Quercetin synergizes with 2-methoxyestradiol inhibiting cell growth and inducing apoptosis in human prostate cancer cells

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Abstract. Lack of effective treatment options for castration-resistant prostate cancer reinforces the great need to develop novel drug therapies. Quercetin is a plant-derived flavonoid that can induce apoptosis in prostate cancer cells. 2-Methoxyestradiol (2-ME) is an endogenous estrogenic metabolite that also has antineoplastic activity. However, these two agents have limited bioavailability. Herein, we explored the antiproliferative and proapoptotic activities of quercetin combined with 2-ME in both androgen-dependent LNCaP and androgen-independent PC-3 human prostate cancer cell lines. Compared to quercetin and 2-ME alone, combining quercetin with 2-ME at appropriate concentrations i) showed synergistic antiproliferative and proapoptotic activities; ii) increased G2/M phase population of cells; iii) decreased the ratio of Bcl-2/Bax significantly. The combination of quercetin and 2-ME is a new clinically relevant treatment regimen which has the potential of enhancing the antitumor effect on prostate cancer and lessening the side effect of either quercetin or 2-ME alone.

Introduction

Prostate cancer is the most common cancer in men, with an estimated 241,740 new cases and 28,170 deaths in 2012 in the United States (1). Cytotoxic chemotherapy based on docetaxel can be used in advanced patients, however, this therapy has only a median overall survival benefit of 2 months (2,3). Lack of effective treatment options for castration-resistant prostate cancer reinforces the great need to develop novel drug therapies that act singly or in combination (4).

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Quercetin (3, 3', 4', 5, 7-pentahydroxyflavone, Que) is a plant-derived flavonoid which displays antioxidant, anti-inflammatory and anticancer properties. It has been reported to induce apoptosis in many human cells, including prostate cancer cells. Although quercetin has already been used to treat advanced prostate cancer as an OTC drug, its clinical use is still limited because of the low bioavailability. Besides modification of its molecular structure to improve bioavailability (5,6), recent studies have been aimed to investigate whether quercetin can enhanced the cytotoxic effect with other chemotherapeutic agents. Several studies demonstrated that the combination of quercetin and classical or new anticancer drugs showed synergistic effect on prostate cancer cells (7-9).

2-Methoxyestradiol (2-ME) is an endogenous metabolite of 17β-estradiol, which does not exhibit direct estrogenic activity. Many studies have shown that 2-ME has antiproliferative, proapoptotic, and anti-angiogenic activity and is considered to have potential clinical benefit in treatment of cancer. It has been reported that 2-ME inhibited growth and induced apoptosis of both androgen-dependent and androgen-independent prostate cancer cells by blocking cells in G2/M phase (10). 2-ME has also been suggested to inhibit angiogenesis by regulating capillary tube formation *in vitro* (11). Sweeney *et al* evaluated the efficacy of 2-ME by administering it orally to hormone refractory prostate cancer patients (12). They found that 2-ME was well tolerated at a dose of 1,200 mg/d, and PSA levels declined or stabilized in a minority of patients. However, the study identified bioavailability as a limiting factor of 2-ME. To overcome this limitation, several studies have investigated whether the efficacy could be enhanced by combining 2-ME with other drugs. Ghosh *et al* reported that combining 2-ME with eugenol inhibited growth of prostate cancer cells and induced apoptosis at lower concentrations than either single agent alone (13).

Despite these promising data, the effects of combining quercetin with 2-ME on prostate cancer cells are as yet unknown. This study was conducted to investigate the antiproliferative and proapoptotic activities of quercetin combined with 2-ME in both androgen-dependent LNCaP and androgen-independent PC-3 human prostate cancer cell lines. The combined antiproliferative effect was analyzed using the multiple drug equations developed by Chou and Talalay (15).

Materials and methods

Cell culture. Two human prostate cell lines, LNCaP and PC-3, were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (Hyclone). Cells were incubated in a humidified CO_2 incubator at 37˚C with 95% air and 5% CO_2.

Drug treatment. Quercetin (Sigma, St. Louis, MO, USA) and 2-methoxyestradiol (Sigma) were dissolved in DMSO (Sigma), and the final concentrations were 3.125-200 μM and 0.3125-10 μM, respectively. DMSO, in equal amounts to the treatment conditions, was added to the media as the control group.

