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ARTICLES

Anti-Proliferative Effects of Evodiamine on Human Prostate Cancer Cell Lines DU145 and PC3

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Abstract Prostate carcinoma is one of the most common malignant tumors and has become a more common cancer in men. Previous studies demonstrated that evodiamine (EVO) exhibited anti-tumor activities on several cancers, but its effects on androgen-independent prostate cancer are unclear. In the present study, the action mechanisms of EVO on the growth of androgen-independent prostate cancer cells (DU145 and PC3 cells) were explored. EVO dramatically inhibited the growth and elevated cytotocixity of DU145 and PC3 cells. The flow cytometric analysis of EVO-treated cells indicated a block of G2/M phase and an elevated level of DNA fragmentation. The G2/M arrest was accompanied by elevated Cdc2 kinase activity, an increase in expression of cyclin B1 and phosphorylated Cdc2 (Thr 161), and a decrease in expression of phosphorylated Cdc2 (Tyr 15), Myt-1, and interphase Cdc25C. TUNEL examination showed that EVO-induced apoptosis was observed at 72 h. EVO elevated the activities of caspase 3, 8, and 9 in DU145 cells, while in PC3 cells only the activities of caspase 3 and 9 were elevated. EVO also triggered the processing of caspase 3 and 9 in both DU145 and PC3 cells. We demonstrate that roscovitine treatment result in the reversion of G2/M arrest in response to EVO in both DU145 and PC3. However, inhibitory effect of roscovitine on EVO-induced apoptosis could only be observed in DU145 rather than PC3. In DU145, G2/M arrest might be a signal for initiation of EVO-triggered apoptosis. Whereas EVO-triggered PC3 apoptosis might be independent of G2/M arrest. These results suggested that EVO inhibited the growth of prostate cancer cell lines, DU145 and PC3, through an accumulation at G2/M phase and an induction of apoptosis. J. Cell. Biochem. 101: 44–56, 2007.

Key words: evodiamine; G2/M arrest; apoptosis; androgen-independent

Prostate cancer is the most commonly diagnosed malignancy in American men over 40 years of age and is the second leading cause of cancer deaths [Godley et al., 1996; Hsing, 1996]. Recently, prostate cancer has become more common in Asia [Chang et al., 1997]. Prostate cancer occurs as an androgen-dependent tumor [Yeap et al., 1999]. Androgen ablation is the standard treatment for patients who present advanced, androgen-dependent metastatic prostate carcinomas [Goolsby, 1998; Beedassy and Cardi, 1999]. However, the high failure rate for castration causes permanent cures and progresses to androgen-independent prostate carcinoma that is resistant to radiation, surgery, and chemotherapy [Mahler and Denis, 1992; Abolhassani and Chiao, 1995; Wilding, 1995]. Because treatment of this stage of prostate cancer has been largely ineffective, eventually the patients would die of an androgen-refractory disease. Hence, it would be important to find promising agents against advanced androgen-independent prostate cancer.

Several microchemicals isolated from natural foods and herbs exerted inhibitory effects on

Abbreviations used: MTT, 3-(2)-2,5-diphenyltetralozium bromide; LDH, lactate dehydrogenase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; EVO, evodiamine; PI, propidium iodide.

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the proliferation of cancer cells [Zi et al., 1998; Panaro et al., 1999; Zi and Agarwal, 1999]. Chinese medicine which contains many chemical compounds exhibited anti-proliferative effects on cancer cells [Li et al., 2000; Yeh et al., 2001, 2003]. Digitalis and bufalin, the cardiac glycosides isolated from Chinese herbs and toad venom, exhibited the anti-proliferative effects on prostate cancer cells via elevation of $[Ca^{2+}]_i$ and apoptosis [Yeh et al., 2001, 2003].

Wu-Chu-Yu is a Chinese herb used for cold hand, migraines, and vomiting. Evodiamine (EVO), one of the major bioactive compounds isolated from Wu-Chu-Yu, has been reported to affect many physiological functions including vasorelaxation, uterotonic action, anoxia, and control of body temperature [Yamahara et al., 1989; Chiou et al., 1992, 1997; Tsai et al., 1995; Choi and Chiou, 1997]. EVO decreased testosterone production via a reduction of 17β-hydroxysteroid dehydrogenase activity in testicular interstitial cells [Lin et al., 1999] and aldosterone secretion via an inhibitory effect on 11β-hydroxylase in glomerulosa cells [Hung et al., 2001]. Meanwhile, EVO inhibited the growth and invasion of the murine colon 26-L5 adenocarcinoma, B16-F10 melanoma and Lewis lung carcinoma [Ogasawara et al., 2001a,b, 2002]. Evidence showed that EVO-induced apoptosis through altering the balance of Bcl-2 and Bax expression in HeLa cells [Fei et al., 2003]. We have demonstrated that EVO inhibited the growth of androgen-dependent prostate cancer cells through an accumulation of cell cycle at G2/M phase and initiation of apoptosis [Kan et al., 2004].

Since EVO might be a compound for anti-metastatic and anti-tumor agent, the inhibition of EVO on the proliferation of androgen-independent prostate cancers was examined. The human prostate cancer cell lines DU145 and PC3 have been used as a model of androgen-independent prostate cancer cells [Stone et al., 1978; Kaighn et al., 1979]. Both DU145 and PC3 cell lines were used to elucidate the anti-proliferative effects and action of EVO on androgen-independent prostate cancer cells.

MATERIALS AND METHODS

Cell Lines and Materials

The prostate cancer cell lines DU145 and PC3 were cultured with Dulbecco's Modified Eagle's Media (DMEM, Gibco Laboratories, Buffalo, Grand Island, NY) containing 50 U/ml potassium penicillin G (Sigma, St. Louis, MO), 50 U/ml streptomycin sulfate (Sigma) and 10% fetal calf serum (FCS, KBH, Israel) as standard media in 5% CO_2/95% air at 37°C. EVO was provided by Dr. L.C. Lin, National Research Institute of Chinese Medicine, Taipei, Taiwan, ROC. The chemical structure of EVO has been shown [Kan et al., 2004].

