streptomycin antibiotic solution (Fished scientific, Pittsburgh, PA). All cells were maintained in humidified incubator containing 5% CO_2 at 37°C.

3.3. MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, MO) assay was performed to check the viability of the cells after drug treatment. 20,000 cells were plated per well of a 96 well plate and after 24h, cells were treated with different concentrations of either curcumin or docetaxel or the combinations. Cell viability was detected at different time points (24, 48 and 72h) by adding 50 µl of 5 mg/ml of MTT solution and incubating at 37°C for 1-3h. The formazan crystals were dissolved in DMSO, and the optical density was measured at 570 nM using a microplate reader (Spectramax M5, Molecular devices, Sunnyvale, CA). Cell treated with only DMSO was used as a control.

3.4. Apoptosis assay

The cells (400,000) were treated with vehicle, curcumin, docetaxel or combination of curcumin and docetaxel and after 48 hours of treatment, the apoptosis was detected by APC annexin V apoptosis detection kit with PI (Biolegend, San Diego, CA). Briefly, the cells were washed once with PBS, pellet was suspended in 100 µL of Annexin V Binding Buffer followed by incubation with annexin V (conjugated with APC) and propidium iodide (PI) in the dark for 15 minutes. The frequency of apoptotic cells was analyzed using a Guava easyCyte HT (EMD Millipore, Billarica, MA) Flow cytometer.

3.5. TUNEL assay

The DNA fragmentation was detected by terminal deoxyuridine triphosphate nick end labelling (TUNEL) staining using the Click-iT® Plus TUNEL Assay *in situ* Apoptosis Detection Kit (Life Technologies, Carlsbad, CA). Briefly, 100,000 cells were plated per well of the 6-well plate and treated with vehicle, curcumin, docetaxel and a combination of curcumin and docetaxel. After 48 hours of treatment, the cells were fixed with 4% paraformaldehyde and TUNEL assay was performed following manufacturer's

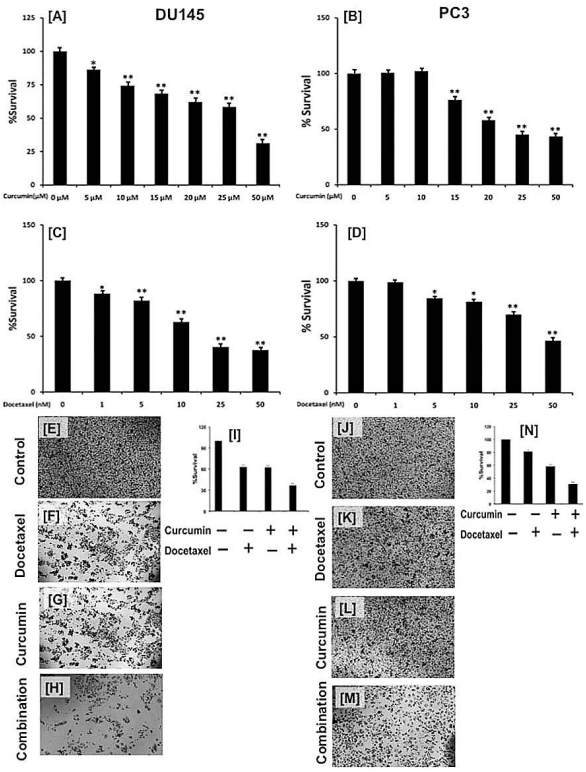

Figure 1. Curcumin enhances the anti-proliferative effect of docetaxel on DU145 and PC3 cells. (i) Cells were grown on 96 well plates and treated with sequential doses of either curcumin (A, B) or docetaxel (C, D) for 48 hours. Thereafter, viability was measured by MTT assay for DU145 (A, C) and PC3 (B, D) after 48 hours. (ii) Cells were treated with either 10 nM docetaxel and 20 μM curcumin or combination of 10 nM docetaxel and 20 μM curcumin. Cell viability was measured by MTT assay after 48 hours of treatment for DU145 (E-I); PC3 (J-N). Images were captured at 100x magnification. Data are represented as a mean ± standard error of the mean of at least three independent experiments and are analyzed by unpaired t-test. ** and * indicate p value≤0.01 and 0.05, respectively.

protocol. TUNEL-positive cells were considered as undergoing apoptosis.

