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Antitumor activity and down-regulation of pro-angiogenic molecules in human prostate cancer cells by a novel thiazolidione compound

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Abstract

BACKGROUND—Current treatments for prostate cancer are effective in many patients with locally advanced disease, but many of these patients eventually have recurrence. It is therefore important to develop alternative therapeutic agents with improved efficacy and tolerability. We recently identified a synthetic thiazolidin compound, 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidione (DBPT), that induces apoptosis in human colon cancer cells, independent of p53 and P-glycoprotein status. Here, we investigated the antitumor properties and mechanisms of action of this compound in human prostate cancer cell lines.

METHODS—The effect of DBPT on cell-cycle progression and apoptosis in LNCaP and DU145 cells was examined by flow cytometry and Western blotting. The effect of DBPT on pro-angiogenic molecules was analyzed by Western blotting and by an enzyme-linked immunosorbent assay.

RESULTS—DBPT inhibited the growth of LNCaP and DU145 cells with 50% inhibitory concentrations ranging from 1.6 to 5.9 μ M. Treating LNCaP and DU145 cells with DBPT led to a time-dependent cell-cycle arrest in the G₂/M phase and increased levels of G₂/M checkpoint proteins, such as cyclin B1, cdc25C, phosphorylated histone H₃, and MPM-2. DBPT induced the phosphorylation of Bcl-xL and Bim, and induced apoptosis, as evidenced by cleavage of caspase and poly (ADP-ribose) polymerase. DBPT also effectively induced apoptosis in Bcl-2-overexpressing DU145 cells. Furthermore, DBPT decreased hypoxia-inducible factor 1 α and vascular endothelial growth factor expression in LNCaP cells under both normoxia and hypoxia.

CONCLUSIONS—DBPT can suppress proliferation, induce apoptosis, and down-regulate pro-angiogenic molecules in prostate cancer cells, and might be useful in treating prostate cancer.

Keywords

novel compound; prostate cancer; apoptosis; G₂/M-phase arrest; VEGF

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INTRODUCTION

Current treatments for prostate cancer, such as radical prostatectomy and radiation therapy, are effective in many patients with locally advanced disease, but many of these patients eventually have recurrence, which can be controlled only temporarily by androgen ablation [1]. Chemotherapeutic agents such as docetaxel and mitoxantrone have improved outcomes for patients with advanced prostate cancer, especially androgen-independent prostate cancer [2, 3], and numerous new antineoplastic agents have shown anticancer activity, either alone or in combination with other agents [4–6]. However, for most prostate cancer patients, currently available agents are only temporarily successful because they frequently lead to resistance or unacceptable levels of toxicity. It is therefore important to develop alternative therapeutic agents with improved efficacy and tolerability.

We recently identified a synthetic small molecule, 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidione (DBPT), that kills cancer cells more effectively than it kills normal human fibroblasts. The antitumor activity of DBPT is independent of p53 and P-glycoprotein status [7]. Thus, DBPT might be useful in cancer therapy. However, the mechanism of the compound's selectivity and initial targets are not yet known. Nevertheless, a preliminary test on various types of cancer cell lines showed that DBPT is effective to prostate cancer cell lines.

The goal of this study was to investigate the molecular mechanisms of DBPT-mediated antitumor activity in prostate cancer cells. To this end, the effects of DBPT on cell viability, cell-cycle progression, apoptosis, and the expression of pro-angiogenic molecules were studied in LNCaP and DU145 cells. We found that DBPT caused growth inhibition and apoptosis in LNCaP and DU145 cells independently of their Bcl-2 status and androgen sensitivity. DBPT also induced cell-cycle arrest in the G₂/M phase and up-regulating the levels of G₂/M checkpoint proteins, such as cyclin B1, cdc25C, histone H₃, and MPM-2. DBPT also induced phosphorylation of Bcl-xL and Bim. Moreover, DBPT diminished hypoxia-mediated VEGF expression in LNCaP cells, suggesting that this compound might be useful for prostate cancer treatment.

MATERIALS AND METHODS

Cells and Culture Conditions

Human prostate cancer LNCaP and DU145 cells were obtained from American Type Culture Collection (Rockville, MD), and routinely cultured in monolayer culture in RPMI 1640 medium or Minimum Essential Medium Eagle supplemented with 10% heat-inactivated fetal calf serum, 25 mM HEPES, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were maintained in the presence of 5% CO₂ and air at 37°C. For hypoxic exposure, cells were placed in a sealed modular incubator (Billups-Rothenberg, Del Mar, CA) flushed with 1% O₂, 5% CO₂, and 94% N₂.

