

# Mammalian Target of Rapamycin Repression by 3,3'-Diindolylmethane Inhibits Invasion and Angiogenesis in Platelet-Derived Growth Factor-D–Overexpressing PC3 Cells

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## Abstract

**Platelet-derived growth factor-D (PDGF-D) is a newly recognized growth factor known to regulate many cellular processes, including cell proliferation, transformation, invasion, and angiogenesis. Recent studies have shown that PDGF-D and its cognate receptor PDGFR- $\beta$  are expressed in prostate tumor tissues, suggesting that PDGF-D might play an important role in the development and progression of prostate cancer. However, the biological role of PDGF-D in tumorigenesis remains elusive. In this study, we found that PDGF-D–overexpressing PC3 cells (PC3 cells stably transfected with PDGF-D cDNA and referred to as PC3 PDGF-D) exhibited a rapid growth rate and enhanced cell invasion that was associated with the activation of mammalian target of rapamycin (mTOR) and reduced Akt activity. Rapamycin repressed mTOR activity and concomitantly resulted in the activation of Akt, which could attenuate the therapeutic effects of mTOR inhibitors. In contrast, B-DIM (BR-DIM from Bioresponse, Inc.; a chemopreventive agent) significantly inhibited both mTOR and Akt in PC3 PDGF-D cells, which were correlated with decreased cell proliferation and invasion. Moreover, conditioned medium from PC3 PDGF-D cells significantly increased the tube formation of human umbilical vein endothelial cells, which was inhibited by B-DIM treatment concomitant with reduced full-length and active form of PDGF-D. Our results suggest that B-DIM could serve as a novel and efficient chemopreventive and/or therapeutic agent by inactivation of both mTOR and Akt activity in PDGF-D–overexpressing prostate cancer. [Cancer Res 2008;68(6):1927–34]**

## Introduction

Platelet-derived growth factor-D (PDGF-D) is a newly recognized growth factor that can regulate many cellular processes, including cell proliferation, transformation, invasion, and angiogenesis by activating its cognate receptor PDGFR- $\beta$  (1, 2). PDGF-D consists of the hydrophobic putative NH<sub>2</sub>-terminal signal peptide, the NH<sub>2</sub>-terminal CUB domain, a hinge region, and the COOH-terminal growth factor domain containing the cystine knot motif (3). Several reports have indicated that the CUB domain of PDGF-D have to be cleaved extracellularly to make the COOH-terminal growth factor

domain active for PDGF-D binding to its receptor (3, 4). It is known that growth factors, such as PDGF and epidermal growth factor, can activate phosphatidylinositol 3-kinase (PI3K)/Akt through activation of receptor tyrosine kinase and thereby associate the mammalian target of rapamycin (mTOR) pathway.

The mTOR protein kinase has emerged as a critical player for controlling many cellular processes, such as cell growth and cell division, by receiving stimulatory signals from Ras and PI3K downstream from growth factors (5). mTOR regulates translation rates and cell proliferation in part by phosphorylating two major targets, the eukaryotic translation initiation factor 4E (eIF4E)–binding protein 1 (4E-BP1) and the ribosomal protein S6 kinases (S6K1 and S6K2). Upon phosphorylation, 4E-BP1 releases from eIF4E, allowing eIF4E to assemble with other translation initiation factors to initiate cap-dependent translation. eIF4E is thought to enhance the translation of transcripts possessing either complex 5'-untranslated region secondary structure and/or upstream open reading frames, which often encode proteins associated with a proliferative response. S6K1 directly phosphorylates the 40S ribosomal protein S6, and then promote ribosome biogenesis (6). Recent studies have shown that S6K and 4E-BP1 regulated by mTOR are required for cell motility, (7) and S6K, a downstream target of the Akt/mTOR pathway, has been shown to inhibit the PI3K/Akt pathway through a negative feedback mechanism (8–13).

