Regulation of FOXO3a/β-Catenin/GSK-3β Signaling by 3,3′-Diindolylmethane Contributes to Inhibition of Cell Proliferation and Induction of Apoptosis in Prostate Cancer Cells*

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Previous studies from our laboratory have shown anti-proliferative and pro-apoptotic effects of 3,3′-diindolylmethane (DIM) through regulation of Akt and androgen receptor (AR) in prostate cancer cells. However, the mechanism by which DIM regulates Akt and AR signaling pathways has not been fully investigated. It has been known that FOXO3a and glycogen synthase kinase-3β (GSK-3β), two targets of activated Akt, interact with β-catenin, regulating cell proliferation and apoptotic cell death. More importantly, FOXO3a, GSK-3β, and β-catenin are all AR coregulators and regulate the activity of AR, mediating the development and progression of prostate cancers. Here, we investigated the molecular effects of B-DIM, a formulated DIM with higher bioavailability, on Akt/FOXO3a/GSK-3β/β-catenin/AR signaling in hormone-sensitive LNCaP and hormone-insensitive C4-2B prostate cancer cells. We found that B-DIM significantly inhibited the phosphorylation of Akt and FOXO3a and increased the phosphorylation of β-catenin, leading to the inhibition of cell growth and induction of apoptosis. We also found that B-DIM significantly inhibited β-catenin nuclear translocation. By electrophoretic mobility shift and chromatin immunoprecipitation assays, we found that B-DIM inhibited FOXO3a binding to the promoter of AR and promoted FOXO3a binding to the p27KIP1 promoter, resulting in the alteration of AR and p27KIP1 expression, the inhibition of cell proliferation, and the induction of apoptosis in both androgen-sensitive and -insensitive prostate cancer cells. These results suggest that B-DIM-induced cell growth inhibition and apoptosis induction are partly mediated through the regulation of Akt/FOXO3a/GSK-3β/β-catenin/AR signaling. Therefore, B-DIM could be a promising non-toxic agent for possible treatment of hormone-sensitive but most importantly hormone-refractory prostate cancers.

Prostate cancer has become a significant health problem in the United States because of its high incidence and mortality (1). Despite an initial efficacy of androgen-deprivation therapy, most patients with prostate cancer progress from androgen-dependent status to hormone-refractory prostate cancer (HRPC)2 for which there is no curative therapy. It is still unclear how the prostate cancer cells progress to androgen independence. However, more evidence indicates that androgen receptor (AR) and Akt pathways participate in the development of HRPC (2, 3).

It has been known that during the progression of prostate cancers from androgen-sensitive status to an androgen-independent stage, the majority of prostate cancer cells still expresses AR, suggesting that AR signaling plays a critical role in the development and progression of prostate cancer (4). In prostate epithelial cells, ligand-free AR is sequestered in the cytoplasm bound to heat shock proteins. Binding of androgen to the AR induces a AR conformational change, which allows dissociation of heat shock proteins. The AR then forms a homodimer and is phosphorylated. This phosphorylation stabilizes the ligand-AR complex and the complex translocates to the nucleus (5). The activated AR then initiates gene transcription by binding to specific androgen-response elements in the promoter regions of target genes. Evidence is emerging showing that phosphorylation of the AR by other molecules in cell signaling pathways can also influence AR transactivation (6).

It has been found that Akt can phosphorylate the AR at Ser-210/213 and Ser-790/791 and transactivate the activity of AR (7, 8). The phosphorylation by Akt also sensitizes the AR to low circulating levels of androgen, such as those present during maximum androgen blockade (9). This sensitization allows low levels of androgen to induce phosphorylation at specific sites required for translocation of the AR to the nucleus. Therefore, Akt is another activator of AR required for androgen-independent survival and growth of prostate cancer cells.

Akt can be activated by the lipid kinase phosphatidylinositol 3-kinase (10). The activated Akt can inhibit apoptosis by direct

The abbreviations used are: HRPC, hormone-refractory prostate cancer; AR, androgen receptor; DIM, 3,3'-diindolylmethane; GSK, glycogen synthase kinase; FOXO, Forkhead transcription factor class O; IGF, insulin-like growth factor; ELISA, enzyme-linked immunosorbent assay; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation.
phosphorylation of its downstream molecules (10). Two of the important targets of Akt-mediated phosphorylation are the Forkhead transcription factor class O (FOXO) and glycogen synthase kinase-3 (GSK-3). The family of FOXO mainly includes FOXO1, FOXO3a, and FOXO4. By binding to the nuclear importer, active FOXO3a translocates to the nucleus, binds to DNA, and promotes the transcription of its target genes, inducing either cell cycle arrest or apoptosis (11). However, activated Akt regulates transcription of FOXO3a target genes through modulation of FOXO3a activity by phosphorylating it at three conserved serine/threonine residues (Thr-32, Ser-253, and Ser-315), leading to the release of FOXO3a from DNA and translocation to the cytoplasm (11, 12). Interestingly, a recent report shows that FOXO3a also promotes the transcription of AR (13). Therefore, Akt/FOXO3a/AR signaling appears to play important roles in the development of hormone-refractory prostate cancer.