3.6. Western blot analysis

The cells were treated as described earlier and were collected after 48 hours and washed with phosphate-buffered saline (PBS). The harvested cells were lysed with ice-cold RIPA buffer containing a protease-phosphatase inhibitor cocktail for 30 minutes with intermittent vortexing after 10 minutes. The lysates were centrifuged at 10,000 rpm for 15 minutes, and supernatants were collected. The protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific, fisher Scientific, Waltham, MA). About 30 μg protein was electrophoresed on 4-12% polyacrylamide gels (Life Technologies, Carlsbad, CA) and transferred to PVDF membrane and probed with primary antibodies (1:500) overnight at 4°C and HRP labeled secondary antibodies (1:2000) at room temperature for 2 hours. The signal was detected using enhanced chemiluminescence (ECL) and Super Signal West Pico substrate (Thermo Fisher Scientific, Waltham, Fisher Scientific, Waltham, fisher Scientific, Waltham, MA). Images were acquired by Image Quant LAS4000 (GE Healthcare-Biosciences, Pittsburgh, PA). Band intensities were quantified, using the Image-J software (NIH) and densitometry values were normalized to the corresponding GAPDH or β-Actin values.

3.7. Statistical analysis

Statistical analysis was performed using the unpaired *t*-tests and one-way analysis of variance (ANOVA) as appropriate. Results are expressed as standard errors of means (±SEM). The p values below 0.05 were considered to be statistically significant.

4. RESULTS

4.1. Curcumin augments the cytotoxic effects of docetaxel in prostate cancer

To explore the synergistic effects and define the efficacious concentrations, sequential doses of curcumin (5, 10, 15, 20, 25 and 50 μM) and docetaxel (1, 5, 10, 25 and 50 nM) were tested independently or in combination for three different time points, and found to be significant cytotoxicity for 48h treated cells. Treatment of both DU145 and PC3 cells with docetaxel and curcumin alone significantly decreased the proliferation compared with the control groups at 48 hours (Figure 1A, 1B, 1C, and 1D). The IC_{50} values for

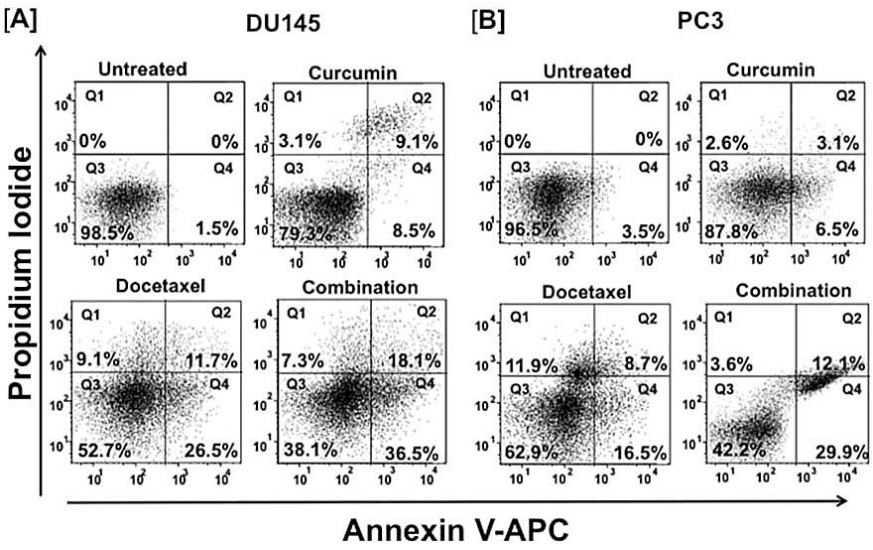

Figure 2. Curcumin enhances the docetaxel-mediated apoptosis in (A) DU145 and (B) PC3. Cells were treated either with docetaxel (10 nM) or curcumin (20 μM) or a combination of docetaxel and curcumin. Apoptosis was evaluated using Annexin V-APC and PI staining followed by flow cytometry analysis. Percentage of early and late apoptotic cells and the necrotic cells are shown as numbers in bold in the flow cytometry chart. Q1 and Q2 (Annexin (+)/(PI (+) represents necrotic and late apoptotic cells. Q3 (Annexin (-) /(PI (-) and Q4 (Annexin (+)/(PI (-) represent viable cells and early apoptotic cells respectively. Data are representative of three independent experiments.