mTOR exists in two distinct complexes (mTORC1 and mTORC2) within the cells: mTORC1 consists of mTOR, G $\beta$ L, raptor, and PRAS40, and mTORC2 contains mTOR, G $\beta$ L, rictor, and SIN1. The raptor-containing complex is sensitive to rapamycin and regulates cell growth and proliferation in part through phosphorylating S6K and 4E-BP1. The rictor-containing complex is not sensitive to rapamycin (14–16). Rapamycin, a specific mTOR inhibitor, interacts with FK506-binding protein 12 (FKBP-12) and subsequently binds to mTOR at a FKBP-12–rapamycin binding domain, resulting in inhibiting the interaction of mTOR with its substrate (17). Rapamycin and its analogues strongly inhibit cell proliferation and induce apoptosis in many tumor cell lines (18, 19), and are known to increase the survival of patients in limited clinical trials (18). However, recent studies have shown that the inhibition of mTOR by rapamycin could lead to the activation of Akt resulting from abrogating feedback inhibition mediated by constitutively activated mTOR, which is likely to attenuate the therapeutic effects of mTOR inhibitors (20–23). These results suggested that mTOR is a target for cancer therapy; however, novel mTOR inhibitors must be developed that will not only inhibit the mTOR pathway but will not activate Akt.

3,3'-Diindolylmethane (DIM), a dimeric product of indole-3-carbinol from cruciferous vegetables, has been shown to inhibit cell growth and induce apoptosis in human prostate cancer cells (24, 25). We have shown that DIM mediates its biological activity

**Note:** D. Kong and S. Banerjee contributed equally to this work.

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via inhibiting PI3K activity and Akt activation (25, 26). However, there is no report showing whether B-DIM-induced inhibition of invasion and angiogenesis could be mediated through down-regulation of the mTOR pathway. In this study, we show that PDGF-D overexpression leads to an increase in mTOR activity and inactivation of Akt through a negative feedback mechanism. The activation of mTOR relays PDGF-D-mediated oncogenic signaling to its downstream targets, S6K and 4E-BP1, to control cell growth, invasion, and angiogenesis. Rapamycin, an inhibitor of mTOR, has been shown to inactivate mTOR signaling but activate Akt, whereas B-DIM treatment inhibits cell growth, invasion, and mTOR activation in PC3 PDGF-D cells without activation of Akt. Moreover, conditioned medium (CM) from PC3 PDGF-D cells treated with B-DIM reduces the tube formation of human umbilical vein endothelial cells (HUVEC). Therefore, we suggest that B-DIM could serve as a novel and efficient chemopreventive and/or therapeutic agent by inhibiting both mTOR and Akt activity, resulting in the inhibition of invasion and angiogenesis in PDGF-D-overexpressing prostate cancer.

## Materials and Methods

**Cell lines and culture.** Prostate cancer cell line PC3 cells and resultant transfected cell lines were maintained in RPMI 1640 (Invitrogen) supplemented with 5% fetal bovine serum (FBS), 2 mmol/L glutamine, 10  $\mu$ mol/L HEPES, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin. All cells were cultured in a 5% CO<sub>2</sub>-humidified atmosphere at 37°C.

**Generation of stable cell lines overexpressing PDGF-D.** Establishment of PDGF-D-overexpressing PC3 cell lines were previously described (27). Briefly, transfection of PC3 cells with pcDNA3-PDGF-D:His and the corresponding empty vector pcDNA3 Neo selected with Geneticin and survived cells were pooled together to rule out artifacts and referred to as PC3 PDGF-D or PC3 Neo.

**Reagent and antibody.** BR-DIM, hereafter termed as B-DIM, a formulated DIM with higher bioavailability, was kindly provided by Dr. Michael Zeligs (BioResponse, Boulder, CO) and was dissolved in DMSO to make 50 mmol/L stock solutions and stored at -20°C in multiple aliquots. Antibody against human PDGF-D was purchased from Invitrogen. Antibody against mTOR, phospho-mTOR (Ser<sup>2448</sup>), p70S6K, phospho-p70S6K (Thr<sup>389</sup>), 4E-BP1, phospho-4E-BP1 (Thr<sup>37</sup>/Thr<sup>46</sup>), Akt, phospho-Akt (Ser<sup>473</sup>), and raptor were purchased from Cell Signaling Technology. The monoclonal antibody to  $\beta$ -actin and PI3K inhibitor, LY294002, were purchased from Sigma-Aldrich. Antibody against human Bcl-2 was from Dako North America, Inc. Rapamycin was obtained from EMB Biosciences, Inc.