Cell Proliferation Assessment

The colorimetric MTT assay [Chung et al., 1999; Kan et al., 2004] was modified and employed to quantify the cell proliferation. Briefly, in the continuous treatment procedure, cells were incubated in 96-well microplates (Falcon, Franklin Lakes, NJ) with DMEM supplemented with 10% FCS. After 24 h the media were removed and replaced by either medium containing different concentrations of drug or a drug-free medium (control condition). After 0, 24, 48, 72, and 96 h, the media were removed and replaced by 50 μl of 1 mg/ml MTT solution (Sigma) in DMEM. Following 4 h incubation in this condition, the plates were centrifuged at 400*g* for 5 min. The MTT solution was removed and replaced by 50 μl DMSO, and the plates were shaken for 3 min. The optical density of each condition was determined using a microplate reader (Dynatech Laboratories, Chantilly, VA) at a wavelength of 570 nm with a reference wavelength of 630 nm. The average OD values obtained from cells challenged for 0 h were indicated as the day 0 control. Then, the proliferation rate could be calculated: proliferation index = (mean OD values of treatment)/(day 0 control). Each experimental condition was repeated by three times.

Cytotoxicity Assessment (by LDH Assay)

The detection of medium lactate dehydrogenase (LDH) activities was performed by the Cytotoxicity Detection Kit purchased from Boehringer Mannheim (Mannheim, Germany) and based on the detection the LDH activity in the culture medium. Briefly, cells were incubated in 96-well microplate (Falcon) as 5×10^3 cells/200 μl/well with DMEM described above supplemented with 10% FCS. After 24 h the media were removed and replaced either by medium containing different concentrations of drug or by a drug-free medium (low control condition). The wells for high control condition

were added the media containing 1% Triton X-100 (Sigma) to determine total cellular LDH. After 6-, 12-, or 24 h treatment 100 μl/well supernatants were removed and transferred into corresponding wells of an optically clear 96-well flat bottom microplates (experimental value). To determine the LDH activities contained in the supernatants, 100 μl reaction mixture was added to each well and incubated with cells for 20 min at room temperature. During this incubation period the microplates were protected from light. The optical density of each condition was determined using a microplate reader (Dynatech Laboratories) at a wavelength of 490 nm with a reference wavelength of 630 nm. According to the OD values, the cytotoxicity could be calculated: cytotoxicity (%) = (experimental value-low control)/(high control-low control).

Flow Cytometric Analysis of Cell Cycle Distribution

Cells were harvested, washed and fixed by 1% formaldehyde (Waco, Osaka, Japan) for 20 min in an iced bath. Then, the fixed cells were added with 70% cold ethanol (Sigma) and maintained at −20°C for 4 h. The cells were washed and stained with 10 μg/ml PI (Sigma) and 10 μg/ml RNase (Sigma) in the dark. The DNA content of cell samples was analyzed by ALTRA cytometer (Backman Coulter Corp., San Diego, CA) with an argon laser turned to the 488 nm for excitation. The red fluorescence of PI was measured at 620 nm.

TUNEL Assay

The apoptosis was assessed by a TUNEL assay with the Apoptosis Detection System kit (Promega Corporation, Madison, WI). The cells treated with EVO for different time intervals were harvested and prepared by the formaldehyde-fixed method which was similar to the method used in the cell cycle analysis. TUNEL assay of fragmented DNA was performed as recommended by the manufacturer (Promega).

Caspase Activity

The cells treated with EVO were harvested and washed twice by PBS. The cell pellets were obtained and the enzyme activities of caspase 3, 8, and 9 were detected by the Caspase Colorimetric Assay System (R&D System, Minneapolis, MN). The protein concentration was measured by Bradford assay.

Western Blotting

In order to observe the protein expression, Western blot was performed by the method described elsewhere [Kan et al., 2004]. After culture under the indicated conditions, cells were harvested and lyzed in RIPA buffer. The aliquots of cell lysate were boiled in SDS sample buffer and Western blots were performed with the antibodies of caspase 3 (IMGENEX, San Diego, CA), 8 (Cell Signaling Technology, MA), -9 (Medical & Biological Laboratories Co., Ltd, Nagoya, Japan), Cdc2 (BD Transduction Laboratories, San Diego, CA), cyclin B1 (Upstate Biotechnology, Charlottesville, VG), phospho-Cdc2 (Thr 161, Cell Signaling), phospho-Cdc2 (Try 15, Cell Signaling), Cdc25C (Cell Signaling), Myt-1 (Santa Cruz, CA), and β-actin (Sigma). The secondary antibodies used for Western Blot were goat anti-mouse (Promega) and goat anti-rabbit (Promega) horseradish peroxidase-labeled antibodies. The signals were visualized by enhanced chemiluminescence detection (ECL, Amersham International, UK).

Histone H1 Kinase Assay

The kinase activity of Cdc2 was measured by incorporation of [γ-^{32}P]ATP into the substrate histone H1 as described previously [Kan et al., 2004]. Briefly, the prostate cancer cells were harvested and lyzed with lysis buffer. The cell lysate was collected for immunoprecipitation and the kinase activity of Cdc2 was further measured. The kinase activity levels on autoradiographs were quantified by a densitometric scanning system.

Statistics

All values are given as the mean ± standard error of mean (SE mean). The treatment means were tested for homogeneity by the one-way analysis of variance, and the difference between specific means was analyzed for significance by Duncan's multiple-range test [Steel and Torrie, 1980]. The difference between two means was considered statistically significant when $P < 0.05$.

RESULTS

Effect of EVO on Cell Proliferation

This is the first report to determine the effect of EVO ($10^{-7} \sim 10^{-4}$ M) on proliferation of both

DU145 and PC3 cells by MTT assay. The cells treated with 0.1% DMSO were used as the control. Figure 1A showed that the growth inhibition of DU145 and PC3 was dependent on the EVO concentration. EVO at 10^{-6} M significantly decreased the proliferation of DU145 and PC3 cells following 24-h incubation (Fig. 1A). A time- and concentration-dependent inhibition of EVO on DU145 and PC3 cell growth was observed at 24 h and persisted for 96 h (Fig. 1A).

EVO on Cytotoxicity

The cytotoxicity of EVO on DU145 and PC3 cells was shown in Figure 1B. After 6-, or 12-h incubation, 8∼12% of cytotoxicity caused by EVO at 10^{-5} M was observed in DU145 cells. After incubation with EVO for 12 h, approximately 10% of cytotoxicity was observed in DU145 cells (Fig. 1B). EVO at 10^{-4} M caused 10% of cytotoxicity during a 6- or 12-h exposure in PC3 cells (Fig. 1B). During 24-h incubation, EVO at the concentration of $10^{-6} \sim 10^{-4}$ M exhibited significant cytotoxicity in DU145 and PC3 cells.