GSK-3, including GSK-3α and GSK-3β, has been implicated in a variety of physiological processes such as induction of apoptosis (10). GSK-3β also controls cell survival through regulation of β-catenin, one of the important molecules in Wnt signaling (14). In the nucleus, β-catenin acts as a transcriptional coactivator and activates genes involved in cell proliferation and survival. GSK-3β binds to β-catenin and induces phosphorylation of β-catenin, which is subsequently ubiquitinated and degraded via the proteosome pathway within the cytoplasmic compartment (14–16). The activity of GSK-3β can be inhibited through phosphorylation at Ser-9 by activated Akt, resulting in nonphosphorylated β-catenin moving into the nucleus. Importantly, GSK-3β and β-catenin are two important AR co-regulators, which regulate transcriptional activity of AR and play important roles in the development of clinical androgen escape by regulation of AR oncogenic functions (3, 17, 18). Moreover, both GSK-3β and β-catenin can interact with molecules (including IKK, p105, etc.) in the NF-kB cell signaling pathway (15, 19, 20), regulating cell survival. Therefore, Akt/GSK-3β/β-catenin/AR signaling may play important roles in the development of HRPC and specific targeting of these signaling pathways could be important for killing HRPC cells.

Studies from our laboratory and others have found that 3,3′-diindolylmethane (DIM) shows potent antiproliferative activities against various cancers including prostate cancer (21–23). We have also reported that B-DIM, a formulated DIM with higher bioavailability in vivo (24), induces growth inhibition and apoptotic cell death in both androgen-sensitive LNCaP and androgen-insensitive C4-2B prostate cancer cells through the down-regulation of AR and Akt (25). However, no studies have been reported to date to elucidate the effect and molecular mechanisms of action of B-DIM on FOXO3a/GSK-3β/β-catenin signaling in prostate cancer cells, especially in HRPC cells. Here we report, for the first time, that B-DIM is a potent agent for inducing growth inhibition and apoptotic cell death in both androgen-sensitive LNCaP and androgen-insensitive C4-2B prostate cancer cells partly mediated through the inactivation of Akt/FOXO3a/GSK-3β/β-catenin/AR signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Reagents, and Antibodies—**Human prostate cancer cell lines including LNCaP and C4-2B cells were maintained in RPMI 1640 (Invitrogen) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO2 atmosphere at 37°C. B-DIM (BioResponse, Boulder, CO; known as BR-DIM and referred as B-DIM) was generously provided by Dr. Michael Zeligs and was dissolved in Me2SO to make a 50 mM stock solution. IGF-1 was purchased from R&D Systems (Minneapolis, MN) and LY294002 was from Sigma. Anti-AR (Santa Cruz Biologicals, Santa Cruz, CA), anti-Akt (Santa Cruz), anti-pAkt Ser-473 (Cell Signaling, Danvers, MA), anti-FOXO3a (Cell Signaling), anti-pFOXO3a Ser-253 (Cell Signaling), anti-pFOXO3a Thr-32 (Cell Signaling), anti-β-catenin (Cell Signaling), anti-p-β-catenin Ser-33/37/Thr-41 (Cell Signaling), anti-FOX-3 (Upstate), anti-karyopherin α (BD Biosciences, San Diego, CA), anti-karyopherin β (BD Biosciences), and anti-β-actin (Sigma) primary antibodies were used for immunoprecipitation, Western blot analysis, or immunofluorescent staining.

**Cell Proliferation Inhibition Studies by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay—**Human LNCaP and C4-2B prostate cancer cells were seeded in 96-well plates. After 24 h, the complete medium was removed and the cells were washed with serum-free medium. The cells were then incubated in serum-free medium supplemented with 100 ng/ml IGF-1, 50 μM B-DIM, or 50 μM B-DIM plus 100 ng/ml IGF-1 for 48 h. Control cells were treated with 0.1% Me2SO (vehicle control). After treatment, the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/ml, Sigma) in medium at 37°C for 2 h and then with isopropyl alcohol at room temperature for 1 h. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (Tecan, Durham, NC) at 595 nm. The growth inhibition of LNCaP and C4-2B cells by B-DIM treatment was statistically evaluated with Student’s t test using GraphPad StatMate software (GraphPad Software Inc., San Diego, CA). Comparisons were made between control and treatment. p value less than 0.05 was used to indicate statistical significance.

**Histone/DNA ELISA for Detection of Apoptosis—**The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in prostate cancer cells treated with B-DIM according to the manufacturer’s protocol. Briefly, LNCaP and C4-2B cells were treated with 100 ng/ml IGF-1, 50 μM B-DIM, or 50 μM B-DIM plus 100 ng/ml IGF-1 for 48 h as described above. The cytoplasmic histone/DNA fragments from these cells were then extracted and incubated in microtiter plate modules coated with anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments followed by color development with ABTS substrate for peroxidase. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (Tecan) at 405 nm. The induction of apoptosis by B-DIM treatment in LNCaP and C4-2B cells was statistically evaluated with Student’s t test using GraphPad StatMate.
**Preparation of Cytoplasmic and Nuclear Lysates**—LNCaP and C4-2B cells were treated with 50 μM B-DIM for 48 and 72 h. After treatment and harvesting, the cells were resuspended in lysis buffer (0.08 M KCl, 35 mM HEPES, pH 7.4, 5 mM potassium phosphate, pH 7.4, 5 mM MgCl₂, 25 mM CaCl₂, 0.15 M sucrose, 2 mM PMSF, 8 mM dithiothreitol) and frozen at −80 °C overnight. The cell suspension was thawed and passed through a 28-gauge needle three times. A small aliquot of the cells were checked for cell membrane breakage using trypan blue. Then, the cell suspension was centrifuged and the supernatant was saved as cytoplasmic lysate. The pellet was suspended in lysis buffer and the nuclei were lysed by sonication. After centrifugation, supernatant was saved as nuclear lysate. The pellet was further lysed in 62 mM Tris-HCl and 2% SDS as membrane lysate. The protein concentration in each sample was measured using Coomassie Plus Protein Assay (Pierce).