**Small interfering RNA and transfection.** PC3 PDGF-D cells were transfected with mTOR, raptor, or control siRNA (100 nmol/L, Santa Cruz Biotechnology) using DharmaFECT3 small interfering RNA (siRNA) transfection reagent (Dharmacon). The medium was removed after 24 hours of transfection and the cells were incubated in serum-free medium for 24 h. The culture medium was collected, centrifuged to remove cellular debris, and stored at -70°C for tube formation assay; the cells were then collected for invasion assay or cell lysates were prepared for Western blot analysis.

**Invasion assay.** Cell invasion was determined using BD BioCoat Tumor Invasion Assay System (BD Bioscience) according to the instruction of the manufacturer. Briefly, PC3 Neo and PC3 PDGF-D cells suspended in serum-free medium containing 10 and 25  $\mu$ mol/L of B-DIM or DMSO and PC3 PDGF-D cells transfected with mTOR, raptor, or control siRNA suspended in serum-free medium were seeded into the upper chamber of the system. Bottom wells were filled with complete medium. After a 24-hour incubation, the cells were stained with 4  $\mu$ g/mL Calcein AM in PBS at 37°C for 1 hour. The fluorescently labeled cells were photographed under a fluorescence microscope. The fluorescence of the invaded cells was read in ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 485/530 nm.

**Matrigel *in vitro* HUVEC tube formation assay.** PC3 Neo and PC3 PDGF-D cells cultured in RPMI 1640 containing 0.5% FBS were treated with B-DIM or DMSO for 48 hours. PC3 PDGF-D cells were transfected with mTOR, raptor, or control siRNA. CM was collected, centrifuged, transferred to fresh tubes, and stored at -70°C. HUVEC tube formation assay was performed as described previously (28).

**Western blot assay.** PC3 Neo and PC3 PDGF-D cells were seeded and incubated for 24 hours in 5% FBS. Cells were washed with PBS and treated with B-DIM in 5% FBS for 48 h. CM was collected from the culture. Cell lysates from different experiments were obtained by scraping the cells from the dishes and washed twice with cold PBS. The cell pellet was suspended in 125 mmol/L Tris-HCl (pH 6.8), sonicated for 10 seconds, and an equal volume of 4% SDS was added. The lysates were boiled for 10 minutes. Protein concentration was determined using BCA protein assay (Pierce). Western blot analysis was performed using CM or cell lysates as described previously (28).

**Cell proliferation studies by WST-1 assay.** PC3 Neo and PC3 PDGF-D cells were seeded in 96-well plates and incubated for 24, 48, 72 hours. Alternatively, PC3 Neo and PC3 PDGF-D cells were seeded in 96-well plates and incubated for 24 hours; the cells were treated with 10 and 25  $\mu$ mol/L B-DIM or 1, 10, and 100 nmol/L rapamycin for 72 hours. Control cells were treated with DMSO as a vehicle control. After incubation, the cells were incubated with cell proliferation reagent WST-1 (Roche Applied Science) in medium for 4 hours at 37°C and 5% CO<sub>2</sub>. The spectrophotometric absorbance was determined by using Ultra Multifunctional Microplate Reader (Tecan) at 450/595 nm.

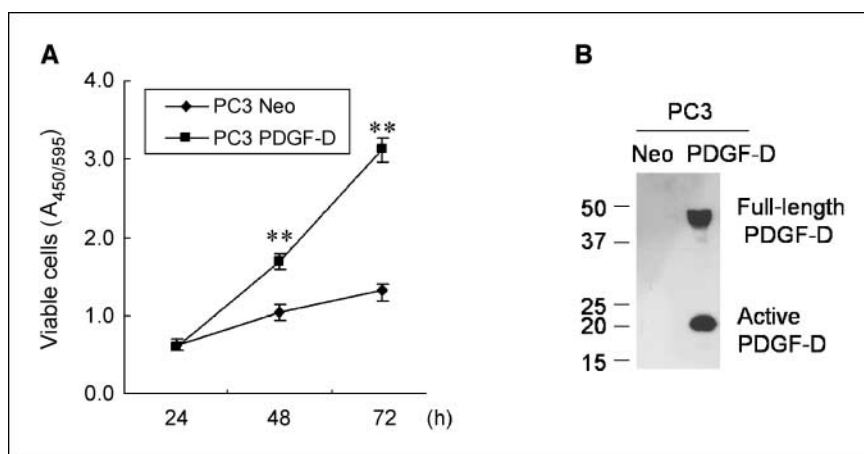
**Data analysis.** Experiments presented in the figures are representative of three or more different repetitions. The data are presented as the mean values  $\pm$  SE. Comparisons between groups were evaluated by a two-tailed Student's *t* test. *P* values <0.05 were considered to be statistically significant.