Cell viability assays and CI calculation. Cells were seeded in 6-well plates at an approximate density of $8x10^4$ cells/ml for LNCaP or $3x10^4$ cells/ml for PC-3 in triplicate. Following attachment (48 and 24 h after seeding for LNCaP and PC-3, respectively), cells were treated with different concentrations of quercetin (3.125, 6.25, 12.5, 25, 50, 100, 200 μM) or 2-ME (0.3125, 0.625, 1.25, 2.5, 5, 10 μM), respectively. Cell viability in the presence and absence of compounds was measured by trypan blue (Sigma) exclusion assay. Briefly, cells were harvested by trypsinization and resuspended in PBS. A small aliquot of cell suspension was added to an equal volume of 0.4% trypan blue and viable (unstained) cells were counted in a hemacytometer. Cells in each well were counted three times. Cell viability is expressed as the percent viable cells after normalizing to total number of cells in the solvent treated control. Data were analyzed with Origin 7.5 software and dose-effect curve of quercetin or 2-ME on LNCaP or PC-3 cells was drawn, respectively. According to the fitted dose-effect curves and IC_{50} values, appropriate concentrations of quercetin (5, 10, 20, 40 μM) and 2-ME (0.5, 1, 3, 5 μM) were selected to compose 16 different combinations using factorial design, which is statistically powered to evaluate effective doses of combination therapy (14). The inhibition rates of cell growth after treatment for 48 h were measured subsequently. Using a non-constant ratio setting, the combination index (CI) values were calculated on the equation stated below (15):

$$CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$$

$(D)_1$ = dose of drug 1 in combination with $(D)_2$; $(Dx)_1$ = dose of drug 1 alone; $(D)_2$ = dose of drug 2 in combination with $(D)_1$; $(Dx)_2$ = dose of drug 2 alone. The CI values are on a continuum with respect to synergy, with values <0.9 indicating synergism and values >1.1 indicating antagonism for a given combination. According to the CI values, Que 10 μM, 2-ME 3 μM, and Que 10 μM with 2-ME 3 μM which showed synergistic activity were used for the subsequent measurements.

Hoechst 33342 and PI double staining. Cells were plated in 35-mm dishes and treated as described above. After treatment for 48 h, morphological changes on the nuclear chromatin of cells undergoing apoptosis were detected by double-staining with Hoechst 33342 (Sigma) and PI (PI, Sigma). Briefly, the cells were incubated with Hoechst 33342 (10 μg/ml) in medium for 30 min at 37˚C, and then incubated with PI (2 μg/ml) for 20 min at 4˚C. Then the cells were examined for morphological changes and were photographed using a Nikon Eclipse TE2000-U fluorescence microscope (Nikon, Tokyo, Japan).

Annexin V binding assay. After treatment for 48 h as described above, the occurrence of apoptosis was determined by Annexin V-FITC Apoptosis Detection kit (Sigma) according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a BD FACSAria flow cytometer (Becton Dickinson, NJ, USA) using FACSDiva 4.1 software. The presence of viable (Annexin V-negative and PI-negative), early apoptotic (Annexin V-positive, PI-negative), late apoptotic and necrotic (Annexin V-positive and PI-positive) cells were recorded. The extent of apoptosis was quantified as percentage of Annexin V-positive and PI-negative cells.

Cell cycle analysis. Cell cycle distribution was analyzed by flow cytometry using PI DNA staining. After treatment for 48 h, cells were harvested and resuspended in ice-cold PBS. Ice-cold 70% ethanol (4 ml) was added in a drop wise manner and cells were stored at 4˚C for 2 h. Then, cells were pelleted by centrifugation for 5 min. The supernatant was removed and cells were resuspended in 1 ml of 0.002% RNAse solution (0.002% RNAse + 0.1% TritonX-100 + 0.01 M PBS) incubated at 37˚C, 5% CO_2 for 30 min. Then 0.1 ml of PI solution (0.05% PI + 1% TritonX-100 + 0.01 M PBS) was added. Subsequently, cells were analyzed by Coulter® Epics XL flow cytometer (Beckman Coulter Inc., CA, USA). Cell cycle distribution was calculated from at least $8x10^4$ cells and was analyzed with MultiCycle software by assigning relative DNA content per cell to sub-G0/G1, G0/G1, S and G2/M fractions.