**Immunoprecipitation**—Nuclear lysate (500 μg) and cytoplasmic lysate (800 μg) were diluted and subjected to immunoprecipitation using 2.5–5 μg of anti-AR, anti-FOXO3a, or anti-β-catenin antibody. The lysate and antibody mixtures were incubated overnight at 4 °C with rotation. After adding 50 μl of Protein G-agarose (Santa Cruz) and incubation for 1 h, the samples were centrifuged. The agarose pellet was then washed three times, resuspended in 50 μl of Laemmli buffer with 2-mercaptoethanol, and boiled for 5 min. Boiled samples were centrifuged and supernatant was used for Western blot analysis.

**Western Blot Analysis**—LNCaP and C4-2B cells were treated with 50 μM B-DIM for 24–72 h. Whole cell lysate of the cells was prepared by sonicating the cell lysed in 62 mM Tris-HCl and 2% SDS. The protein concentration was measured by BCA Protein Assay (Pierce). Immunoprecipitates and whole cell lysates were subjected to 10 or 14% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The membranes were incubated with the indicated primary antibodies, and subsequently incubated with appropriate secondary antibody conjugated with peroxidase (Bio-Rad) or TrueBlot secondary antibody (eBioscience, San Diego, CA). The signal was then detected using the chemiluminescent detection system (Pierce) and quantified with AlphaEaseFC software (Alpha Innotech, San Leandro, CA).

**Immunofluorescence Staining**—LNCaP and C4-2B cells were plated on coverslips in each well of a 6-well plate. Cells were then treated with 50 μM B-DIM for 24–72 h. Cells were then fixed with acetone for 15 min, rinsed with phosphate-buffered saline, and incubated with 1% bovine serum albumin in phosphate-buffered saline for 30 min. The cells were then incubated with anti-FOXO3a or anti-β-catenin antibody at 4 °C overnight. After washing with phosphate-buffered saline, the cells were incubated with fluorescein isothiocyanate-conjugated (1:100, Santa Cruz) or Texas Red-conjugated (1:100, Santa Cruz) secondary antibody at 37 °C for 1 h and washed with phosphate-buffered saline. Cell images were observed under a fluorescent microscope.

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSA was conducted to measure the activity of FOXO3a binding to AR or p27Kip1 promoter in B-DIM-treated and untreated cells. LNCaP and C4-2B cells were treated as described above. Following treatment, cells were harvested and incubated in ice-cold cell lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 mg/ml benzamidine) on ice. After 15 min, Nonidet P-40 was added to the cell suspension at a final concentration of 0.3% and the samples were vortexed vigorously for 20 s. After centrifugation, the supernatant was saved as cytoplasmic protein and the nuclear pellet was incubated in ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 mg/ml benzamidine) on ice for 30 min. After centrifugation, the supernatant was saved as nuclear protein and the protein concentration was measured using the BCA Protein Assay (Pierce). 8 μg of nuclear protein was assembled with 10 mM Tris-HCl, 50 mM NaCl, 6 mM dithiothreitol, 0.5% Tween 20, 0.08 mg/ml poly(dI)-poly(dC), and 0.01 μM IR Dye 700 or 800-labeled oligonucleotide (LI-COR, Lincoln, NE), which contains AR or p27Kip1 promoter sequences with a consensus FOXO3a DNA binding site. The sequences of oligonucleotides are as follows: 5’-ATTATGTCCCTTTCACGCTT-3’ for AR; 5’-ATGTTAGCTTTTACCCAC-3’ for p27Kip1 (13, 26, 27). After incubation at room temperature for 30 min, the samples were loaded on a pre-run 4.5 (for AR) or 8% (for Kip1) polyacrylamide gel and electrophoresis was continued at 100 volts for 60 min. The signal was then detected and quantified by using Odyssey Infrared Imaging System (LI-COR). Supershift assay using FOXO3a antibody was also conducted to confirm the specificity of FOXO3a DNA-binding activity.