Chemicals and Antibodies

DBPT was obtained from ChemBridge Corporation (San Diego, CA). The chemical structure of DBPT is shown in Figure 1. DBPT was dissolved in dimethyl sulfoxide to a concentration of 10 mM and stored at 4°C as a master stock solution. The JNK-specific inhibitor SP600125 was purchased from Calbiochem (La Jolla, CA), dissolved in dimethyl sulfoxide, stored at –20°C, and protected from light. An equal volume of dimethyl sulfoxide (<0.1%) had no obvious effect on cell viability and was used as a control. The following antibodies were used in Western blot analysis: cyclin A, caspase-3, Bax, Bcl-2, cdc2p34, cdc25C, and VEGF (Santa Cruz Biotechnology, Santa Cruz, CA); cyclin B1 (Neomarkers, Fremont, CA); phosphohistone

H₃ and MPM-2 (Upstate Biotechnology, Lake Placid, NY); caspase-8 (MBL International, Woburn, MA); poly (ADP-ribose) polymerase (PARP), Bcl-xL, and cytochrome *c* (BD Pharmingen, San Diego, CA); hypoxia-inducible factor (HIF)-1 α and HIF-1 β (BD Biosciences, San Diego, CA); caspase-9, phosphorylated JNK, JNK, and phosphorylated c-Jun (Cell Signaling Technology, Beverly, MA); Bim (Calbiochem, La Jolla, CA); and β -actin (Sigma-Aldrich, St. Louis, MO).

Cell Proliferation Assay

The antiproliferative effects of DBPT on LNCaP and DU145 cells were determined by the XTT assay. Cells ($5\text{--}10 \times 10^3$ cells in 100 μl of culture medium/well) were seeded in 96-well flat-bottomed plates and treated the next day with DBPT at the indicated concentrations. After 72 h, cell viability was determined by colorimetric assay with the tetrazolium dye XTT using a Cell Proliferation Kit II (Roche Diagnostics, Indianapolis, IN) as described previously [7]. The experiments were performed at least three times for each cell line.

Flow Cytometry Analysis

For detection of cell-cycle progression and apoptosis, fixed cells were resuspended in phosphate-buffered saline containing 10 $\mu\text{g/ml}$ propidium iodide (PI; Roche Diagnostics) and 10 $\mu\text{g/ml}$ RNase A (Sigma-Aldrich) at 37°C for 30 min. Cell-cycle analysis was performed using an Epics Profile II flow cytometer (Beckman Coulter, Fullerton, CA) with MultiCycle software (Phoenix Flow Systems, San Diego, CA). The accumulation of sub-G₁ cells, an indicator of DNA fragmentation and apoptosis, was used to quantify apoptosis.

Western Blot Analysis

Whole-cell extracts and cytosolic extracts were prepared as described previously [8,9]. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Briefly, equal amounts of proteins (30–50 μg) were subjected to electrophoresis under reducing conditions on 8–12.5% (w/v) polyacrylamide gels and then transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membranes were incubated with a primary antibody followed by a peroxidase-linked secondary antibody, which was detected using the ECL Western blotting system (Amersham).

Plasmid Transfection

The plasmid containing Bcl-2 cDNA was provided by Dr. Timothy J. McDonnell (The University of Texas M. D. Anderson Cancer Center) [10]. Plasmid transfection was performed using FuGENE6 reagent (Roche Diagnostics), and transfected cells were selected in the presence of 500 $\mu\text{g/ml}$ G418.

Enzyme-Linked Immunosorbent Assay for VEGF

The VEGF concentration in media from DBPT-treated LNCaP cells was determined by using an enzyme-linked immunosorbent assay kit for VEGF protein (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's protocol. The results were expressed as the concentration of VEGF (pg/ml) per microgram of protein in each well.

RESULTS

DBPT Inhibited Proliferation of Human Prostate Cancer Cells

To test whether DBPT is active against prostate cancer cells, we determined the cell growth inhibitory effect of DBPT on cells from two human prostate cancer cell lines, LNCaP and DU145. The cells were treated with DBPT at various concentrations for 72 h and examined for cell viability by using an XTT assay. DBPT inhibited the growth of LNCaP and DU145

cells in a dose-dependent manner (Fig. 2A). The 50% inhibitory concentrations (IC₅₀ values) in LNCaP and DU145 cells were 1.6 and 5.9 μ M, respectively. Treatment with 2 μ M DBPT inhibited the growth of LNCaP cells in a time-dependent manner (Fig. 2B).