Western blot experiments. After treated with indicated concentrations of drugs for 48 h, total cell proteins were extracted from cells with RIPA Lysis buffer (Applygen Inc., Beijing, China) added with protease inhibitor (Roche, Switzerland) according to the manufacturer's instructions. Cellular protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, USA) according the manufacturer's instructions. Equivalent amounts of protein samples (80 μg) were separated with 15% SDS-PAGE gels and transferred to nitrocellulose membranes (Pall, NY, USA). Following primary antibodies: Bcl-2 (Santa Cruz, CA, USA), Bax (Santa Cruz) and β-actin (Santa Cruz) were used. After binding to indicated secondary antibodies, an enhanced chemiluminescence (Pierce) blotting analysis system (Geldoc, Bio-Rad, CA, USA) was used for antigen-antibody detection.

Statistical analysis. Data were expressed as means ± SD. Between-group comparisons were analyzed by ANOVA using SPSS Statistics 17.0. P-values <0.05 were regarded as a significant difference.

Results

Inhibition of cell growth. The effects of quercetin or 2-ME alone on cell viability in LNCaP or PC-3 prostate cancer cells were measured, respectively. As shown in Fig. 1, the inhibi-

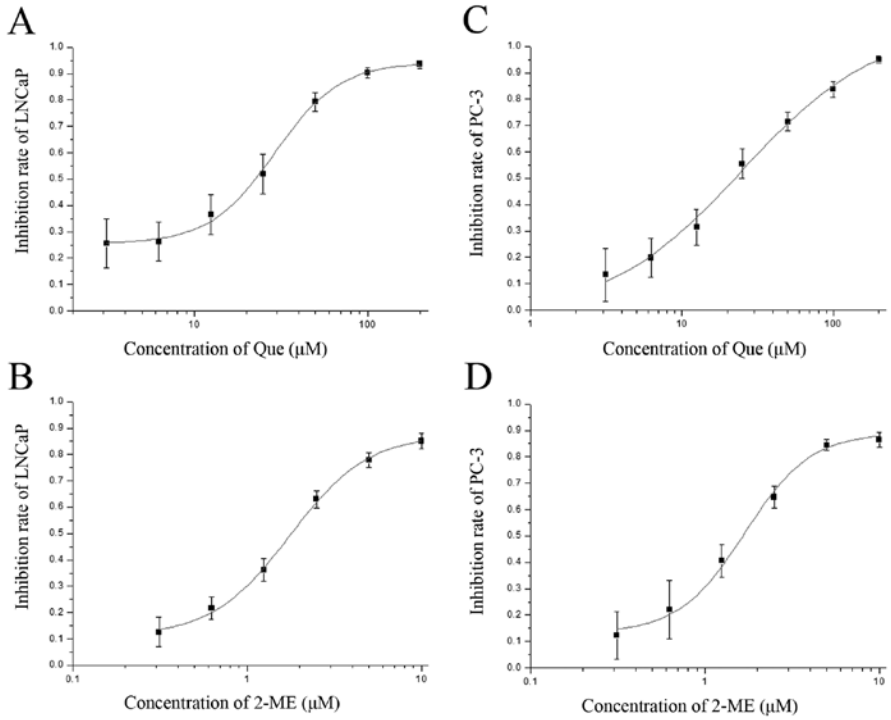

Figure 1. Dose-effect curves of quercetin or 2-ME on LNCaP (A and B) or PC-3 (C and D) cells. Cells were cultured as described in Materials and methods and treated with varying combinations of quercetin (3.125, 6.25, 12.5, 25, 50, 100, 200 μM) or 2-ME (0.3125, 0.625, 1.25, 2.5, 5, 10 μM). After treatment for 48 h, inhibition rate of cell growth was measured. Dose-effect curves were fitted and IC_{50} values were calculated using origin 7.5 software.