Effects of EVO on the Cell Cycle

The cell cycle distribution was assessed after treatment with various doses of EVO for 24 h by flow cytometry. EVO ($10^{-6} \sim 10^{-5}$ M) caused the accumulation of cell cycle at G2/M phase in both DU145 and PC3 cells (Fig. 2A). The increase in G2/M population by EVO was accompanied by a significant decrease in both G1 and S phase (Fig. 2A). We analyzed the DNA histogram and illustrated the results in Figure 2B. After 24 h treatment, DU145 cells that arrested at G2/M phase were elevated with increased concentrations of EVO by 16, 28, and 78%, respectively (Fig. 2B, upper panel). Similar results were observed in PC3 cells. EVO at 10^{-7}, 10^{-6}, and 10^{-5} M caused the G2/M arrest by 12, 33, and 75%, respectively (Fig. 2B, lower panel).

Effects of EVO on the Expression of Regulators of Cell Cycle

Treatment of DU145 cells with $10^{-6} \sim 10^{-5}$ M EVO for 12 h resulted in an increase in cyclin B1 expression (Fig. 3A). However, there was no difference in the protein expression of Cdc2 (Fig. 3A). EVO altered the expression of phosphorylated forms of Cdc2 including Thr161-phosphorylated Cdc2 (phosphorylated at threonine 161) and Tyr15-phosphorylated Cdc2 (phosphorylated at tyrosine 15). Phospho Cdc2 (Thr161) gradually increased after EVO treatment for 12 h (Fig. 3). The expression of phospho Cdc2 (Tyr15) was diminished by EVO treatment (Fig. 3A). Myt-1 is a kinase which phosphorylated Cdc2 on Tyr15 and resulted in Cdc2

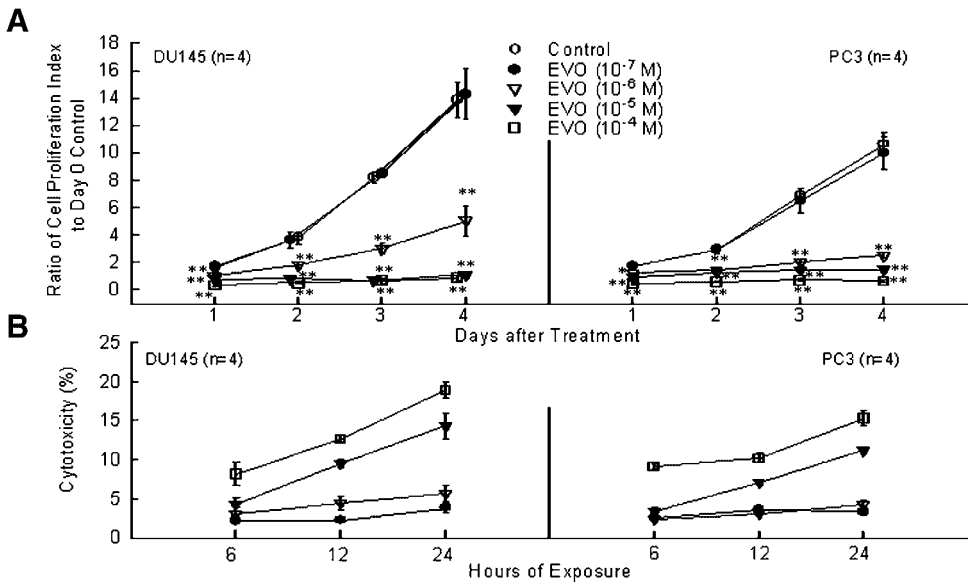

Fig. 1. **A**: Effects of different concentrations of EVO on the proliferation of DU145 and PC3 cells by MTT assay. **B**: The cytotoxicity caused by different concentrations of EVO in prostate cancer cells was determined by LDH assay. Each point represents the mean ± SE mean. *, **, $P < 0.05$ and $P < 0.01$ compared to control group (EVO = 0 M), respectively.

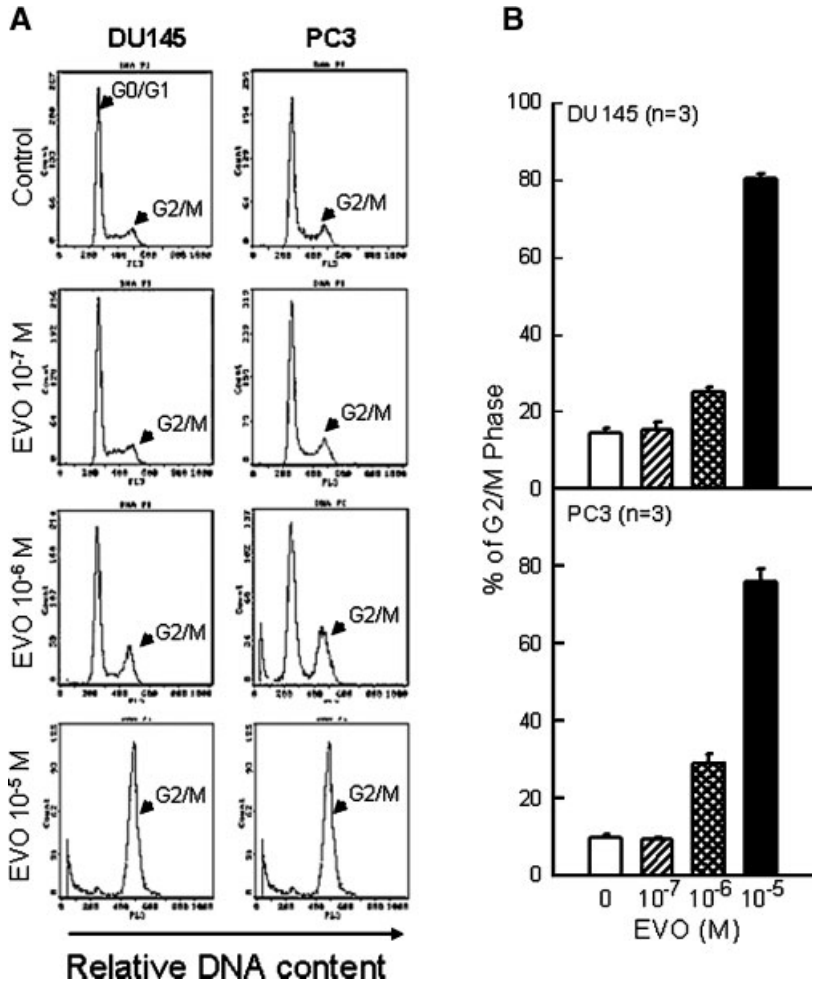

Fig. 2. **A**: Detection of EVO-induced cell cycle distribution in DU145 and PC3 cells. After exposure of EVO ($10^{-7} \sim 10^{-5}$ M) for 24 h, the DNA histogram was measured by flow cytometry. In each DNA histogram, the x-axis indicated as the cell count and y-axis indicated as the relative DNA content (the fluorescence intensity of propidium iodide). The arrows showed the cell population in the state of G2/M phase (4n DNA content). The cell population in G2/M phase was markedly elevated following the treatment of evodiamine. **B**: Dose-dependent analysis of G2/M arrest induced by EVO in DU145 and PC3 cells. The results were expressed as the mean of triplicates. Each value represents mean ± SE mean.