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIP assay was performed to test the effect of B-DIM on FOXO3a binding to AR or p27Kip1 promoter under an in vivo environment. Briefly, LNCaP and C4-2B cells were treated with 50 μM B-DIM for 48 h. Formaldehyde was then added to the cells at a final concentration of 1%. After formaldehyde cross-linking the reaction was quenched with 125 mM glycine, the cells were incubated in cell lysis buffer (5 mM HEPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF) on ice for 10 min. The nuclei were pelleted by centrifugation and resuspended in nuclear lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF). After incubation on ice for 10 min, the nuclear lysates were sonicated to generate an average DNA size of ~600 bp and the protein concentration was measured using BCA Protein Assay (Pierce). 1000 μg of each sample was made between control and treatment. p value less than 0.05 was used to indicate statistical significance.
sample were diluted and 200 μg of diluted sample were kept as input. The remaining sample was incubated with FOXO3a antibody (Santa Cruz) at 4 °C overnight. The FOXO3a antibody-bound AR and p27KIP1 promoter DNA complexes were pelleted with Protein G–agarose at 4 °C for 2 h. After washing and elution, the immunoprecipitated complexes and input were reversed from cross-linking by adding RNase and 0.3 M NaCl and heating at 65 °C for 5 h. The input DNA and immunoprecipitated DNA were then precipitated with ethanol. After being treated with proteinase K and purified with phenol/chloroform, the purified DNA was precipitated again with ethanol. Real-time and conventional PCRs were performed using purified DNA (input DNA for β-actin and FOXO3a immunoprecipitated DNA for AR or p27KIP1 promoter with FOXO3a binding site) and the following primers: AR promoter (forward, 5′-AGGCCCTCCCCAAATGACATG-3′; reverse, 5′-GCACAGCACAACATCATAGG-3′), p27KIP1 promoter (forward, 5′-GTCCCTTCTCAGCTGCATCAT-3′; reverse, 5′-GGAACCAACCTTCGTTCAT-3′), β-actin (forward, 5′-CCACACTGTGCCCATCTACG-3′; reverse, 5′-AGGATCTCATGAGTGTCAGTCGCTG-3′). For real-time PCR, data were analyzed according to the comparative Ct method and the amount of FOXO3a-bound AR or p27KIP1 promoter DNA was normalized by input β-actin DNA. For conventional PCR, PCR product was visualized by agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Inhibition of FOXO3a Phosphorylation by B-DIM—We previously reported that B-DIM significantly inhibited the phosphorylation of Akt and the expression of AR in LNCaP and C4-2B prostate cancer cells (25). To further investigate how B-DIM suppresses the expression of AR through the inhibition of Akt phosphorylation, we tested the effects of B-DIM on FOXO3a, one of the main downstream target genes of Akt. We observed that phosphorylation of Akt and FOXO3a was up-regulated by IGF-1 and down-regulated by LY294002 in prostate cancer cells as expected (Fig. 1, A and C). We found that B-DIM significantly inhibited the phosphorylation of FOXO3a in C4-2B and LNCaP cells (Fig. 1B). Moreover, B-DIM abrogated the up-regulation of Akt and FOXO3a phosphorylation induced by IGF-1 (Fig. 1, A–C). Furthermore, we found that B-DIM significantly inhibited the phosphorylation of FOXO3a at Thr-32 and Ser-253 and decreased the ratio of p-FOXO3a over FOXO3a in both the cytoplasm and nucleus of prostate cancer cells (Fig. 1C), suggesting that B-DIM could help to retain a greater amount of FOXO3a in the activated form to inhibit cancer cell growth. By immunoprecipitation, we found that B-DIM significantly inhibited the formation of FOXO3a and Akt complex in the cytosol and nucleus of prostate cancer cells (Fig. 1D), consistent with Akt inactivation and the subsequent decrease of FOXO3a phosphorylation by B-DIM treatment. We also found that B-DIM significantly decreased the formation of the FOXO3a and AR complex, which could inhibit FOXO3a activity in both cytoplasmic and nuclear components (Fig. 1D), suggesting that a greater level of active FOXO3a proteins will exert their anti-proliferative and pro-apoptotic effects on B-DIM-treated cells. It has been known that activated Akt also phosphorylates and inactivates GSK-3β, leading to the dephosphorylation of β-catenin and cancer cell survival. β-Catenin also interacts with FOXO3a to regulate cell growth. Therefore, we further investigated the effects of B-DIM on GSK-3β/β-catenin signaling.

Increased GSK-3β Binding to β-Catenin and Induction of β-Catenin Phosphorylation by B-DIM—By Western blot analysis, we found that B-DIM significantly decreased the level of β-catenin protein in the cytosol and nucleus of C4-2B cells (Fig. 2, A and B). More importantly, we found that B-DIM enhanced the phosphorylation of β-catenin and increased the ratio of p-β-catenin over β-catenin in the nucleus of C4-2B cells (Fig. 2B). By immunoprecipitation, we discovered that GSK-3β binding to β-catenin was increased upon B-DIM treatment (Fig. 2C), consistent with the increase in the phosphorylation of β-catenin. We also found that B-DIM inhibited FOXO3a binding to β-catenin (Fig. 2C). In LNCaP cells, we also observed similar effects of B-DIM on the down-regulation of the β-catenin level and up-regulation of β-catenin phosphorylation (data not shown). These results suggest that B-DIM treatment leads to a greater level of inactivated β-catenin and less of activated β-catenin in prostate cancer cells, causing a decrease in the
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A. Control | IGF-1 | LY294002
\[\begin{array}{llll}
\text{Total lysate} & \beta\text{-Catenin} \\
1.1 & 0.49 & 0.56
\end{array} \]
\[\beta\text{-Catenin in total lysate}\]
\[\beta\text{-actin in total lysate}\]
\[1 \text{ NA} 0.69\]