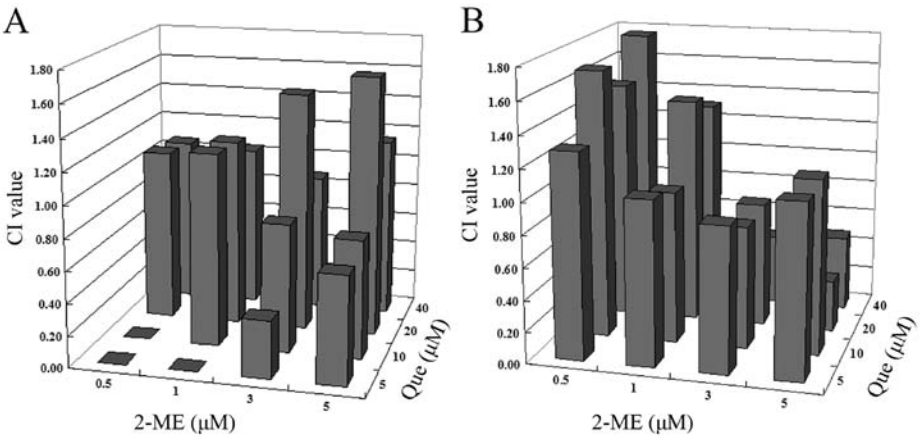

Figure 2. CI values for the combinations of quercetin and 2-ME on LNCaP (A) or PC-3 (B) cells. Cells were cultured as described in Materials and methods and treated with varying combinations of quercetin (5, 10, 20, 40 μM) and 2-ME (0.5, 1, 3, 5 μM). After treatment for 48 h, cell viability was measured and the CI values were determined using the equation described in Materials and methods. In (A), CI values for combinations of Que 5 μM + 2-ME 0.5 μM, Que 5 μM + 2-ME 1 μM, and Que 10 μM + 2-ME 0.5 μM were not available using the present method.

tion rate of LNCaP or PC-3 cells treated with varying doses of quercetin or 2-ME showed a dose-dependent increase. For LNCaP cells, the IC_{50} values of quercetin and 2-ME were 23.29 μM and 1.89 μM, respectively. For PC-3 cells, the IC_{50} values of quercetin and 2-ME were 22.12 μM and 1.74 μM, respectively. Then the effects of 16 combinations of quercetin (5, 10, 20, 40 μM) and 2-ME (0.5, 1, 3, 5 μM) on cell growth were measured. CI values were calculated and shown in Fig. 2. The combination treatments demonstrated CI values of 0.36-2.18 and 0.32-1.77 for LNCaP and PC-3 cells, respectively. According to the CI values, lower dose of quercetin (5 and 10 μM) with higher dose of 2-ME (3 and 5 μM) for LNCaP cells showed synergistic activity, whereas for PC-3 cells, besides the combination of Que 10 μM and 2-ME 3 μM, higer dose of quercetin (20 and 40 μM) with higher dose of 2-ME (3 and 5 μM) showed synergistic activity. The combination of Que 10 μM and 2-ME 3 μM which showed synergism (CI=0.81 for LNCaP; CI=0.78 for PC-3) was used for the subsequent measurements.

Morphological detection of apoptosis by Hoechst 33342 and PI staining. Normal cells stained with Hoechst showed normal

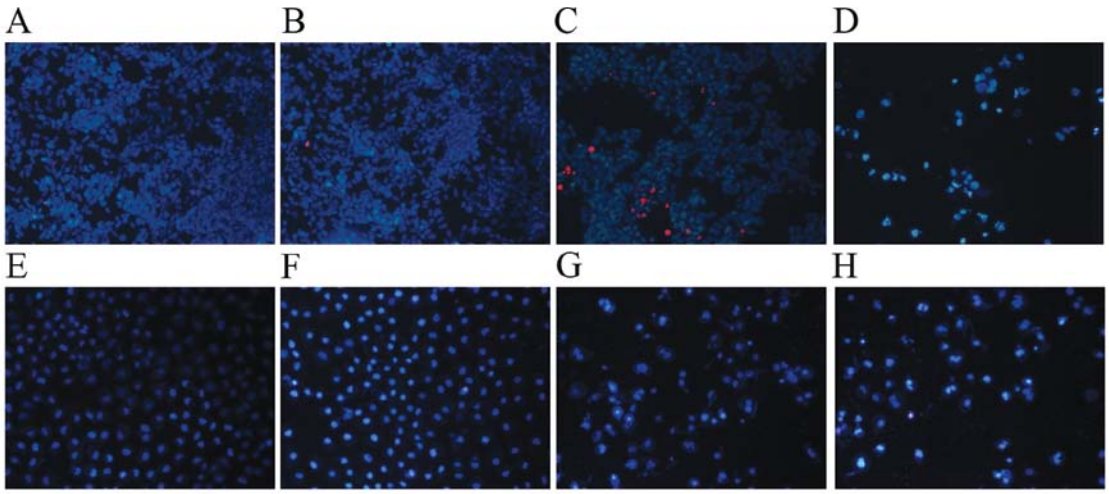

Figure 3. Hoechst 33342/PI double staining in LNCaP (A-D) or PC-3 (E-H) cells. (A and E) Control cells; (B and F) cells were treated with Que 10 μM for 48 h; (C and G) cells were treated with 2-ME 3 μM for 48 h; (D and H) cells were treated with Que 10 μM + 2-ME 3 μM for 48 h. Cells were photographed using a fluorescence microscope (x200). Representative data of three independent experiments are shown.