docetaxel and curcumin were 19.2 nM and 32.3 μM for DU145, and 46 nM and 36.1 μM for PC3 respectively at 48 hours. A combined treatment of 20 μM curcumin and 10 nM docetaxel significantly decrease (2.7 fold) the proliferation compared to docetaxel (1.5 fold) and curcumin (1.6 fold) alone in DU145, whereas in PC3 cells combined treatment decrease (3.2 fold) fold compared to docetaxel (1.2 fold) and curcumin (1.7 fold) at 48 hours (Figure 1E-1N). Based on these results, we used curcumin 20 μM and docetaxel 10 nM for DU145 and PC3 cell lines for 48 hours treatment to evaluate the synergistic effects of curcumin and docetaxel.

4.2. Curcumin in combination with docetaxel enhances the apoptosis in PCa cells

To evaluate the synergistic effects of curcumin and docetaxel on apoptosis, DU145 and PC3 cell lines were treated with 20 μM curcumin and 10 nM docetaxel alone, or in combination of 20 μM curcumin and 10 nM docetaxel for 48 hours and upon treatment, cells were stained with Annexin V-APC and PI and analyzed by flow cytometry. On the flow cytometry charts, the Q1 represents necrotic cells, whereas, Q2, Q3, and Q4 refer to late apoptotic, viable cells and early apoptotic respectively (Figure 2A and 2B). As depicted in Figure 2A and 2B, the curcumin (20 μM) and docetaxel (10 nM) apparently shift the cells from Q3 to Q4 and Q2 demonstrated the early and late apoptotic cells, however, the combinatorial treatment (20 μM curcumin and 10 nM docetaxel) further increases the number of apoptotic cells. The early and late apoptotic cells increased up to 36.5 and 18.1% for DU145 treated with the combination (20 μM curcumin and 10 nM docetaxel), compared to 8.5 and 9.1% with curcumin (20 μM), and 26.5 and 11.7% with docetaxel (10 nM) alone (Figure 2A). The combined treatment (20 μM curcumin and 10 nM docetaxel) of PC3 increased the early and late apoptotic cells up to 29.9 and 12.1% compared to 6.5 and 3.1% with curcumin (20 μM) and 16.5 and 8.7% with docetaxel (10 nM) alone (Figure 2B). These results indicate that curcumin enhances the docetaxel-mediated apoptosis in DU145 and PC3 cells.

4.3. Curcumin synergizes the apoptosis induced by docetaxel in PCa cells

To affirm the induction of apoptosis with curcumin and docetaxel alone or in combination in DU145 and PC3 cells, we detected the DNA fragmentation and nuclear condensation as apoptotic markers by TUNEL and DAPI co-staining assay at 48 hours. As shown in Figure 3A and 3B, treatment with the combination of curcumin and docetaxel was significantly higher DNA fragmentation and nuclear condensation compared to curcumin and docetaxel-treated groups alone, which confirm its effectiveness docetaxel-mediated apoptosis.