B. Cyto | Nu | Total
\[\begin{array}{llll}
\beta\text{-Catenin} & \beta\text{-actin} \\
1 \text{ NA} 1 & 3.07 1 & 4.21
\end{array} \]
\[\beta\text{-Catenin in cytoplasm}\]
\[\beta\text{-actin in cytoplasm}\]
\[1 \text{ NA} 1 \text{ NA}\]

C. IP: \beta\text{-Catenin}
\[\beta\text{-Catenin}\]
\[\beta\text{-Catenin}\]

IP: FOXO3a
\[\text{GSK3β}\]

FIGURE 2. B-DIM inhibited the expression of \(\beta\)-catenin and induced phosphorylation of \(\beta\)-catenin. A, C4-2B cells were treated with 100 ng/ml IGF-1, 50 \(\mu\text{M}\) B-DIM, or 20 \(\mu\text{M}\) LY294002 for 48 h. Total, cytoplasmic (Cyto), and nuclear (Nu) lysates from each sample were subjected to Western blot analysis. The ratio of \(\beta\)-catenin over \(\beta\)-actin was calculated by standardizing the ratios of each control to the unit value. B, C4-2B cells were treated with 50 \(\mu\text{M}\) B-DIM for 48 h. Total lysate from each sample was subjected to Western blot analysis (C, control; D, B-DIM treatment). The ratios of \(\beta\)-catenin over \(\beta\)-actin and p-\(\beta\)-catenin over \(\beta\)-catenin were calculated by standardizing the ratios of each control to the unit value. C, C4-2B cells were treated with 50 \(\mu\text{M}\) B-DIM for 48 h. Total lysates from each sample were subjected to immunoprecipitation (IP) and Western blot analysis (C, control; D, B-DIM treatment).

activated \(\beta\)-catenin in the nucleus of prostate cancer cells. Therefore, we further investigated the location of \(\beta\)-catenin and FOXO3a altered by B-DIM treatment.

Inhibition of \(\beta\)-Catenin Nuclear Localization by B-DIM—Indeed, we observed a lower level of \(\beta\)-catenin protein in the nucleus after B-DIM treatment (Fig. 2, A and B). By immunofluorescent staining, we found that B-DIM significantly inhibited the nuclear localization of \(\beta\)-catenin; however, no significant change in the nuclear localization of FOXO3a was observed upon B-DIM treatment (Fig. 3A). To confirm these results, we also tested the expression of karyopherin, one of the nuclear importins, and the interaction of \(\beta\)-catenin with karyopherin in B-DIM-treated and un-treated prostate cancer cells. We found that B-DIM significantly decreased the level of karyopherin protein in the cytoplasm and nucleus of C4-2B cells (Fig. 3B). More importantly, we found that B-DIM significantly inhibited karyopherin binding to \(\beta\)-catenin, consistent with the data from immunofluorescent staining, demonstrating the inhibitory effects of B-DIM on the nuclear translocation of \(\beta\)-catenin. However, we did not observe such a significant inhibitory effect of B-DIM on the binding of karyopherin to FOXO3a (Fig. 3C). We also observed similar effects of B-DIM on the relocation of \(\beta\)-catenin in LNCaP cells (data not shown). Because FOXO3a as a transcription factor could regulate the transcription of its downstream genes including AR and p27KIP1, we tested the effects of B-DIM on the transcriptional activity of AR and p27KIP1 regulated by FOXO3a.

Inhibition of FOXO3a-mediated AR Transcription by B-DIM—By EMSA, we found that B-DIM inhibited the activity of FOXO3a protein binding to AR promoter DNA in C4-2B and LNCaP cells (Fig. 4, A and B), suggesting the inhibitory effect of B-DIM on the FOXO3a-induced transcription of AR. We also conducted ChIP assay with conventional and real-time PCR to confirm this effect of B-DIM in a live cell environment. We found that B-DIM treatment decreased FOXO3a protein binding to AR promoter in vivo in C4-2B (Fig. 4, C and D) and LNCaP (data not shown) prostate cancer cells. By Western blot analysis, we indeed observed the down-regulation of AR expression by B-DIM treatment (Fig. 1A), consistent with the results from EMSA and ChIP assay.

Stimulation of FOXO3a-mediated p27KIP1 Expression by B-DIM—In contrast with AR, using EMSA we found that B-DIM significantly increased the activity of FOXO3a protein binding to p27KIP1 promoter DNA in C4-2B cells (Fig. 5A). ChIP assay confirmed EMSA data, showing that B-DIM significantly enhanced FOXO3a protein binding to the p27KIP1 promoter in vivo in prostate cancer cells (Fig. 5, B and C). Furthermore, we found an increased level of p27KIP1 protein after B-DIM treatment (Fig. 5D), consistent with the data from the EMSA and ChIP assay. We also observed similar results in LNCaP cells (data not shown). Because FOXO3a, GSK-3β, and \(\beta\)-catenin all are important factors that regulate cell survival and apoptotic cell death, we tested the effects of B-DIM on cell proliferation and apoptosis.