inactivation. EVO decreased the expression of Myt-1 expression in DU145 cells after an 18-h exposure (Fig. 3A). Furthermore, the expression of Cdc25C was changed in response to EVO treatment (Fig. 3A). After 12 h of EVO exposure, the expression of interphase Cdc25C was attenuated and slower migration form of phosphatase Cdc25C was present in DU145 cells.

The similar effects of EVO on PC3 cells were shown in Figure 3B. Exposure of EVO markedly altered the expressions of cyclin B1 and Cdc2 (Fig. 3B). We further investigated the expression of phosphorylated forms of Cdc2. The elevated expressions of Thr161-phosphorylated Cdc2 and reduced Tyr15-phosphorylated Cdc2 were observed following 18-h exposure of EVO (Fig. 3B). Treatment of PC3 cells with $10^{-6} \sim 10^{-5}$ M EVO for 12 h resulted in a decrease in Myt-1 expression. In contrast to DU145 cells, EVO caused a decrease in the expression of interphase Cdc25C after 18 h treatment. However, the slower migration form of Cdc25 could not be observed in PC3 cells (Fig. 3B). The expression of β-actin was employed as an internal control to normalize the Western blot signals. The altered expression of cyclin B1, phospho-Cdc2 (Thr161 and Tyr15), Cdc25C, and Myt-1 caused by EVO in DU145 and PC3 cells could be extended for 24 h (Fig. 3A,B).

Since EVO enhanced the protein expression of Thr161-phosphorylated Cdc2 and decreased the protein expression of Tyr15-phosphorylated

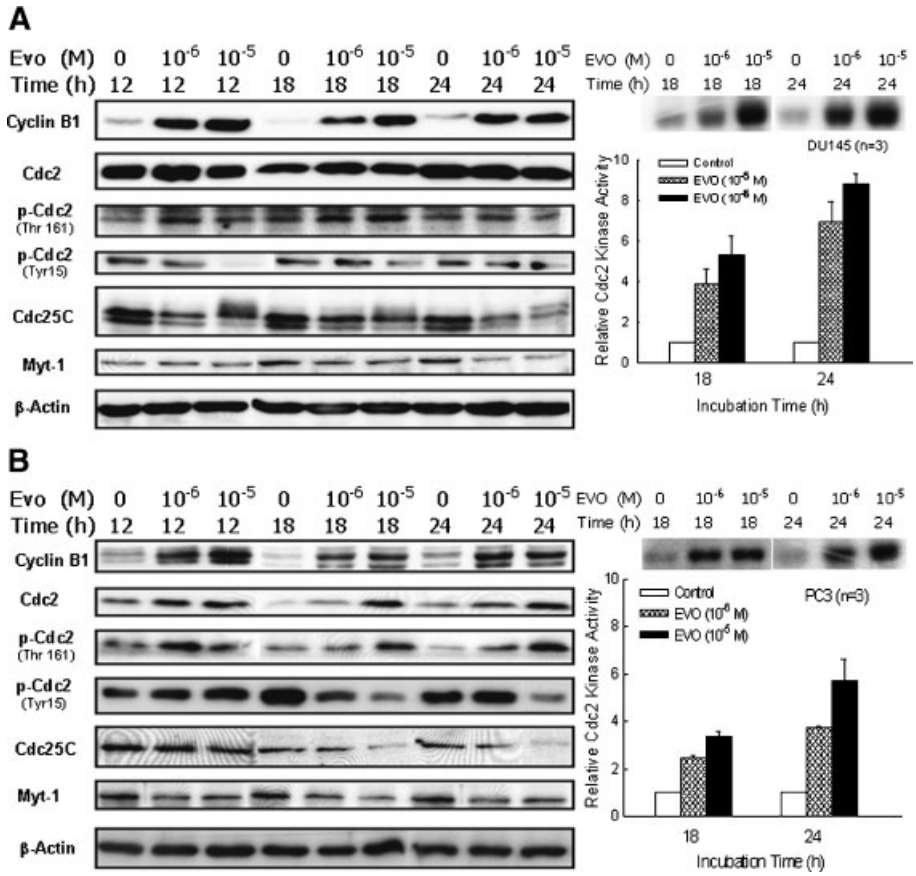

Fig. 3. Expression of cell cycle-regulating molecules after EVO exposure in prostate cancer cells. **A**: The DU145 cells were treated with EVO and the cell lysate was subjected to Western blot for analyzing the levels of cyclin B1, Cdc2, phospho-Cdc2 (Thr 161), phospho-Cdc2 (Tyr 15), Cdc25C, Myt-1, and β-actin. As shown in (A) right panel, the kinase activity of Cdc2 was measured by [γ-^{32}P] ATP incorporation into the substrate histone H1. **B**: The expression of cell cycle-regulating molecules were also examined after EVO exposure in PC3 cells. Furthermore, the kinase activity of Cdc2 affected by EVO in PC3 cells was also measured. Each value represents mean ± SE mean of three independent experiments.

Cdc2, we further determined if the activity of Cdc2 kinase was up-regulated by EVO in prostate cancer cells. The control cells displayed a low Cdc2 kinase activity. The kinase activity was increased in cells treated with EVO ($10^{-6} \sim 10^{-5}$ M) for different time periods in DU145 and PC3 cells (Fig. 3A,B). The elevated activity of Cdc2 kinase in response to EVO concentrations could be observed during 18-h exposure and persisted to 24 h (Fig. 3A,B).