## Results

**PDGF-D significantly increases PC3 cell proliferation.** PDGF-D is a potent stimulator of cell proliferation, chemotaxis, and transformation. To assess the mitogenic activity of PDGF-D, we examined the growth rates of PC3 Neo and PC3 PDGF-D cells under normal growth conditions. PC3 PDGF-D exhibited an increase of 1.6- and 2.4-fold compared with PC3 Neo after 48 and 72 hours of incubation, respectively (Fig. 1A). Figure 1B shows that PDGF-D expression and active form of PDGF-D in PC3 PDGF-D cells were higher than PC3 Neo (negligible or not at detection level). These results show that exposure of PC3 cells to PDGF-D accelerates cell proliferation. Because growth factor is known to activate the mTOR pathway to control cell proliferation and survival, we investigated the state of mTOR and cell survival factor in PC3 PDGF-D cells.

**PDGF-D markedly up-regulates the mTOR pathway, enhances survival factor Bcl-2, and results in inactivation of Akt.** Because PDGF-D significantly increases PC3 cell proliferation, and growth factors could activate the mTOR pathway via activation of PI3K/Akt, resulting in stimulation of cell growth and proliferation, we tested whether the mTOR pathway is activated in PDGF-D-overexpressing PC3 cells. To assess the activity of the mTOR pathway, we detected phosphorylated mTOR (p-mTOR) and its two downstream targets phosphorylated 4E-BP1 (p-4E-BP1) and phosphorylated S6K (p-S6K). We found that PDGF-D markedly increased the levels of p-mTOR, p-4E-BP1, and p-S6K. We also found that Bcl-2, a main prosurvival factor, was significantly enhanced in PC3 PDGF-D cells (Fig. 2A and B). Moreover, PDGF-D also increased the activity of mTOR signaling in PDGF-D-overexpressing LNCaP cells (data not shown). These results suggest that the mTOR pathway and Bcl-2 could contribute to prostate cancer cell proliferation induced by prolonged stimulation via activation of PDGF-D signaling.

**Figure 1.** PDGF-D promotes proliferation of PC3 cells. **A**, PC3 Neo and PC3 PDGF-D cells were seeded in 96-well plates in 5% FBS and incubated for 24, 48, and 72 h. The cells were incubated with cell proliferation reagent WST-1 in medium for 4 h at 37°C and 5% CO<sub>2</sub>. The spectrophotometric absorbance was determined by using Ultra Multifunctional Microplate Reader at 450/595 nm. *Points*, mean (*n* = 6); *bars*, SE. \*\*, *P* < 0.01 compared with control. **B**, full-length and active forms of PDGF-D in the culture medium from PC3 Neo and PC3 PDGF-D cells incubated in medium containing 5% FBS for 48 h.