Inhibition of Cell Proliferation and Induction of Apoptosis by B-DIM through Regulation of Akt/FOXO3a/\(\beta\)-Catenin/GSK-3β/AR Signaling—By 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, we found that IGF-1, which activates Akt, promoted cell proliferation in LNCaP and C4-2B cells (Fig. 6, A and B). Importantly, B-DIM inhibited cell proliferation and abrogated IGF-1-induced cell proliferation (Fig. 6, A and B). By apoptosis assay, we observed that IGF-1 protected cancer cells from apoptosis (Fig. 6, C and D). However, B-DIM-induced apoptosis and abrogated IGF-1-mediated protection of LNCaP and C4-2B cells from apoptosis. These results are consistent with our observation on the alterations of Akt and FOXO3a caused by IGF-1 or B-DIM treatment (Fig. 1, A and C), respectively.
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Regulation of FOXO3a/GSK-3β

...were subjected to immunoprecipitation (IP) and/or Western blot analysis (C, control; D, B-DIM treatment; Cyto, cytoplasmic; Nu, nuclear). The ratio of karyopherin α over β-actin was calculated by standardizing the ratios of each control to the unit value.

FIGURE 3. B-DIM inhibited the nuclear translocation of β-catenin but did not alter FOXO3a nuclear translocation. A, C4-2B cells were treated with 50 μM B-DIM for 48 h. The samples were subjected to immunofluorescent staining using anti-β-catenin (a and b) and anti-FOXO3a (c and d) antibodies. (a and c, control; b and d, B-DIM treatment). B and C, C4-2B cells were treated with 50 μM B-DIM for 48 h. Total, cytoplasmic, and nuclear lysates from each sample were subjected to immunoprecipitation (IP) and/or Western blot analysis (C, control; D, B-DIM treatment; Cyto, cytoplasmic; Nu, nuclear). The ratio of karyopherin α over β-actin was calculated by standardizing the ratios of each control to the unit value.

suggesting that the inhibition of cell proliferation and induction of apoptosis by B-DIM is partly mediated by the deregulation of Akt/FOXO3a/GSK-3β/β-catenin/AR signaling pathways.

DISCUSSION

FOXO3a is a main molecule in the Akt signaling pathway. It plays important roles in the control of cell survival and apoptotic cell death through transcriptional regulation of genes that are critically involved in the processes of cell cycle and apoptosis (28). The main regulatory event that controls FOXO3a-induced transcription is phosphorylation or dephosphorylation of FOXO3a. The ratio of p-FOXO3a over FOXO3a is one of the factors that determine whether cancer cells will survive or undergo apoptotic cell death. From our series of studies, we found that B-DIM significantly inhibited the phosphorylation and activation of Akt, resulting in lower levels of p-FOXO3a (Fig. 7). More importantly, B-DIM inhibited the phosphorylation of FOXO3a and abrogated the inactivation of FOXO3a induced by IGF-1 in both cytoplasmic and nuclear compartments, causing less p-FOXO3a and more FOXO3a in the nucleus compared with control. However, we did not find any significant change in FOXO3a binding to one of the nuclear import protein, karyopherin. These results suggest that B-DIM regulates the activity of FOXO3a through the regulation of FOXO3a phosphorylation rather than the regulation of FOXO3a nuclear importing. It has been known that FOXO3a regulates the transcription of p27KIP1 and AR by binding to their promoters (13, 29). Interestingly, using EMSA and ChIP assay, we found that B-DIM induced FOXO3a binding to the p27KIP1 promoter and suppressed FOXO3a binding to the AR promoter, suggesting its effects on up-regulation of p27KIP1 and down-regulation of AR. Indeed, we found that p27KIP1 expression was increased and AR expression was decreased upon B-DIM treatment. Up-regulation of p27KIP1 promoter activity and protein expression by B-DIM-mediated FOXO3a regulation could lead to the growth inhibition and apoptotic cell death that we have observed in prostate cancer cells. It has been reported that FOXO3a activates AR expression (13), whereas AR inactivates FOXO3a (30), forming a regulatory loop to maintain a balance between AR and FOXO3a. The decreased levels of AR protein and AR binding to FOXO3a as observed by Western blot analysis and immunoprecipitation clearly suggest that B-DIM could interrupt the above mentioned regulatory loop, resulting in the inhibition of AR transcription (Fig. 7). In addition, alteration of the p27KIP1 protein level by B-DIM treatment could also be due to the altered activity of proteasome as observed in other agent treatments (31) and, as such, require further investigation.