4.4. Curcumin enhances pro-apoptotic marker expression in docetaxel-treated PCa cells

To evaluate the synergistic effects of curcumin and docetaxel in DU145 and PC3 cells; cellular apoptosis was evaluated by analysis of the expression of anti- and pro-apoptotic protein markers and PARP

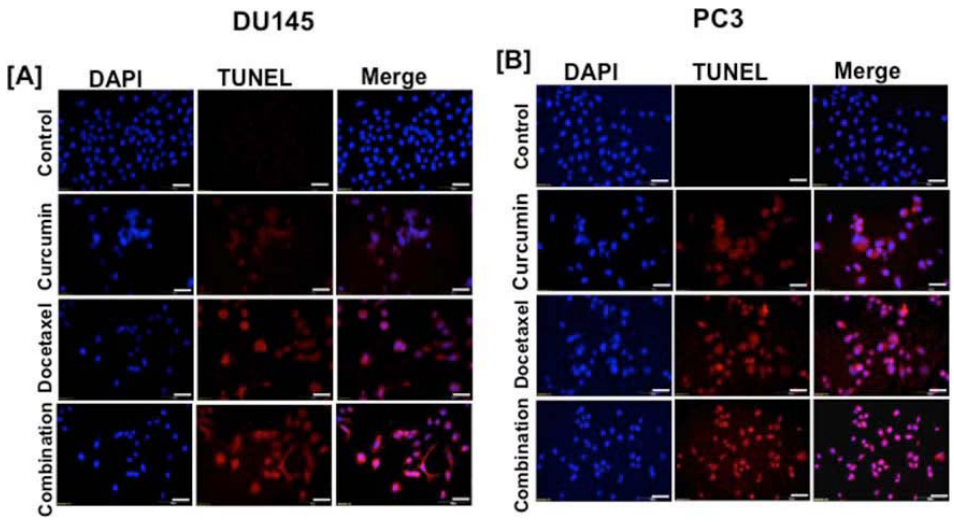

Figure 3. Curcumin enhances the docetaxel-mediated apoptosis in (A) DU145 and (B) PC3 cells. Cells were treated with 10 nM docetaxel and 20 μM curcumin or a combination of 10 nM docetaxel and 20 μM curcumin. TUNEL assay was done after 48h of treatment. The TUNNEL (red staining) indicates the fragmented nuclei of the apoptotic cell. DAPI (blue staining) indicates intact nuclei. The images were captured at 200x magnification. Data are representative of three independent experiments.

cleavage. DU145 and PC3 cells were treated with curcumin (20 μM) and docetaxel (10 nM) alone or the combination with curcumin (20 μM) and docetaxel (10 nM) followed by pro-survival and pro-apoptotic marker expressions were analyzed by western blot analysis. Our data indicates that combined treatment with curcumin with docetaxel down-regulates the expression of the anti-apoptotic proteins BCL-2, BCL-XL and MCL-1 in DU145 and PC3 cells (Figure 4A and 4B). Whereas, the expression of the pro-apoptotic markers BAK and BID were significantly up-regulated in curcumin with docetaxel treated group compared to curcumin and docetaxel-treated group alone (Figure 4A and 4B). The expression of proliferating cell nuclear antigen (PCNA) for tumor development (16) was significantly inhibited in curcumin with docetaxel treated group compared to curcumin and docetaxel-treated group alone (Figure 4A and 4B). Next, we tested the degradation of PARP as an indicator of apoptosis. As shown in Figure 4A and 4B, the combined treatment with curcumin and docetaxel in DU145 and PC3 cells enhanced proteolysis of PARP compared to curcumin and docetaxel alone.

4.5. Curcumin blocks NF-κB activation in docetaxel-treated PCa cells

To explore the synergistic effects of curcumin and docetaxel on NF-κB expression; cells were treated with curcumin (20 μM) and docetaxel (10 nM) or with the combination for 48 hours and were subjected to western blot analysis. The expression of NF-κB protein was down-regulated in the nuclear extract of the cells with curcumin and docetaxel-treated group compared with the cells treated with docetaxel and curcumin alone (Figure 5A and 5B). The combined treatment of curcumin and docetaxel significantly reduced the expression of the proliferation marker CDK-1 and inflammatory marker COX-2 compared to the cells treated with docetaxel alone (Figure 5A and 5B).