EVO-Induced Apoptosis

EVO not only brought about G2/M phase arrest but also induced apoptosis in LNCaP cells (2). Apoptosis was examined by increasing intensity of TUNEL staining which detected DNA fragmentation in cells treated with EVO. The apoptosis in DU145 and PC3 cells was shown by flow cytometry (Fig. 4A). During 72-h exposure, various concentrations of EVO dose-dependently increased the numbers of TUNEL-positive cells in DU145 and PC3 cells (Fig. 4B). The levels of apoptotic cells caused by EVO in DU145 and PC3 cells were further quantified and illustrated in Figure 4B.

Caspases exist as inactive zymogens in normal and survival cells and perform proteolytic processing upon activation during apoptosis. Hence, it would be interesting to explore which caspase was processed and cleaved during the course of EVO-induced apoptosis. Procaspase 9 (45 kDa) was cleaved into its active form after EVO treatment for 24 h in both cells (Fig. 5A). It was indicated by the appearance of a 37-kDa band representing an intermediate cleavage product of caspase 9 (Fig. 5A). Meanwhile, EVO caused the cleavage of procaspase 8 (57 kDa) and 3 (32 kDa) in DU145 cells (Fig. 5A).

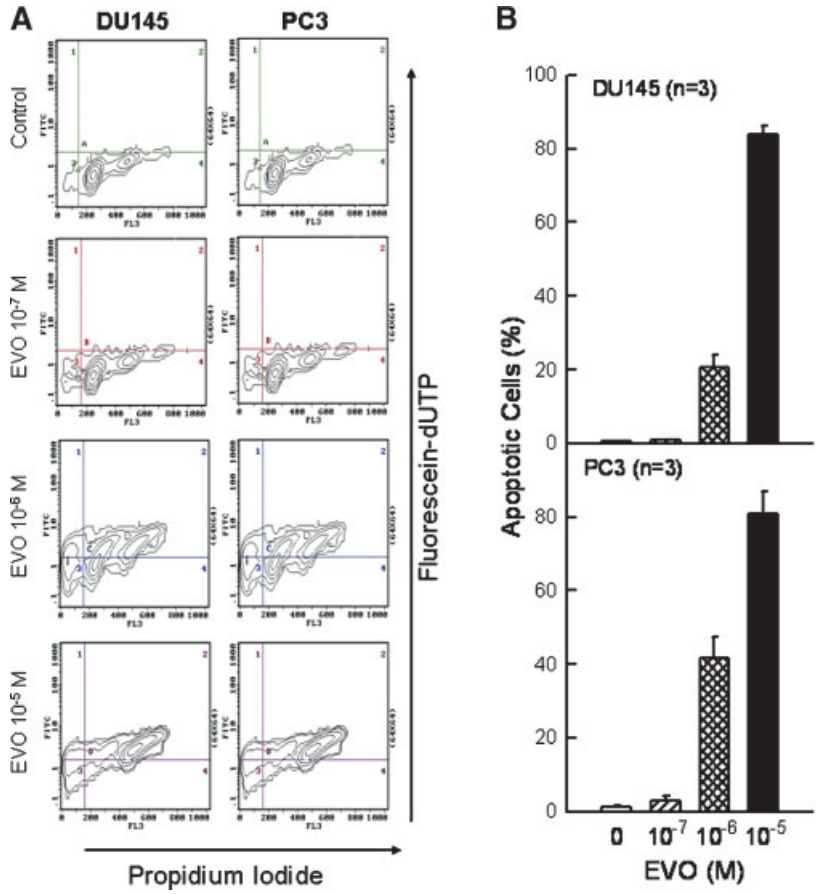

Fig. 4. **A**: EVO-induced apoptosis in DU145 and PC3 cells. Cells were treated with EVO for 72 h and evaluated for DNA fragmentation by using TUNEL technique. In each panel, the x-axis was the propidium iodide (PI) fluorescence intensity and the y-axis was the fluorescein isothiocyanate (FITC) fluorescence intensity of the cells in sample population. The cell appeared on the upper right quadrant were indicated as the apoptotic cells. Exposure of EVO promoted the apoptosis of DU145 and PC3 cells dose-dependently. **B**: The percentage of apoptotic cells caused by EVO in DU145 and PC3 cells were quantified and illustrated. The results were expressed as the mean of triplicates. Each value represents mean ± SE mean.

The proteolytic fragments of caspase 8, indicated by 41 kDa and caspase 3, indicated by 17 kDa were present following EVO exposure (Fig. 5A). As similar to DU145 cells, EVO caused the intermediate cleavage product of caspase 9 and processing of procaspase 9 and 3 in PC3 cells (Fig. 5B). However, the proteolytic fragments of caspase 3 could not be observed in PC3 cells (Fig. 5B). Concentration- and time-dependent caspases processing in response to EVO in DU145 and PC3 cells were persisted for 72 h (Fig. 5A,B).

EVO at the concentrations from $10^{-6} \sim 10^{-5}$ M significantly increased the activities of caspase 3 and caspase 9 in DU145 and PC3 cells after an exposure of 18 h (Fig. 5C). The activation of caspase 3 and 9 by EVO was elevated with an increase in treatment period (Fig. 5C). EVO caused a dose-dependent elevated level of caspase 8 activity in DU145 cells, rather than PC3 cells (Fig. 5C). The high level of caspase activities induced by EVO were persisted for 48 h (Fig. 5C).

The Role of Cdc2 in EVO-Induced Apoptosis

Since treatment of EVO caused both G2/M arrest and apoptosis in human prostate cancer cells. The timing for triggering G2/M arrest and apoptosis by EVO was quite different. The G2/M arrest was earlier than apoptosis after treatment of EVO. Abnormal G2/M arrest might be a required signal for EVO-induced apoptosis. To directly understand the effects of G2/M arrest on EVO-triggered apoptosis, a highly selective Cdc2 kinase inhibitor, roscovitine (RV, Calbiochem, La Jolla, CA) was applied. Prostate cancer cells were pretreated with 5×10^{-5} M of roscovitine for 4 h prior to EVO treatment for

24 h. The cell cycle distribution and apoptosis were monitored. Pretreatment of DU145 cells with roscovitine suppressed the G2/M phase arrest induced by EVO and rescued EVO-triggered apoptosis (Fig. 6A). In PC3 cells, pretreatment with roscovitine did decrease the accumulation of cell cycle in G2/M phase, however, did not markedly diminish the EVO-induced apoptotic cells (Fig. 6B).