β-Catenin is an important molecule in the Wnt signaling pathway (32). However, it also interacts with GSK-3β to regulate cell proliferation through activation of the Akt signaling pathway. It has been known that activated Akt phosphorylates and inactivates GSK-3β, causing degradation of GSK-3β (33). If Akt is inactivated, GSK-3β can phosphorylate and inactivate β-catenin, leading to a decrease in nuclear translocation of β-catenin (3). In most cancer cell lines, activated Akt causes more β-catenin to translocate to the nucleus and bind to T-cell factor, stimulating the expression of genes that are related to cell proliferation and survival (3, 32, 34). In this study, we found that B-DIM treatment increased GSK-3β binding to β-catenin, suggesting that B-DIM could up-regulate the phosphorylation of β-catenin. Indeed, we observed higher levels of p-β-catenin and lower levels of β-catenin in the nucleus of B-DIM-treated C4-2B and LNCaP cells compared with control. Moreover, B-DIM decreased the protein level of karyopherin, which has been known to be a main nuclear import protein to carry important nuclear regulatory factors into the nucleus (35, 36), suggesting that B-DIM could inhibit the nuclear translocation of nuclear regulatory factors. Indeed, we previously reported that B-DIM inhibited AR nuclear translocation (25). In the present study, we observed that β-catenin nuclear translocation was decreased upon B-DIM treatment. In the nucleus, β-catenin interacts with T-cell factor and further regulates the transcription of genes in the Wnt signaling pathway (32, 37). Therefore, our results clearly suggest that B-DIM could down-regulate Wnt signaling and inhibit cell survival through the regulation of GSK-3β/β-catenin signaling (Fig. 7).

It has been known that Akt, FOXO3a, β-catenin, and GSK-3β interact with each other to regulate cell survival and...
apoptosis (38–40). Moreover, all of them act as AR regulators or co-regulators that influence AR transactivation through regulation of phosphorylation and transcription of AR (13, 17, 18). It is obvious that there is significant cross-talk between Akt, FOXO3a, GSK-3β, β-catenin, and AR. Therefore, the Akt/FIGURE 4. B-DIM inhibited FOXO3a binding to the promoter of AR. A and B, C4-2B (A) and LNCaP (B) cells were treated with 30 or 50 μM B-DIM for 24, 48, and 72 h. Nuclear proteins from each sample were subjected to EMSA using AR oligonucleotide. The arrow indicates specific DNA binding. The density of each signal was calculated by standardizing each control to the unit value). C and D, C4-2B cells were treated with 50 μM B-DIM. The samples were then subjected to ChIP assay using real-time PCR (C, bars, mean ± S.E.; n = 3) or conventional PCR (D: ARP, using AR promoter primers; C, control; D, B-DIM treatment). FOXO3a/GSK-3β/β-catenin/AR signaling pathway appears to play critical roles in the control of cell growth and apoptosis. In the present study, we found that B-DIM elicits its effects on each of these molecules at every step of this signaling pathway. The effect of B-DIM could begin from the inhibition of Akt activation and cause a subsequent series of alterations of signal transduction in this signaling pathway, finally leading to the inhibition of AR transactivation in both androgen sensitive and hormone refractory prostate cancers (Fig. 7). More importantly, it has been found that GSK-3β functions as a critical regulator in the development of androgen-independent prostate cancer by regulation of the oncogenic function of AR (3). In
addition, β-catenin-mediated Wnt signaling also interacts with AR and plays important roles in the development and progression of prostate cancer (41). We found that B-DIM could regulate these molecules, cause down-regulation of AR transactivation, and result in the decreased level of PSA as we reported previously (25), suggesting that B-DIM could be very effective for the treatment of prostate cancer.

It has been well known that Akt controls cell proliferation and apoptosis. Growth factor IGF-1 has been found to activate Akt (42, 43), suggesting that IGF-1 could induce cell growth and inhibit apoptosis. Indeed, we found that IGF-1 treatment caused an increase in the amount of p-Akt, induced cell proliferation, and decreased the number of apoptotic cells. However, B-DIM abrogated IGF-1-induced Akt activation and cell proliferation and caused more apoptotic cell death. B-DIM also affected Akt downstream genes including FOXO3a and GSK-3β due to inactivation of Akt, resulting in the down-regulation of both β-catenin and AR.

These results suggest that B-DIM inhibited cell proliferation and induced apoptotic cell death partly through the regulation of Akt/FOXO3a/GSK-3β/β-catenin/AR signaling. Based on our molecular mechanistic pre-clinical data, we believe that B-DIM could be a promising non-toxic agent for the possible treatment of androgen-dependent but most importantly hormone-refractory prostate cancers.