DBPT Induced G₂/M-Phase Arrest and Changes in the Expression of G₂/M Check Proteins

To elucidate the mechanisms of DBPT-induced growth inhibition in prostate cancer cells, we used flow cytometry to perform a cell-cycle analysis of LNCaP and DU145 cells treated with DBPT. The treatment caused an accumulation of cells at the G₂/M phase in a time-dependent manner, indicating that DBPT induced cell-cycle arrest at the G₂/M phase. The peak values of G₂/M phase arrest were 50.4% for LNCaP and 54.0% for DU145 cells after 12 h of DBPT treatment (Fig. 3A). To evaluate molecular changes in cells treated with DBPT, we examined the effect of DBPT on the expression of proteins related to the G₂/M phase in LNCaP and DU145 cells (Fig. 3B). Western blotting showed a noticeable reduction of cyclin A and a transient increase of cyclin B1 after DBPT treatment. In addition, a more slowly migrating form of cdc25C phosphatase was detected in cells after 12–24 h of treatment. The levels of phosphorylated histone H₃ and MPM-2, two mitotic markers [11,12], were dramatically higher 12–24 h after treatment with DBPT. These results further indicated that DBPT induced G₂/M-phase arrest.

DBPT Induced Apoptosis thorough Caspase Activation

The cell-cycle analysis also indicated apoptosis induction in prostate cancer cells by DBPT (Fig. 3A). Therefore, we further evaluated DBPT-induced apoptosis in prostate cancer cell lines by flow cytometry and Western blotting assays. DBPT induced a marked, time-dependent increase in the percentage of the sub-G₁ population in LNCaP (60.6% with 3 μ M DBPT) and DU145 cells (71.5% with 10 μ M DBPT) 48 h after treatment (Fig. 4A). In addition, cleavage of caspase-3, caspase-8, caspase-9, and PARP were observed in LNCaP and DU145 cells treated with DBPT after 24 h or 48 h (Fig. 4B). This demonstrated that DBPT effectively induces apoptosis in prostate cancer cells.

DBPT Altered the Expression of Some Antiapoptotic and Proapoptotic Proteins

To investigate the molecular mechanisms of DBPT-induced apoptosis, we evaluated changes in the levels of Bcl-xL, Bcl-2, Bax, and Bim proteins after treatment with DBPT. LNCaP and DU145 cells were treated with DBPT at 3 μ M and 10 μ M, respectively, for 12 h and 24 h, and whole-cell lysates were subjected to Western blotting (Fig. 5). Using anti-Bcl-xL antibody, we detected a marked mobility shift of Bcl-xL in both LNCaP and DU145 cells 12 h and 24 h after DBPT treatment, indicating that Bcl-xL had been phosphorylated. In addition, Bim also underwent a transient mobility shift in DBPT-treated cell extracts from LNCaP and DU145 cells. No obvious changes were seen in Bcl-2 and Bax expression in LNCaP and DU145 cells treated with DBPT.

Bcl-2 Overexpression Increased DBPT-induced apoptosis in DU145 cells

It has been reported that Bcl-2 overexpression causes resistance to various apoptotic stimuli such as androgen ablation, radiation, and chemotherapeutic agents in prostate cancer cells [13–15]. To further pinpoint whether Bcl-2 overexpression alters the response to DBPT-induced apoptosis in DU145 cells, we investigated the effect of DBPT on apoptosis in stably Bcl-2-overexpressing DU145 cells (DU145/Bcl-2). Western blotting analysis demonstrated a markedly higher expression of Bcl-2 in two DU145/Bcl-2 clones, when compared with parental DU145 cells (Fig. 6A). Treatment of the DU145/Bcl-2 clones and the parental DU145 cells with 10 μ M DBPT resulted in apoptosis induction in both cells, as shown by an increase in the sub-G₁ population after 48 h (Fig. 6B), and detection of cytochrome *c* release (Fig. 6C).