4.6. Curcumin synergizes the inhibition of oncogenic receptor tyrosine kinases and activation of tumor suppressor gene p53 in the docetaxel treated prostate cancer

To study the synergism further; the combined effect of curcumin and docetaxel was explored on the expression of receptor tyrosine kinases, phosphoinositide 3-kinase (PI3K)/phospho-AKT, epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor type 2 (HER2). The combined treatment of curcumin and docetaxel reduced the expression of PI3K, phospho-AKT, EGFR and HER2 in both DU145 and PC3 cells compared to docetaxel or curcumin treated group alone (Figure 5A and 5B). Curcumin synergizes the effect of docetaxel by increasing the expression of p53 compared to cells treated with curcumin or docetaxel alone.

5. DISCUSSION

The present study demonstrates that the combination of curcumin and docetaxel targets multiple signaling molecules associated with pro-survival, pro-apoptotic and proliferation. Thus curcumin-docetaxel combination treatment could be useful for blocking mCRPC cancer cell proliferation and apoptosis. Docetaxel is the most widely used chemotherapeutic drug for the treatment of mCRPC and long-term usage results in acquired resistance and toxicity (17). The combination therapy with natural

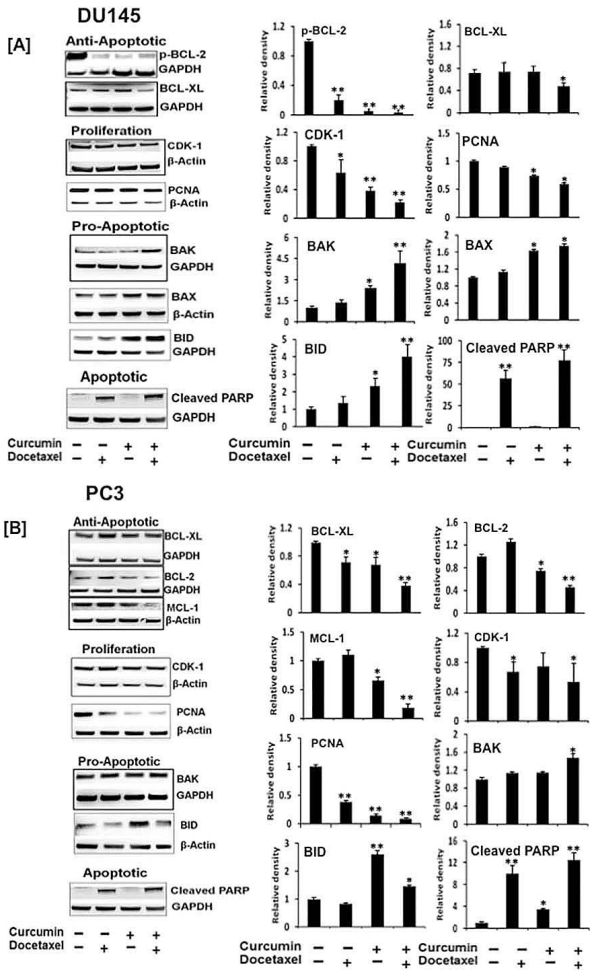

Figure 4. Curcumin decreases the expression of the proliferative markers and enhances the expression of pro- and anti-apoptotic markers in docetaxel-treated (A) DU145 and (B) PC3 cells. Cells were treated with curcumin and docetaxel, or the combination of curcumin and docetaxel for 48h. The expressions of proliferative, anti- and pro-apoptotic markers were analyzed by western blots. Equal amounts of protein were loaded and separated by 1D gel electrophoresis. GAPDH and β-Actin were used as an internal control. The immunoblots are shown in the left panel and the densitometry of the proteins in right panel. Data are representative of three independent experiments. Data are represented as a mean ± standard error of the mean of three independent experiments and analyzed by unpaired t-test. ** and * indicate p value≤0.01 and 0.05, respectively.