REFERENCES

1. Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., Smigal, C., and Thun, M. J. (2006) CA-Cancer J. Clin. 56, 106–130
2. Chen, C. D., Welsbie, D. S., Tran, C., Baek, S. H., Chen, R., Vessella, R., Rosenfeld, M. G., and Sawyers, C. L. (2004) Nat. Med. 10, 33–39
3. Mulholland, D. J., Dedhar, S., Wu, H., and Nelson, C. C. (2006) Oncogene 25, 329–337
4. Heinlein, C. A., and Chang, C. (2004) Endocr. Rev. 25, 276–308
5. Edwards, J., and Bartlett, J. M. (2005) BJU Int. 95, 1320–1326
6. Lee, H. J., and Chang, C. (2003) Cell Mol. Life Sci. 60, 1613–1622
7. Lin, H. K., Yeh, S., Kang, H. Y., and Chang, C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7200–7205
8. Wen, Y., Hu, M. C., Makino, K., Spohn, B., Bartholomeusz, G., Yan, D. H., and Hung, M. C. (2000) Cancer Res. 60, 6841–6845
9. Rochette-Egly, C. (2003) Cell. Signal. 15, 355–366
10. Franco, T. F., Hornik, C. P., Segev, L., Shostak, G. A., and Sugimoto, C. (2003) Oncogene 22, 8983–8998
11. Burgering, B. M., and Kops, G. J. (2002) Trends Biochem. Sci. 27, 352–360
12. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Joo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) Cell 96, 857–868
13. Yang, L., Xie, S., Jamaluddin, M. S., Altuwaijri, S., Ni, J., Kim, E., Chen, Y. T., Hu, Y. C., Wang, L., Chuang, K. H., Wu, C. T., and Chang, C. (2005) J. Biol. Chem. 280, 33558–33565
14. Kikuchi, A. (1999) Cell Signal. 11, 777–788
15. Deng, J., Xia, W., Miller, S. A., Wen, Y., Wang, H. Y., and Hung, M. C. (2004) Mol. Carcinog. 39, 139–146
16. Yost, C., Torres, M., Miller, J. R., Huang, E., Kimelman, D., and Moon, R. T. (1996) Genes Dev. 10, 1443–1454
17. Salas, T. R., Kim, J., Vakar-Lopez, F., Sabichi, A. L., Troncoso, P., Jenster, G., Kikuchi, A., Chen, S. Y., Shemshedini, L., Suraokar, M., Logothetis, C. J., DiGiovanni, J., Lippman, S. M., and Menter, D. G. (2004) J. Biol.
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Chem. 279, 19191–19200
18. Truica, C. I., Byers, S., and Gelmann, E. P. (2000) Cancer Res. 60, 4709–4713
19. Demarchi, F., Bertoli, C., Sandy, P., and Schneider, C. (2003) J. Biol. Chem. 278, 39583–39590
20. Lamberti, C., Lin, K. M., Yamamoto, Y., Verma, U., Verma, I. M., Byers, S., and Gaynor, R. B. (2001) J. Biol. Chem. 276, 42276–42286
21. Firestone, G. L., and Bjeldanes, L. F. (2003) J. Nutr. 133, 2448S–2455S
22. Nachshon-Kedmi, M., Yannai, S., Haj, A., and Fares, F. A. (2003) Food Chem. Toxicol. 41, 745–752
23. Hong, C., Kim, H. A., Firestone, G. L., and Bjeldanes, L. F. (2002) Carcinogenesis 23, 1297–1305
24. Anderton, M. J., Manson, M. M., Verschoyle, R., Gescher, A., Steward, W. P., Williams, M. L., and Mager, D. E. (2004) Drug Metab. Dispos. 32, 632–638
25. Bhuiyan, M. M., Li, Y., Banerjee, S., Ahmed, F., Wang, Z., Ali, S., and Sarkar, F. H. (2006) Cancer Res. 66, 10064–10072
26. Tran, H., Brunet, A., Griffith, E. C., and Greenberg, M. E. (2003) Sci. STKE 2003, RE5
27. Huang, H., and Tindall, D. J. (2006) Future Oncol. 2, 83–89
28. Hu, Y., Wang, X., Zeng, L., Cai, D. Y., Sabapathy, K., Goff, S. P., Firpo, E. J., and Li, B. (2005) Mol. Biol. Cell 16, 3705–3718
29. Li, P., Lee, H., Guo, S., Unterman, T. G., Jenster, G., and Bai, W. (2003) Mol. Cell. Biol. 23, 104–118
30. Nam, S., Smith, D. M., and Dou, Q. P. (2001) J. Biol. Chem. 276, 13322–13330
31. Clevers, H. (2006) Cell 127, 469–480
32. Salas, T. R., Reddy, S. A., Clifford, J. L., Davis, R. J., Kikuchi, A., Lippman, S. M., and Menter, D. G. (2003) J. Biol. Chem. 278, 41338–41346
33. Kuemmerle, J. F. (2005) Am. J. Physiol. 288, G101–G110
34. Mosammaparast, N., and Pemberton, L. F. (2004) Trends Cell Biol. 14, 547–556
35. Chook, Y. M., and Blobel, G. (2001) Curr. Opin. Struct. Biol. 11, 703–715
36. Chong, Z. Z., Li, F., and Maisie, K. (2006) Curr. Neurovasc. Res. 3, 107–117
37. Rosas, M., Dijkers, P. F., Lindemans, C. L., Lammers, J. W., Koenderman, L., and Coffer, P. J. (2006) J. Leukocyte Biol. 80, 186–195
38. Segrelles, C., Moral, M., Lara, M. F., Ruiz, S., Santos, M., Leis, H., Garcia-Escudero, R., Martinez-Cruz, A. B., Martinez-Palacio, J., Hernandez, P., Ballestin, C., and Paramio, J. M. (2006) Oncogene 25, 1174–1185
39. Terry, S., Yang, X., Chen, M. W., Vacherot, F., and Buttyn, R. (2006) J. Cell. Biochem. 99, 402–410
40. Prueitt, R. L., Boersma, B. J., Howe, T. M., Goodman, J. E., Thomas, D. D., Ying, L., Pfister, C. M., Yfantis, H. G., Cottrell, J. R., Lee, D. H., Remaley, A. T., Hofseth, L. J., Wink, D. A., and Ambs, S. (2007) Int. J. Cancer 120, 796–805
41. Lawlor, M. A., and Rotwein, P. (2000) Mol. Cell. Biol. 20, 8983–8995