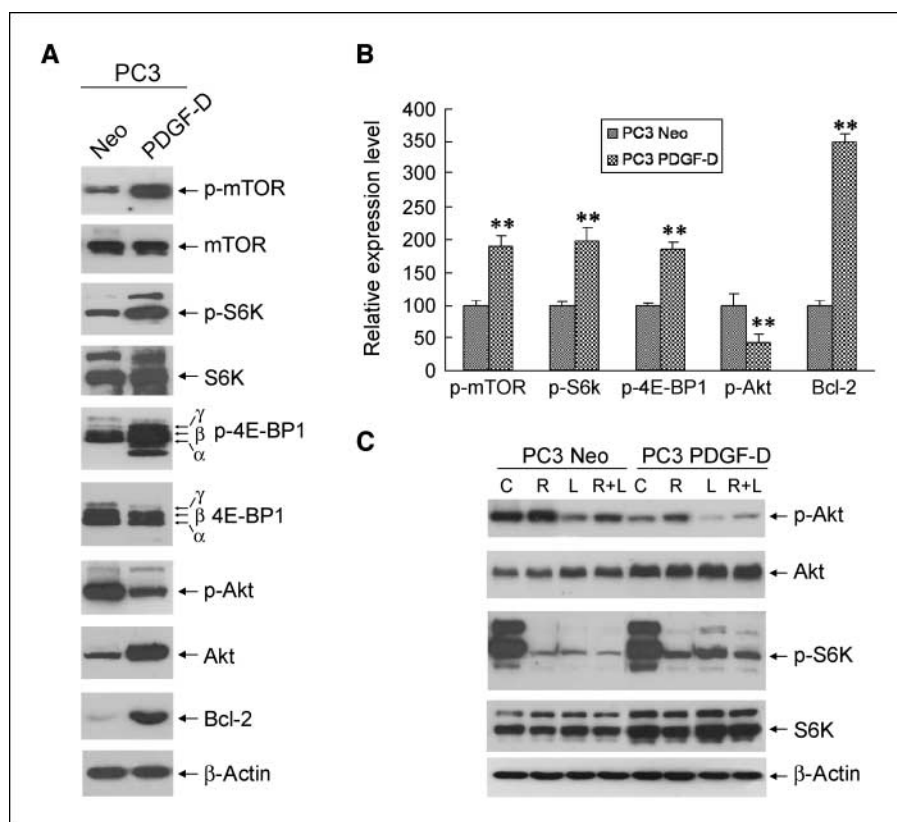


The serine/threonine protein kinase Akt is a major upstream regulator of mTOR. We sought to determine whether Akt activity was altered in PDGF-D-overexpressing PC3 cells. Interestingly, we found that p-Akt was reduced in PDGF-D-overexpressing PC3 cells in higher passages (from passages 10 to 30; Fig. 2A and B) but not in earlier passage (passage 7, data not shown). Because the total Akt expression was up-regulated in PDGF-D-overexpressing PC3 cells, we tested whether the inhibition of Akt activity was due to the activation/hyperactivation of the mTOR pathway in PC3 PDGF-D cells. For this experiment, we tested the effects of rapamycin, a mTOR inhibitor, on Akt phosphorylation. Rapamycin at 10 nmol/L increased phosphorylation of Akt in PC3 Neo and especially in PC3 PDGF-D cells, suggesting that inactivation of Akt is attributed to a negative feedback regulation mediated by the mTOR pathway

(Fig. 2C). PI3K is an upstream regulator of Akt; thus, we sought to determine whether the rapamycin-induced activation of Akt was dependent on PI3K. As shown in Fig. 2C, LY294002, a PI3K inhibitor, reversed the rapamycin-induced activation of Akt in PC3 Neo and especially in PC3 PDGF-D cells, suggesting that prolonged exposure of cells to PDGF-D activates the mTOR pathway, which, in turn, represses Akt activity in PC3 PDGF-D cells through a PI3K-dependent manner. These results suggest that prolonged exposure to PDGF-D-induced hyperactivation of mTOR is responsible for inactivation of Akt.