DBPT had little effect on JNK activation in prostate cancer cells

We have previously showed that JNK activation is crucial for DBPT-induced apoptosis in colon cancer cells [7]. To investigate whether DBPT activates the JNK signaling pathway in prostate cancer cells, LNCaP or DU145 cells were treated with DBPT for 24 h and whole-cell lysates were analyzed by Western blotting with phosphorylated JNK, JNK, and phosphorylated c-Jun antibodies (Fig. 7A). Phosphorylated JNK was not detected in DBPT-treated LNCaP and DU145 cells. Treatment with DBPT resulted in only slight increase of phosphorylated c-Jun in DU145 cells, but not in LNCaP cells, suggesting that DBPT-mediated apoptosis in prostate cancer cells might be JNK-independent. We also treated LNCaP or DU145 cells with DBPT in the presence or absence of the JNK inhibitor SP600125 for 24 h and analyzed the sub-G1 population by flow cytometry (Fig. 7B). Interestingly, pretreatment with JNK inhibitor SP600125 dramatically blocked DBPT-induced apoptosis in DU145 cells, but not in LNCaP cells. The percentages of apoptotic cells in DU145 cells after treatment with 20 μ M SP600125, 10 μ M DBPT, or both were 1%, 19%, and 3%, respectively. The mechanism by which SP600125 blocked DBPT-mediated apoptosis in DU145 cells is not clear.

DBPT Down-Regulates Hypoxia-Induced VEGF Expression in LNCaP Cells

HIF-1 is a proangiogenic transcription factor stabilized and activated under hypoxia. HIF-1 activates angiogenesis via transcriptional activation of VEGF, as well as other genes. Consequently, it is an important tumor survival factor [16]. To investigate whether DBPT influences the expression of pro-angiogenic molecules, we examined the effects of DBPT treatment on HIF-1 α and VEGF proteins in LNCaP cells under normoxia and hypoxia. DBPT reduced the levels of the HIF-1 α protein detected in a whole-cell extract preparation when the cells were treated with 5 μ M DBPT under both normoxic and hypoxic conditions (Fig. 8A). HIF-1 β protein was slightly down-regulated by DBPT under hypoxia. VEGF levels were dramatically decreased in a dose-dependent manner under both normoxic and hypoxic conditions. Moreover, VEGF protein levels in the conditioned medium from DBPT-treated LNCaP cells were decreased in a dose-dependent manner under both normoxic and hypoxic conditions (Fig. 8B). To further confirm the effects of DBPT-induced apoptosis on the down-regulation of HIF-1 α and VEGF protein expression under hypoxia, we examined the apoptotic ratio in DBPT-treated LNCaP cells under the same experimental conditions. DBPT induced G₂/M-phase arrest at 5 μ M; however, it did not markedly affect apoptosis under the experimental conditions (Fig. 8C). This indicated that the down-regulations in HIF-1 α and VEGF protein expression were not caused by the increase in DBPT-induced apoptosis.

DISCUSSION

We found that DBPT is potential antitumor agent for prostate cancer cells. The cytotoxicity assay showed that it was effective in both androgen-sensitive LNCaP cells and androgen-insensitive DU145 cells, which suggests that the cytotoxic effect of DBPT in prostate cancer cells is independent of androgen sensitivity. In addition, our study showed that DBPT induces apoptosis through caspase activation in prostate cancer cells. This is consistent with our previous data showing that DBPT induced apoptosis in colon cancer cell lines, in part, through caspase-dependent pathways [7]. Our previous data also suggested that DBPT might have a larger therapeutic window than that of paclitaxel. The IC₅₀ value for DBPT in human colon cancer DLD-1 cells and normal human fibroblasts were 1.6 μ M and 22.9 μ M, respectively. In contrast, the IC₅₀ value for paclitaxel in those two cells were 10 nM and 10.6 nM, respectively [7,17]. Thus, DBPT might be a safe anticancer agent. Nevertheless, the mechanism of the selectivity between normal cells and cancer cells DBPT action remains unknown.

Interestingly, our cell-cycle analysis showed that DBPT caused an accumulation of cells at the G₂/M-phase and regulated the expression of G₂/M checkpoint proteins, specifically increasing

the levels of phosphorylated MPM-2 and histone H₃ in prostate cancer cells. This result is consistent with the observation in DLD1 cells after treatment with DBPT which led to cell-cycle arrest at mitotic phase with up regulation of cyclin B1, cdc25C, Bcl-2, and histone H₃ [18]. The changes in expression and phosphorylation are consistent with mitotic arrest [11, 12]. In addition, Bcl-xL has been found to be phosphorylated in mitosis after treatment with microtubule-interacting agents such as paclitaxel and 2-methoxyestradiol [19]. Consistent with this, our study also showed that Bcl-xL is phosphorylated after DBPT treatment. Moreover, it has recently been seen that Bim is phosphorylated in mitosis [20,21]. Our study showed that DBPT causes phosphorylation of Bim in LNCaP and DU145 cells. Thus, these findings suggest that DBPT may be able to induce mitotic arrest in prostate cancer cells. One recent study showed that the expression of Bim is related to apoptosis induction, and phosphorylation of Bim may be sufficient to cause Bax-induced apoptosis [22]. It is certainly possible that phosphorylation of Bim may be involved in DBPT-induced apoptosis in prostate cancer cells. Our data showed that DBPT-mediated G₂/M-phase arrest occurred earlier (6 h after treatment) than apoptosis induction (12 h after treatment). However, it remains to be determined whether these two events (G₂/M-phase arrest and apoptosis) are coupled.