compounds is being popular because of the minimum adverse effects on healthy cells. Curcumin is a dietary polyphenol, which has the enormous potential to be used for cancer prevention and treatment. The anti-cancer effects of curcumin are primarily mediated through different oncogenic molecules associated with the cell proliferation, transformation, invasion, angiogenesis and chemoresistance (18-21). Curcumin also enhanced the efficacy of docetaxel treatment in different cancers (3,22, 23). Our study suggests that curcumin considerably enhances the efficacy of docetaxel treatment in androgen-independent PCa cells by inhibiting proliferation and inducing apoptosis through modulation of tumor-suppressor proteins, transcription factors and oncogenic protein kinases compared to docetaxel or curcumin alone. Moreover, curcumin not only synergizes with docetaxel to enhance the cell death but also reduces the effective concentration of docetaxel in PCa cells. The synergistic effects of curcumin and docetaxel on PCa cells are further confirmed by the nuclear fragmentation and flow cytometry assay.

The transcription factor, NF-κB contributes towards the development and progression of cancer by regulating the expression of the genes associated with the cell-survival, proliferation, apoptosis, angiogenesis and metastasis (24). Current studies demonstrated the synergism between curcumin and docetaxel in enhancing the anti-proliferative and pro-apoptotic effects in PCa cells through NF-κB. The elevated levels of NF-κB associated with the different types of human cancer imposing the resistance to chemotherapy (25, 26). The combined treatment of curcumin and docetaxel effectively reduces the expression (data not shown) as well as nuclear translocation of NF-κB thus

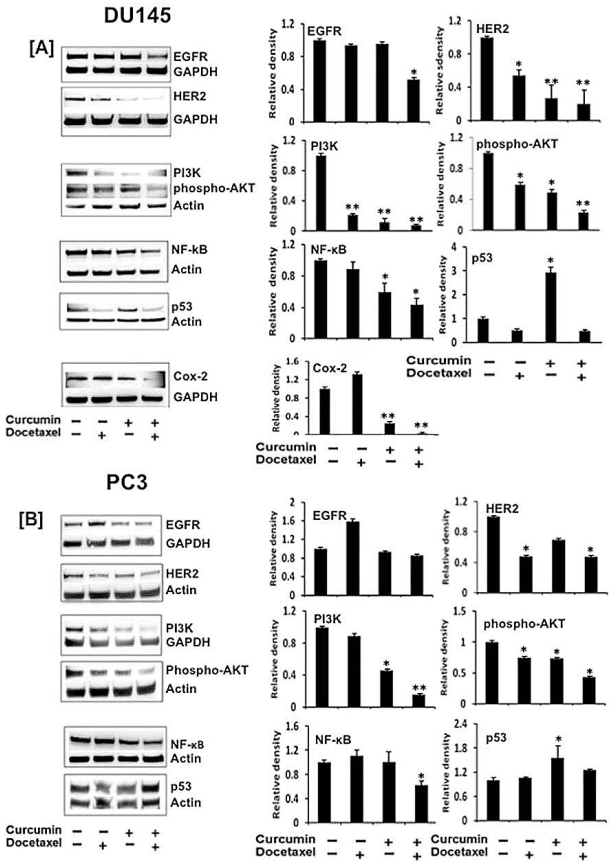

Figure 5. Curcumin with docetaxel down-regulates the expression of RTKs, oncogenic kinases, tumor suppressor protein and inflammatory marker in (A) DU145 and (B) PC3. Cells were treated with curcumin and docetaxel or the combination of curcumin and docetaxel for 48h. The expressions of markers were monitored by western blot analysis. Equal amount of protein was loaded; GAPDH and β-Actin were used as internal control. The immunoblots are shown in the left panel, and the densitometry of the proteins are shown in right panel. Data are representative of three independent experiments. Data are represented as a mean ± standard error of the mean of three independent experiments and analyzed by unpaired t-test. ** and * indicate p value≤0.01 and 0.05, respectively.