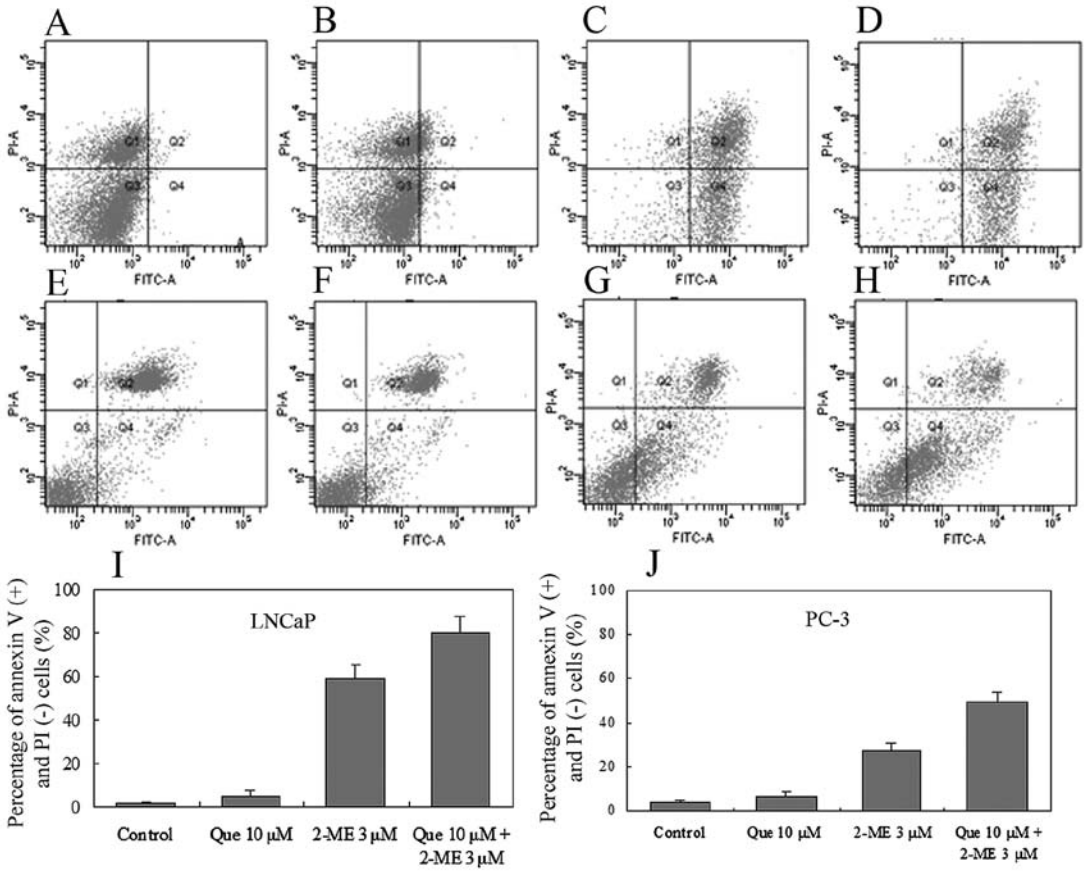

Figure 4. Apoptosis induced by quercetin, 2-ME or combination in LNCaP (A-D) or PC-3 (E-H) cells. (A and E) control cells; (B and F) cells were treated with Que 10 μM for 48 h; (C and G) cells were treated with 2-ME 3 μM for 48 h; (D and H) cells were treated with Que 10 μM + 2-ME 3 μM for 48 h. Data are presented as means ± SD from triplicate of percentage of Annexin V-FITC positive and PI negative and cells (I and J).

morphology of nuclei (blue), while apoptotic cells showed nuclear condensation or nuclear fragmentation (bright blue). PI can penetrate the cytoplasmic membrane of late apoptotic and necrotic cells (pink). As shown in Fig. 3, in both LNCaP and PC-3 cells, slight nuclear condensation or nuclear fragmentation were observed in control cells and cells treated with Que (10 μM) alone. In contrast, a large number of cells showed these morphological changes after treatment with 2-ME 3 μM or with the combination of Que 10 μM and 2-ME 3 μM.