DISCUSSION

Men with metastatic prostate cancer respond to androgen ablation therapy rapidly with improvement of bone pain, regression of soft tissue metastases, and decrease in serum prostate antigen [Petrylak, 1999]. Unfortunately, androgen ablation is not curative and patients succumb to their disease after 12–18 months [Mahler and Denis, 1992]. It is necessary to develop new chemicals against prostate cancer, especially hormone independent prostate cancer.

Previous studies indicated that EVO possessed many physiological functions [Hung et al., 2001; Chiou and Chen, 2002]. Furthermore, EVO showed an anti-tumor activity on cancer cells [Ogasawara et al., 2001, 2002; Fei et al., 2003], especially androgen-dependent prostate cancer cells [Kan et al., 2004]. To examine the effective concentrations of EVO on cell proliferation, a broad range of concentrations ($10^{-7} \sim 10^{-5}$ M) was used. Administration of EVO ($10^{-6} \sim 10^{-4}$ M) for 24 h, the growth of both DU145 and PC3 cells was inhibited in a dose-dependent manner and be persisted at least 4 days. EVO also caused high cytotoxicity in DU145 cells as compared with PC3 cells. Since lower concentrations of EVO exhibited an anti-tumor activity, $10^{-6} \sim 10^{-5}$ M, instead of 10^{-4} M of EVO were employed for mechanism studies.

The flow cytometric examination indicated that EVO caused G2/M phase arrest in DU145 and PC3 cells in a concentration-dependent manner. The cell cycle progression is regulated by activation and inactivation in different

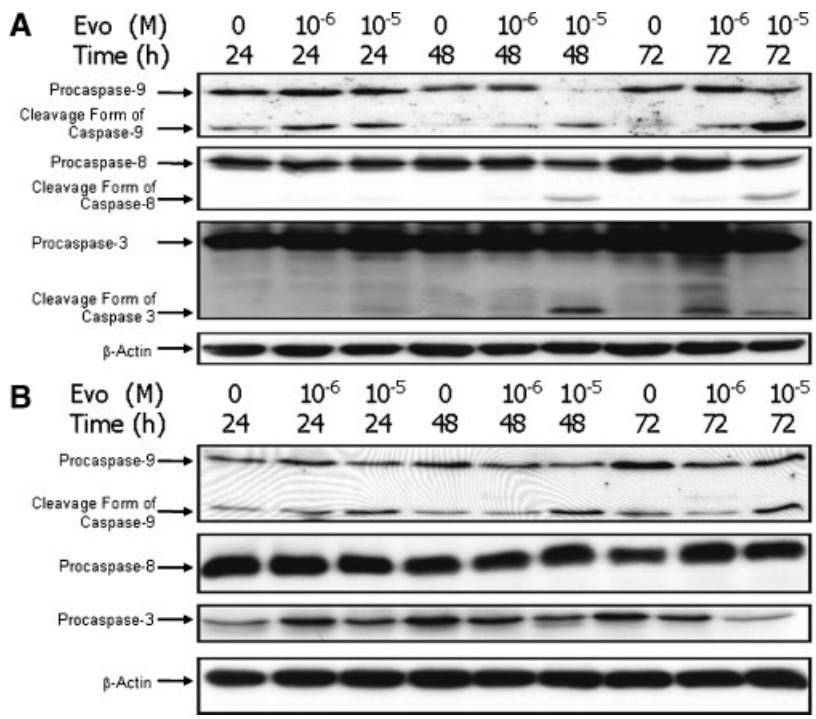

Fig. 5. Analysis of EVO-induced caspase processing in prostate cancer cells. Cells were treated with EVO and preformed for Western Blot analysis of caspase 3, 8, and 9. Reduction in the band intensity of procaspase is corresponding to procaspase processing. **A**: Caspases processing in response to EVO in DU145 cells. EVO caused the caspase 3, 8, and 9 processing and the cleavage forms of caspase 3, 8, and 9. **B**: Analysis of EVO-induced procaspase processing in PC3 cells. The procaspase processing of caspase 3 and 9 were occurred at 24 h of EVO treatment and the proteolytic fragments were detected following EVO treatment for 24 h. However, the procaspase 8 processing and proteolytic fragments did not be observed by EVO treatment. The expression of β-actin was employed to normalize the signals. **C**: The caspase activities were measured in the cytosolic lysate extracted from DU145 and PC3 cells treated with EVO for indicated time periods. Each value represents mean ± SE mean.

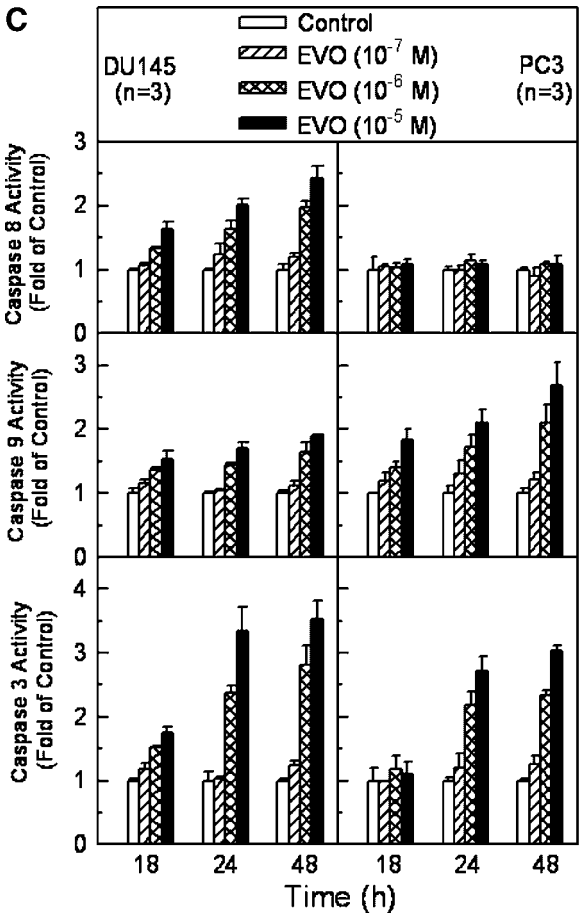

Fig. 5. (*Continued*)