In this study, we also investigated whether JNK activation is required for the apoptosis induction by DBPT as we have observed in colon cancer cells [7,18]. Our data showed that treatment with DBPT only induced minimal activation of JNK in prostate cancer cell lines tested. Interestingly, a JNK specific inhibitor, SP600125, blocked DBPT-induced apoptosis in DU145 cells. A possible explanation is that DBPT-mediated apoptosis in prostate cancer cells might through a JNK-independent pathway that can also be inhibited by SP600125. In fact, SP600125 is known to inhibit other kinases such as PKB/AKT and PKC α [23]. This result is also consistent with our previous observation that, although functional blockage of JNK inhibited apoptosis by DBPT in DLD1 cells, JNK activation alone is not sufficient to induce apoptosis, suggesting other death signals may also be involved. Further characterization of the molecular mechanisms of DBPT induced apoptosis in prostate cancer cells may allow us to identify the second death signal by DBPT.

Bcl-2 protein has been correlated with aggressive histology and high rates of recurrence in prostate cancers [24,25] and contributes to the phenotype of androgen-resistant prostate cancers [26]. In addition, it has been shown that Bcl-2 overexpression makes prostate cancer cells resistant to various treatments, including radiation, hormonal ablation, and chemotherapy [13–15,27]. These findings prompted us to test the effect of DBPT on apoptosis in Bcl-2-overexpressing DU145 cells. Interestingly, we found that DBPT induced apoptosis in both the parental DU145 cells and DU145/Bcl-2 cells with cytochrome *c* release. This suggests that DBPT induces apoptosis in prostate cancer cells independently of their Bcl-2 status and that DBPT may have potential against androgen-insensitive refractory tumors.

Recently, antiangiogenic therapy has been seen as a promising approach to slowing tumor growth, and the development of angiogenesis inhibitors has become an attractive novel strategy [28]. HIF-1 is a well-known oxygen-regulated transcriptional factor that plays a critical role in cell survival, cell-cycle progression, and angiogenesis [29,30], and its HIF-1 α subunit plays a key role in inducing VEGF production under hypoxic conditions [31]. One recent study showed that acute hypoxia up-regulates aggressiveness and survival properties such as HIF-1 α and VEGF protein expression in LNCaP cells [32]. Consistent with this, our study also demonstrated that acute hypoxia dramatically increased both VEGF protein expression and levels of HIF-1 α protein. We also found that DBPT down-regulated HIF-1 α and VEGF protein levels in a dose-dependent manner under hypoxic conditions, but it had little effect on apoptosis induction in LNCaP cells. This suggests that DBPT down-regulates hypoxia-mediated VEGF production in prostate cancer cells with little cytotoxic effect. However, the exact molecular actions by which DBPT down-regulates hypoxia-mediated HIF-1 α and VEGF protein

expression remain unclear. Due to limited supply of the compound, we are not able to determine antiangiogenic activity of DBPT in vivo at this time. Nevertheless, results from our in vitro study suggested that further studies with this compound or its analogs are warranted in order to explore their potential clinical applications.

CONCLUSIONS

In summary, our study demonstrated that the small molecule DBPT has potential against prostate cancer LNCaP and DU145 cells by antiproliferative and proapoptotic effects and by down-regulation of pro-angiogenic molecules. Further study on DBPT and its analogs may lead to development of new anti-prostate cancer therapeutics.

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Fig. 1.
Chemical structure of 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidione (DBPT)

Fig. 2.

Effect of DBPT on cell viability in prostate cancer LNCaP and DU145 cells. **(A)** Cells were treated with various concentrations of DBPT for 72 h, and the viability was determined by XTT assay. Cells treated with dimethyl sulfoxide were used as a control, with their viability set at 100%. Each data point represents the mean $\pm$ SD of three independent experiments. **(B)** Time course of cell viability for LNCaP cells treated with the indicated concentrations of DBPT for 24, 48, and 72 h. Each data point represents the mean $\pm$ SD of three independent experiments.