Apoptotic effect is induced by quercetin with 2-ME treatment. We examined whether the combination treatment for 48 h could enhance the apoptotic effect in LNCaP and PC-3

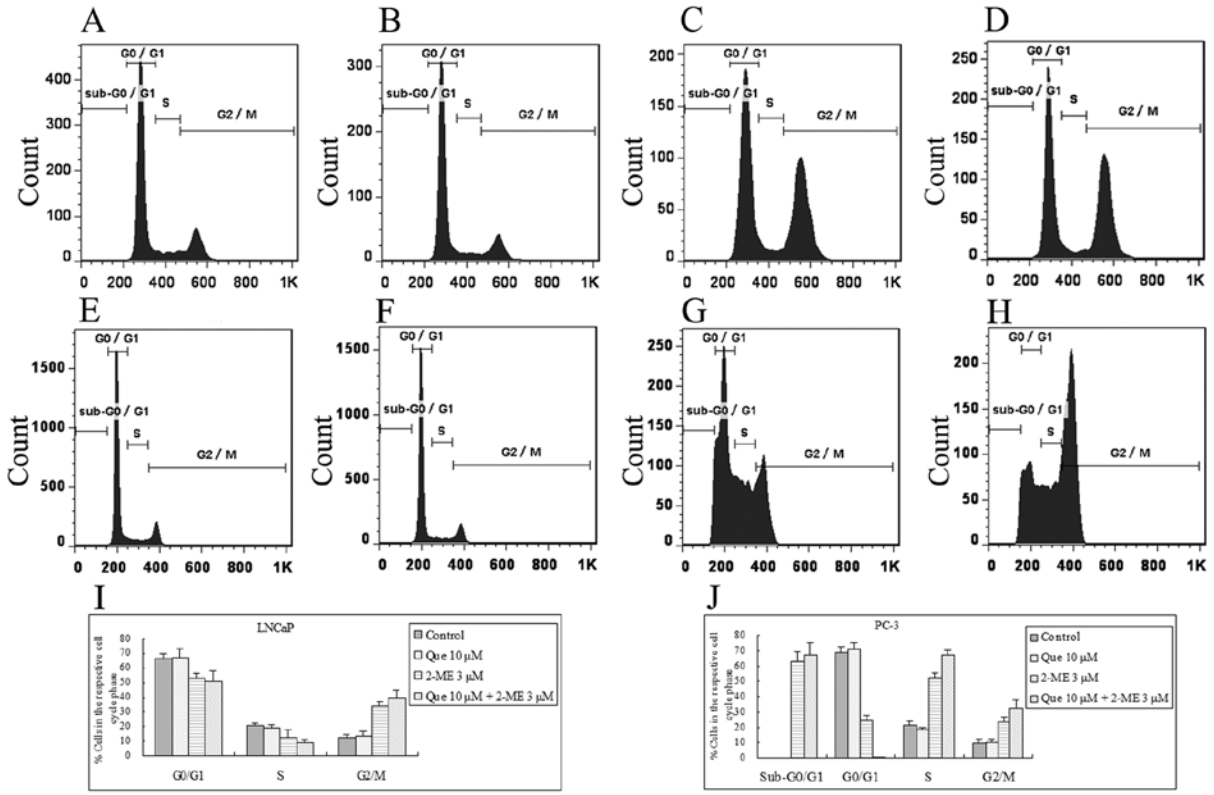

Figure 5. Effect of Que, 2-ME or combination on cell cycle distribution in LNCaP (A-D) or PC-3 (E-H) cells. (A and E) Control cells; (B and F) cells were treated with Que 10 μM for 48 h; (C and G) cells were treated with 2-ME 3 μM for 48 h; (D and H) cells were treated with Que 10 μM + 2-ME 3 μM for 48 h. Distribution of cells in various phases was determined by flow cytometry. Representative histograms are shown in the top panel (A-H) and graphic presentation of the data shown in the bottom panel (I and J) is an average of three independent experiments.

cells using Annexin V binding assay. As shown in Fig. 4, the percentage of Annexin V (+) and PI (-) cells in the combination group were significantly increased (80.2±7.1% for LNCaP; 49.2±4.5% for PC-3; P<0.05) as compared to the control (1.3±0.5% for LNCaP; 3.8±1.1% for PC-3), quercetin alone (4.2±2.6% for LNCaP; 6.4±2.6% for PC-3) and 2-ME alone (58.9±6.4% for LNCaP; 27.3±3.4% for PC-3) for both LNCaP and PC-3 cells.