classes of cyclins, cyclin dependent kinase (CDK), and some regulatory proteins [Draetta, 1990]. The Cdc2 activity controls the progression from G2 phase to M phase [Taylor and Stark, 2001]. The phosphorylation of Cdc2 occurs on three regulatory sites as following: threonine 14, tyrosine 15, and threonine 161 [Graves et al., 2000]. During S and G2 phases of cell cycle, Cdc2 is kept inactivated by phosphorylation on Thr 14 and Tyr15 mediated by Wee-1 and Myt-1 kinases [Booher et al., 1997; Liu et al., 1997; Palmer et al., 1998]. In late G2 phase, the Cdc25C phosphatase dephosphorylates Cdc2 on both Thr14 and Tyr15. Also, cdk-activating kinase (CAK) phosphorylates Cdc2 on Thr161, leading to the activation of Cdc2/cyclin B complex [Devault et al., 1995]. The formation and activation of Cdc2/cyclin B complex is associated with G2/M phase transient and M phase entrance [Glotzer et al., 1991]. At the end of M phase, Cdc2/cyclin B complex is rapidly degraded by ubiquitinization [Glotzer et al., 1991]. Several chemicals have been identified to be able to inhibit the proliferation of cancer cells by blockade of cell cycle at G2/M phase and induction of apoptosis [Ling et al., 1998, 2002]. Paclitaxel and arsenic trioxide (As_2O_3), the anti-tubulin agents, inhibit the cell growth and cause cell cycle to arrest at M phase during the activation of Cdc2 kinase and accumulation of cyclin B1 in cancer cells [Donaldson et al., 1994; Ling et al., 1998, 2002]. In the present study, we found that EVO treatment not only caused an increase in the protein levels of cyclin B1 and phospho-Cdc2 (Thr 161, the active form of Cdc2) but also diminished the expression of phospho-Cdc2 (Try 15, the inactive form of Cdc2) in prostate cancer cells. Moreover, histone H1 kinase assay revealed that exposure of EVO activated Cdc2 kinase activity in a dose- and time-dependent manner. These results indicated that EVO treatment increased the activation of cyclin B1/Cdc2 complex. The pattern of EVO-induced activation of G2/M phase associated regulators, cyclin B1 and Cdc2 might be similar to that of paclitaxel as well as As_2O_3. Therefore, these results suggested that EVO might induce the cell cycle arrest at M phase rather than at G2 phase.

Moreover, it has been well documented that Cdc2 activation at the onset of mitosis results from the concurrent inhibition of Wee-1 and Myt-1 and activation of Cdc25C phosphatase. Wee-1 and Myt-1 are the negative regulators of Cdc2 which phosphorylated Cdc2 at Thr14 and Tyr 15. The activity and expression of both Myt-1 and Wee-1 decline during mitosis, therefore, contributing to the fall in the inhibitory phosphorylation of Cdc2 [Palmer et al., 1998]. The activation of Cdc2 is accompanying by the Cdc25C. Activated Cdc25C dephosphorylates Cdc2 on Thr14 and Tyr 15 and also trigger the activation of Cdc2/cyclin B complex [Palmer et al., 1998]. Activated Cdc25C requires dephosphorylation of Cdc25C at Ser 216, resulting in dissociation of 14-3-3 protein [Graves et al., 2000; Takizawa and Morgan, 2000]. The dephosphorylated Cdc25C will be further activated by extensive phosphorylation of its amino terminus [Peng et al., 1997; Graves et al., 2000; Takizawa and Morgan, 2000]. These are important steps for activation of the cyclin B1-associated kinase and entry into mitosis of the cell cycle. Previous reports indicated that several chemicals regulated the Cdc2/cyclinB