Fig. 3. DBPT induced cell-cycle arrest at the G₂/M phase and regulated G₂/M phase-related proteins in prostate cancer LNCaP and DU145 cell lines. **(A)** Cell-cycle distribution of DBPT-treated LNCaP (3 μM) and DU145 (10 μM) cells at the indicated times. Cells were harvested and fixed with ice-cold 70% ethanol and then resuspended in PBS containing 10 μg/ml of propidium iodide and 10 μg/ml of RNase A at 37°C for 30 min for flow cytometric analysis. The peaks represent G₁ and G₂/M phases of the cell cycle. **(B)** LNCaP and DU145 cells were treated with DBPT for the indicated times, and proteins in whole-cell lysates were analyzed by Western blot analysis with the indicated antibodies. β-actin was used as the loading control.

Fig. 4.

DBPT induced apoptosis in prostate cancer cells through caspase activation. **(A)** Cells were treated with DBPT at 3 μ M (LNCaP) or 10 μ M (DU145) for the indicated times and stained with propidium iodide. The percentage of cells in sub-G1 phase was determined by flow cytometry. Data represent the mean $\pm$ SD of three independent experiments. **(B)** Cells were treated with DBPT at 3 μ M (LNCaP) or 10 μ M (DU145) for the indicated times, and whole-cell extracts were analyzed by Western blotting. Cleavages of caspases and PARP were detected 24 and 48 h after DBPT treatment. Arrowheads indicate cleavage proteins. β -actin was used as a loading control.

Fig. 5.

Effects of DBPT on the expression of proapoptotic and antiapoptotic proteins. Cells were treated with DBPT at 3 μ M (LNCaP) or 10 μ M (DU145) for 12 or 24 h. Whole-cell extracts were then analyzed by Western blotting with the indicated antibodies. Arrows indicate cleavage proteins. β -actin was used as a loading control.

Fig. 6.

Cytotoxic effects of DBPT in Bcl-2-overexpressing DU145 cells. **(A)** DU145 cells stably transfected with Bcl-2 plasmid and parental DU145 cells were analyzed by Western blot analysis with anti-Bcl-2 antibody. β -actin was used as a loading control. **(B)** Bcl-2-overexpressing DU145 (clones Bcl-2 #1 and Bcl-2 #2) and parental DU145 cells were treated with 10 μ M DBPT for 24 or 48 h, and the percentage of cells in the sub-G₁ phase was determined by flow cytometry. Each value represents the mean $\pm$ SD of three independent experiments. **(C)** Cells were treated with 10 μ M DBPT for 24 h, and then cytosolic extract was subjected to Western blotting with anti-cytochrome c antibody. β -actin was used as a loading control.

Fig. 7.

Minimal effect of DBPT on JNK activation in the prostate cancer cells. **(A)** Cells were treated with DBPT at 3 μM (LNCaP) or 10 μM (DU145) for 24 h. Whole-cell lysates were analyzed for total and active (phosphorylated) JNK, and phosphorylated c-Jun by Western blotting. β-actin was used as a loading control. **(B)** Effect of JNK inhibitor (SP600125) on DBPT-induced apoptosis. Cells were treated with DBPT at 3 μM (LNCaP) or 10 μM (DU145) in the presence or absence of 5 or 20 μM SP600125 for 24 h. The percentage of cells in sub-G1 phase was determined by flow cytometry. Data represent mean ± SD from two independent experiments performed in triplicate.

Fig. 8. DBPT down-regulated hypoxia-induced HIF-1 activity and VEGF expression in DBPT-treated LNCaP cells. **(A)** LNCaP cells were treated with DBPT at the indicated concentrations for 16 h and then incubated in normoxic or hypoxic conditions for an additional 6 h. Whole-cell lysates were analyzed by Western blotting with the indicated antibodies. β -actin was used as a loading control. **(B)** The conditioned media from LNCaP cells treated with DBPT were analyzed for VEGF expression. Data represent the mean $\pm$ SD of three independent experiments. **(C)** Cell-cycle distribution of DBPT-treated LNCaP cells under hypoxic conditions and under the experimental conditions shown in Fig. 8A. Cells stained with propidium iodide were analyzed by flow cytometric analysis. The peaks represent the G₁ and G₂/M phases of the cell cycle. Cells treated with dimethyl sulfoxide were used as a control.