Effect of quercetin with 2-ME treatment on the cell cycle. The cell cycle was examined after treatment. As shown in Fig. 5, the combination of quercetin and 2-ME treatment for 48 h resulted in a significant increase in G2/M phase cells (40.0±4.4% for LNCaP; 32.1±5.8% for PC-3; P<0.05) as compared to the control (12.8±1.6% for LNCaP; 9.8±2.1% for PC-3), quercetin alone (13.3±3.7% for LNCaP; 10.4±1.4% for PC-3) and 2-ME alone (33.7±3.1% for LNCaP; 23.2±3.6% for PC-3). For PC-3 cells, the combination treatment resulted in a significant increase in the S phase cells (67.4±3.2%, P<0.05) as compared to the control (21.9±2.3%), quercetin alone (18.2±1.4%) and 2-ME alone (52.3±2.8%), with a significant decrease in the G0/G1 phase cells (0.5±0.2%, P<0.05) as compared to the control (68.3±4.1%), quercetin alone (71.4±3.7%) and 2-ME alone (24.5±3.5%). A sub-G0/G1 peak appeared in the 2-ME alone and combination treated PC-3 cells, however, there was no statistical significance between the percentages of cells in sub-G0/G1 phases (63.1±5.9% for 2-ME group; 67.6±5.3% for combination group).

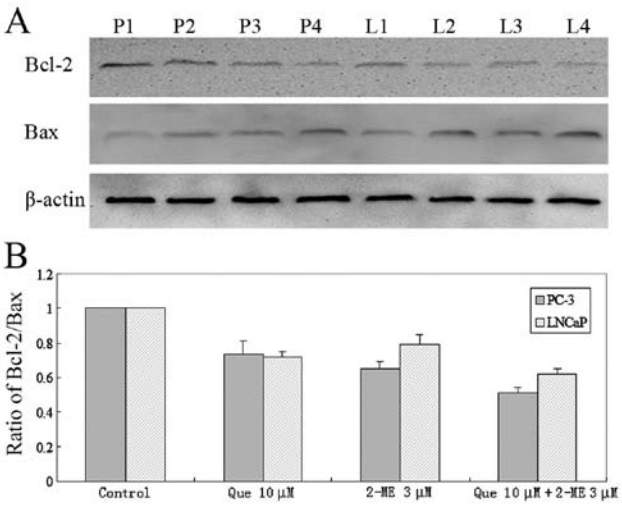

Figure 6. Effect of Que, 2-ME or combination on Bcl-2 and Bax expression in PC-3 or LNCaP cells. (P1 and L1) control cells; (P2 and L2) cells were treated with Que 10 μM for 48 h; (P3 and L3) cells were treated with 2-ME 3 μM for 48 h; (P4 and L4) cells were treated with Que 10 μM + 2-ME 3 μM for 48 h. Expression of Bcl-2 and Bax was determined by western blot. Representative blots are shown in the top panel (A) and graphic presentation of the data is shown in the bottom panel (B) is an average of three independent experiments.

Effect of quercetin with 2-ME treatment on expression of Bcl-2 and Bax in prostate cancer cells. We investigated whether the combination of quercetin and 2-ME treatment for 48 h could induce apoptosis through regulation of anti-apoptotic

Bcl-2 and proapoptotic Bax. As shown in Fig. 6, the expression of anti-apoptotic protein Bcl-2 decreased after treated with quercetin and 2-ME alone or in combination, whereas the expression of the proapoptotic protein Bax increased. The combination treatment of quercetin and 2-ME resulted in a significant decrease of Bcl-2/Bax ratio (0.514±0.028 for PC-3; 0.618±0.034% for LNCaP; $P<0.05$) as compared to the control (1 for PC-3; 1 for LNCaP), quercetin alone (0.734±0.073 for PC-3; 0.718±0.032 for LNCaP) and 2-ME alone (0.647±0.043 for PC-3; 0.792±0.058 for LNCaP). These data indicate a potential role for Bcl-2/Bax ratio in drug-induced apoptosis in prostate cancer cells.

Discussion

Most patients with late prostate cancer treated with chemotherapy have temporary responses, associated with reduced quality of life due to the side effects of drugs (16). We aimed to evaluate new chemotherapeutic combinations based on two promising drugs for the treatment of prostate cancer. Drug combination has several advantages. Firstly, each drug has specific anticancer mechanism, leading to a wider spectrum of anticancer mechanism when combined. Secondly, certain drug may synergize with another. Finally, combination therapy with lower doses of each drug may be safer for long-term treatment and may lessen the drug-specific side-effect.