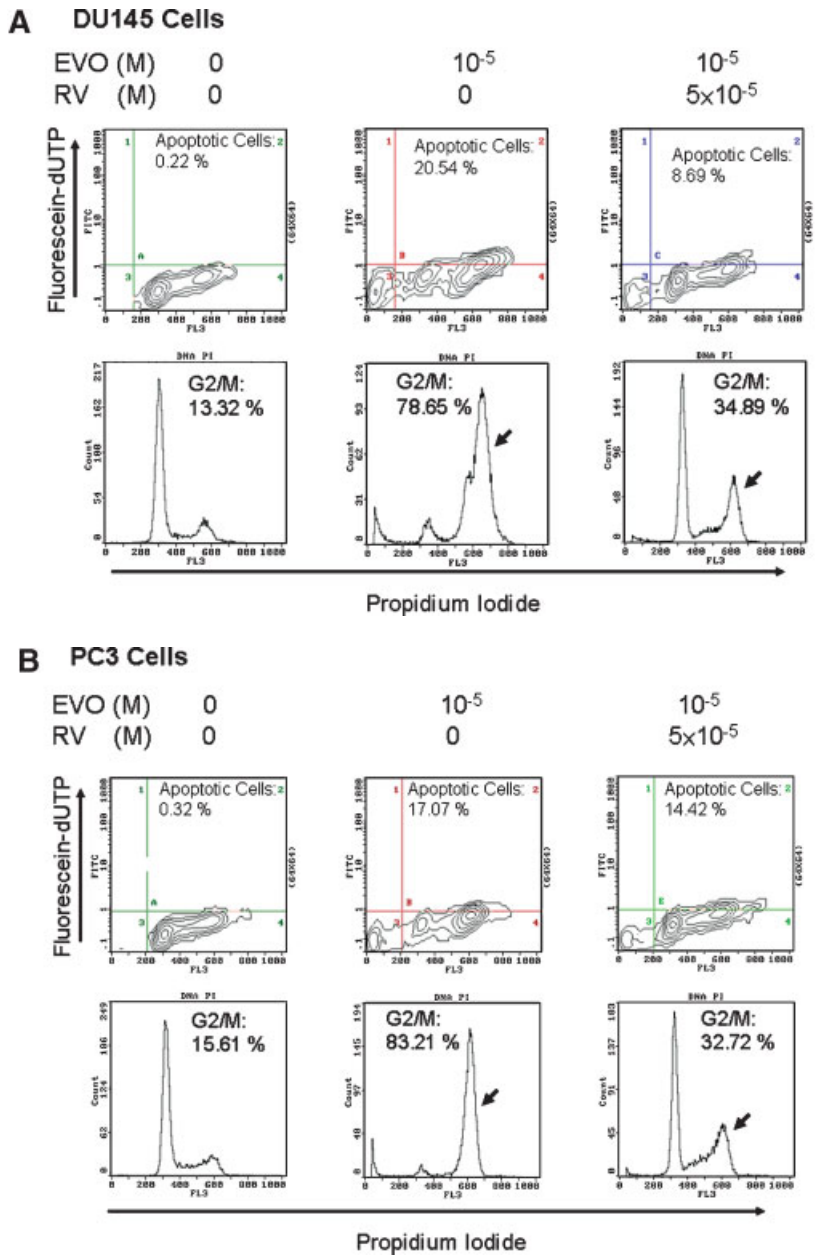

Fig. 6. Effects of the Cdc2 inhibitor on cell cycle and apoptosis. Prostate cancer cells were pretreated with or without roscovitine (5×10^{-5} M) and then stimulated with EVO (10^{-5} M) for 24 h. Both the cell cycle distribution and apoptosis were examined by flow cytometer. The x-axis showed the propidium iodide (PI) fluorescence intensity. The y-axis of the upper panel and lower panel showed the fluorescein isothiocyanate (FITC) fluorescence intensity and the cells count in sample population, respectively. In the upper panel, the cells appeared on the upper right quadrant indicated as the apoptotic cells. In DNA histograms, the arrows indicated the G2/M cell population. Based on the analysis of flow cytometry, the cell cycle distribution and apoptosis of DU145 were illustrated in (**A**). Meanwhile, the results of PC3 were shown in (**B**). Results are representative of three experiments.

complex and arrest cell cycle in G2-M phase through Cdc25C and Myt-1 proteins. The elevated hyperphosphorylation of Cdc25C and declined unphosphorylated Cdc25C (interphase Cdc25C) in human cancer cells were parallel with the activation of Cdc2/cyclinB after exposure of chemicals [Chen et al., 2002; Chang et al., 2004; Kuo et al., 2004; Singh et al., 2004]. Griseofulvin, an anti-fungal agent caused the down-regulation of Myt-1 protein expression, contributed the abnormal G2-M cell cycle arrest [Ho et al., 2001]. The expression of Myt-1 was also declined after EVO exposure. Also, salvinal, a microtubule inhibitor, accumulated the

tumor cells at G2/M phase by up-regulation of cyclin B1 levels and Cdc25C phosphorylation [Chang et al., 2004]. According to our results, the changed patterns of Cdc25C in response to EVO treatment were very different between DU145 and PC3 cells. EVO caused the slower migration form of Cdc25C (Cdc25C phosphorylation form) and diminished the expression of unphosphorylated Cdc25C (the interphase Cdc25C) in DU145 cells. Only the diminished expression of unphosphorylated Cdc25C (the interphase Cdc25C) was observed in PC3 cells. The hyperphosphorylated Cdc25C (mitotic form) was observed in neither DU145 nor PC3 cells. Thus, EVO, like griseofulvin and salvinal, may cause efficient activation of Cdc2 by resulting in activation of Cdc25C and down-regulation of Myt-1.

Apoptosis is a highly programmed and regulated process for elimination of abnormal or damaged cells. To examine whether the EVO induce apoptosis, the TUNEL assay was used. The marked induction of DNA fragmentation indicated that prolonged exposure of EVO caused apoptosis. Caspases involved in apoptosis are generally divided into two categories, the initiator caspases (caspase 2, 8, 9, and 10) involving regulatory events and effector caspases (caspase 3, caspase 7) responsible for cell disassembly events [Ranger et al., 2001]. Activated initiator caspases lead to activate the effector caspases and then execute the apoptosis [Budihardjo et al., 1999]. The current identified caspases are intracellular cysteine proteases that initially are produced as inactive zymogens (procaspases). Activation of caspases includes proteolysis of procaspases and cleavage of multiple substrates [Shi, 2001]. Since EVO-induced apoptosis in DU145 and PC3 cells, we further investigated the cellular mechanism(s) responsible for EVO-induced apoptosis, and found that the activities of caspase 3, 8, and 9 were enhanced after EVO treatment in DU145 cells. However, EVO caused the elevated levels of caspase 3 and 9 activities rather than caspase 8 activity in PC3 cells. Moreover, Western blot examination showed that the proteolytic processes of caspase 3 and 9 were also observed following EVO treatment. Activation of caspase 9 and formation of Apaf/cytochrome c complex have been shown to be essential for the mitochondria-initiated apoptosis [Budihardjo et al., 1999]. Besides, caspase 8 activation by forming death-inducing signal complex (DISC) is also required for apoptosis [Muzio et al., 1996]. Therefore, our data suggested that the mitochondrial dysfunction and the formation of DISC might be the upstream intracellular signals that were associated with EVO-induced apoptosis in DU145 cells. Only the mitochondrial dysfunction involved in the apoptosis that was induced by EVO in PC3 cells.

Abnormal regulation in the expression and/or activation of cyclin, CDK and regulatory factors resulted in blockage of cell cycle progression and causing apoptosis [Donaldson et al., 1994; Shi et al., 1994; Pines, 1995; Ling et al., 1998, 2002]. Our data indicated that EVO caused an accumulated expression of cyclin B1 and active form of cyclin B/Cdc2 kinase before undergoing apoptosis. G2/M arrest and improper expression of cyclin B1 and Cdc2 and activation of Cdc2 kinase caused by EVO might promote apoptosis. We, therefore, determined the significance of G2/M arrest or activation of Cdc2 kinase in EVO-triggered apoptosis by administration of Cdc2 kinase inhibitor (Fig. 6A,B). In the recent report, roscovitine was utilized to inhibit Cdc2 kinase activity and blocked the accumulation of G2/M phase, which suggests the practicable application of these kinase inhibitors in Cdc2-expressed cells [Dutertre et al., 2002; Takano et al., 2004]. In both DU145 and PC3 cells, roscovitine significantly reduced the EVO-induced G2/M arrest. Attenuation of the EVO-triggered apoptosis could be observed after roscovitine treatment in DU145 cells. The data suggested that EVO could lead to inappropriate activation of G2/M phase-regulators as a trigger for initiation of apoptosis signaling in DU145 cells. By contrast, promoting apoptosis of PC3 cells triggered by EVO did not be abolished or diminished in the present of roscovitine treatment. These results suggested that the apoptosis of PC3 cells triggered by EVO was independent of G2/M arrest or Cdc2 activation.

In summary, the present study demonstrated that EVO caused [1] G2/M phase arrest that was parallel with the altered expression of cyclin B1, phospho-Cdc2 including Cdc2 (Thr 161, Tyr15), Cdc25C and Myt-1, and [2] apoptosis which was mediated by the activation of caspase 3 and 9 in androgen-independent human prostate cancer cells.

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