reducing the expression of proliferation markers CDK-1 (27) and PCNA (DNA replication and repair) (28) in DU145 cells, which are controlled through the NF-κB pathway (21, 29). In PC3 cells, the effect was not prominent for CDK1 but the down-regulation of PCNA was achieved in the cells treated with both curcumin and docetaxel. The NF-κB up-regulates the BCL-2 family proteins in cancer cells, which indicate the anti-apoptotic and chemo-resistance property (30-32). Previous studies demonstrated that docetaxel-induced apoptosis in DU145 cells is independent of both BCL-2 and pro-apoptotic caspases (7). Our data shows that the expression of BCL-2 did not change with docetaxel (data not shown), but the down-regulation of p-BCl-2 (33) was observed in DU145 cells treated with curcumin and docetaxel together (Figure 4A and 4B). The down-regulation of the other apoptosis suppressor proteins (BCL-XL and MCL-1) in PCa cells treated with the combination of both drugs compared to either of the drugs alone further indicates the synergism of curcumin and docetaxel in inducing apoptosis via NF-κB signaling pathway (Figure 5A and 5B) (34). On the other hand, the up-regulation of the pro-apoptotic markers (BAK and BID) (35) in PCa cells treated with both drugs supports the synergistic effect of curcumin and docetaxel in inducing apoptosis. Moreover, our results demonstrate that the docetaxel-mediated apoptosis of both DU145 and PC3 are independent of pro-apoptotic caspases. However, cleavage of PARP indicates DNA degradation in the cells treated with docetaxel alone or with the combination of curcumin and docetaxel through other pathways (36). The down-regulation of PCNA, a marker of DNA replication and repair, and up-regulation of PARP cleavage further substantiate the synergistic effect of curcumin and docetaxel on promoting apoptosis in PCa.

The present study also suggests that treatment with docetaxel in combination with curcumin down-regulates the expression of HER2 and EGFR resulting inhibition of the expression of PI3K kinase and phospho-AKT, which suppressed the NF-κB expression thus blocking proliferation and inducing apoptosis. Human epidermal growth factor (HER2)

and EGFR belong to the growth factor receptor tyrosine kinases (RTKs) family and notable for having the ability to activate the androgen receptor signaling by a ligand-independent mechanism in the androgen-independent stage of advanced PCa (37, 38). In the absence of androgen, HER2 induces proliferation and inhibits apoptosis thus promoting resistance to existing therapy (39). The HER2 dimerize with EGFR to become active and both the receptors play an important role in the development and progression of cancer (40). The RTKs recruits phosphoinositide 3-kinase (PI3K), which is a major signaling component in activation of the serine-threonine kinase AKT (41). The PI3K-AKT pathway is a primary regulator of a cell survival pathway through activating the key transcription factors, e.g. NF-κB (21). Curcumin has been shown to suppress the HER2 and EGFR expression in different cancers (42, 43) and suppress the expression of PI3 kinase and phosphorylation of AKT in PCa cells (44).

Current study establishes that curcumin synergizes with docetaxel in reducing NF-κB expression thus reducing the expression of COX-2 in DU145 cells. No constitutive COX-2 expression was detected in PC3 cells. Cyclooxygenase 2 (COX-2) is a key mediator of inflammatory processes, which plays an important role in different steps of tumor progression by promoting cell proliferation and blocking apoptosis (45). The expression of COX-2 is modulated by NF-κB signaling (46). Curcumin is a potent anti-inflammatory agent and known to inhibit the expression of COX-2 in cancer cells (21, 23).

The combined treatment of curcumin with docetaxel induces the expression of p53 and suppresses the expression of NF-κB thus promoting apoptosis in PCa. The p53, a tumor suppressor gene is often inactivated in cancer and its down-regulation leads to cancer progression. Previous studies suggested that in a variety of cancer cells, curcumin induces apoptosis by inhibition of NF-κB and activation of p53 (21).

In summary, our data demonstrates that curcumin enhances the therapeutic efficacy of docetaxel by inhibiting proliferation and inducing apoptosis in PCa. Moreover, down-regulation of transcription factor NF-κB, COX-2, RTKs, and oncogenic kinases PI3K and phospho-AKT by combined treatment reveal the multifaceted potential of curcumin in augmenting the efficacy of docetaxel treatment in PCa. Further investigation into the mechanistic aspects of the synergism between curcumin and docetaxel may lead to a novel therapeutic approach for advanced PCa treatment.

6. ACKNOWLEDGMENTS

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