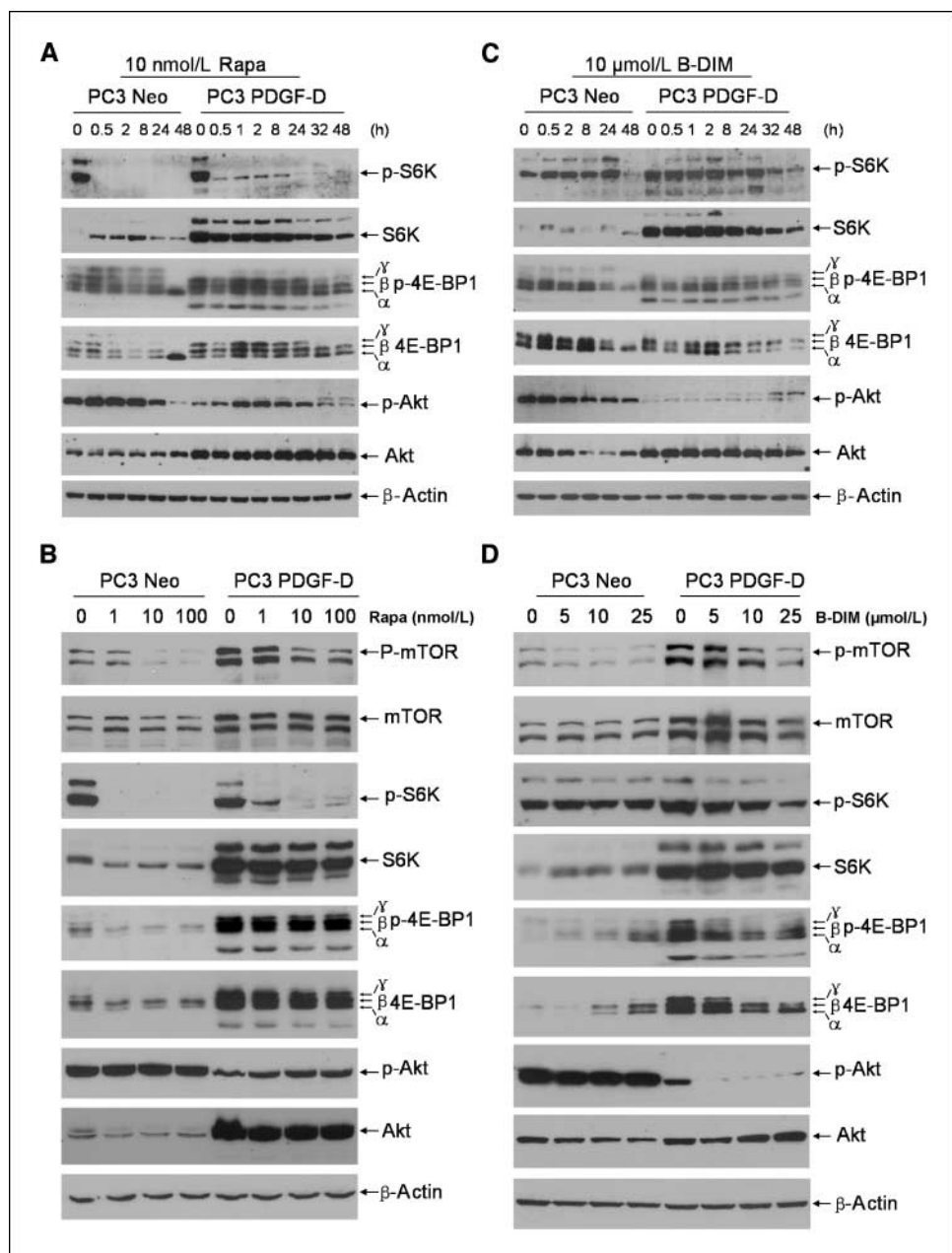
**Rapamycin inhibits mTOR activity and induces activation of Akt, and B-DIM represses the mTOR pathway without activation of Akt in PDGF-D-overexpressing PC3 cells.** Rapamycin, a specific inhibitor of mTOR, is thought to exert its effect on

**Figure 2.** PDGF-D induces activation of mTOR pathway and survival factor Bcl-2 expression concomitant with inactivation of Akt. **A**, PC3 Neo and PC3 PDGF-D cells were seeded in 100-mm plates in 5% FBS. After 48 h, cell lysates were prepared and equal amounts of proteins were subjected to gel electrophoresis. Western blot analysis was performed using antibody against phospho-mTOR (Ser<sup>2448</sup>), mTOR, phospho-p70S6K (Thr<sup>389</sup>), S6K, phospho-4E-BP1 (Thr<sup>37</sup>/Thr<sup>46</sup>), 4E-BP1, phospho-Akt (Ser<sup>473</sup>), Akt, and Bcl-2.  $\beta$ -Actin protein was used as loading control. **B**, quantitative analysis of expression of phospho-mTOR, phospho-p70S6K, phospho-4E-BP1, phospho-Akt, and Bcl-2 in PC3 Neo and PC3 PDGF-D cells was performed and relative density of bands was normalized to  $\beta$ -actin (PC3 Neo with assigned value of 100%). *Columns*, mean (*n* = 4); *bars*, SE. \*\*, *P* < 0.01 compared with PC3 Neo. **C**, PC3 Neo and PC3 PDGF-D cells were seeded and incubated for 24 h. Cells were treated with 10 nmol/L rapamycin (R), 10  $\mu$ mol/L LY294002 (L), 10 nmol/L rapamycin combined with 10  $\mu$ mol/L LY294002 (R+L), or DMSO control (C) for 8 h. Following incubation, cell lysates were prepared and equal amounts of proteins were subjected to gel electrophoresis. Western blot analysis was performed using antibody against phospho-Akt, Akt, phospho-p70S6K (Thr<sup>389</sup>), and p70S6K.  $\beta$ -Actin protein was used as loading control.