The combination of two drugs may generate synergism, additive effect or antagonism, which directly impacts the rationality and efficacy of the treatment. In this study, 4 different doses of each drug were selected based on the dose-effect curves, thus 16 different drug combinations were composed using factorial design, which could be used to study the fixed-dose combinations of two drugs (14). Our data showed that lower dose of quercetin (<10 μM) with higher dose of 2-ME (>3 μM) for LNCaP cells, higher dose of quercetin (>20 μM) with higher dose of 2-ME (>3 μM) and Que 10 μM with 2-ME 3 μM for PC-3 cells showed synergistic activity. These data suggested that combination of quercetin and 2-ME at appropriate concentrations had the potential for greater antiproliferative activity. These data were consistent with the published results showing interaction between quercetin or 2-ME and other chemotherapeutic agents (8,13,17).

Previous studies have shown that both quercetin alone and 2-ME alone can induce apoptosis in prostate cancer cells (10,18). In our experiments, typical morphological changes of apoptotic cells were more evident in the combination group. Annexin V and PI staining showed that combination of quercetin and 2-ME induced a significant increase of apoptosis as compared to the drugs administered alone in both LNCaP and PC-3 cells. The results were consistent with a study treating human hepatoma cell lines with quercetin and 2-ME (19).

Vijayababu *et al* demonstrated that quercetin blocked G2/M transition and induced apoptosis in PC-3 cells (20). Kumar *et al* reported that 2-ME treatment arrested prostate cancer cells in G2/M phase, with a concomitant decrease in G1 population (10). Our data showed that for LNCaP cells, although a relative lower dose of quercetin (10 μM) could not alter the cell cycle distribution obviously, its treatment with a higher dose of 2-ME (3 μM) could induce a slight increase in G2/M phase as compared to 2-ME alone. For PC-3 cells, as compared to the drugs administered alone, combination of quercetin and 2-ME arrested cells in S phase and G2/M phase, with a significant decrease in G0/G1 population. These data showed that the combination of quercetin and 2-ME could arrest LNCaP cells in G2/M phase and PC-3 cells in S phase and G2/M phase, thus blocking the progress of cells to G0/G1 phase and inducing apoptosis. This advantage of drug combination was consistent with the cell proliferation data.

The mitochondrial pathway of apoptosis is mediated by the Bcl-2 family proteins whose members include proapoptotic Bax and Bak and anti-apoptotic Bcl-2, Bcl-X_L, and Mcl-1. Bcl-2 family proteins regulate the passage of small molecules, such as cytochrome *c*, Smac/Diablo, and apoptosis-inducing factor, which activates caspase cascades (18,21). The activation of caspases is counteracted by anti-apoptotic members, because these proteins heterodimerize with proapoptotic members and interfere with release of cytochrome *c* by pore-forming proteins (Bid, Bik) (22,23). The relative ratio of proapoptotic and anti-apoptotic proteins determines the sensitivity or resistance of cells to various apoptotic stimuli (21,24,25). Vijayababu *et al* showed that quercetin treatment significantly increased the expression of Bax and decreased the expression of Bcl-2 in a dose-dependent manner in PC-3 cells (20). Lee *et al* revealed that the apoptotic processes of LNCaP cells induced by quercetin were mediated by the dissociation of Bax from Bcl-xL and the activation of caspase families (18). Basu *et al* suggested that the phosphorylation of Bcl-xL induced by 2-ME might oppose the anti-apoptotic function of Bcl-xL to permit prostate cancer cells to undergo apoptosis (26). In the present study, we demonstrated that both a low dose of quercetin (10 μM) alone and a high dose of 2-ME (3 μM) alone can increase the expression of Bax and decrease the expression of Bcl-2 in both LNCaP and PC-3 cells after treatment for 48 h, furthermore, their combination enhanced these effects, with the ratio of Bcl-2/Bax decreasing more obviously.

In this study, we explored the potential treatment of quercetin combined with 2-ME in both androgen-dependent LNCaP and androgen-independent PC-3 human prostate cancer cell lines. Our findings showed synergistic inhibition of cell proliferation in both LNCaP and PC-3 cells, which have implications to clinicians. Since the regulation of proapoptotic Bax and anti-apoptotic Bcl-2 correlated well with the combination treatment to inhibit cell growth and inducing cell apoptosis, the combination treatment was more effective in inducing apoptosis through Bcl-2/Bax-dependent mechanism than the single drug. Although the detail mechanism involved in anticancer activity needs further investigation, this study provided evidence that the treatment of quercetin combined with 2-ME enhanced the apoptotic action in human prostate cancer cells and raised the possibility of using these two anticancer drugs to treat prostate cancer.

Acknowledgements

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