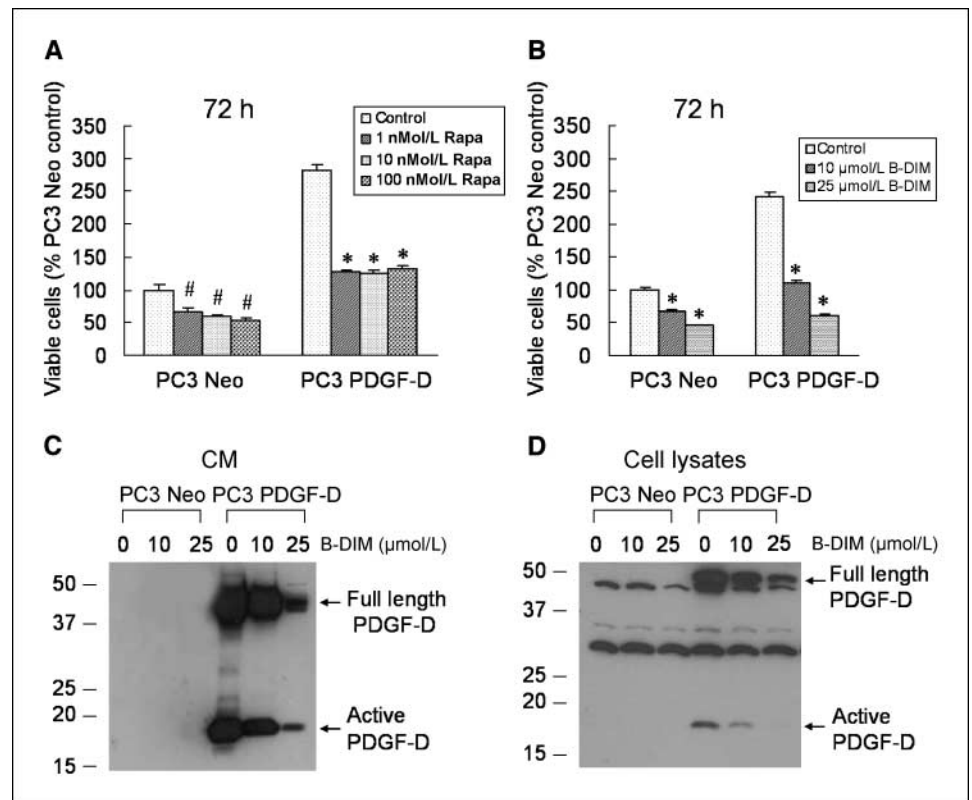
mTOR activity by inhibiting the interaction of mTOR with its substrate. In this study, we have shown that rapamycin inhibited p-S6K and p-4E-BP1 in PC3 Neo and especially in PC3 PDGF-D cells. However, rapamycin resulted in activation of Akt in PC3 Neo and PC3 PDGF-D cells (Fig. 3A and B). Interestingly, rapamycin treatment down-regulated phosphorylation of mTOR at site Ser<sup>2448</sup> in PC3 Neo and PC3 PDGF-D cells (Fig. 3B). These results support previous studies showing that mTOR is one of the substrates for S6K, and phosphorylation of mTOR at Ser<sup>2448</sup> is mediated by S6K (29, 30). Thus, rapamycin inhibits S6K1 activity, which, in turn, reduces the phosphorylation of mTOR at Ser<sup>2448</sup>. These results suggest that mTOR is a target for cancer therapy. However, the activation of Akt mediated by rapamycin treatment is likely to attenuate the therapeutic effects of mTOR inhibitors, suggesting that novel mTOR inhibitors must be developed that will not only inhibit the mTOR pathway but will also not activate Akt.

DIM has been shown to inhibit the growth of tumor cells through induction of apoptosis and G<sub>1</sub> cell cycle arrest in human prostate cancer cells (25). We sought to determine whether B-DIM inhibits the mTOR pathway. In this study, we found that B-DIM treatment inhibited phosphorylation of 4E-BP1 and S6K in PC3 PDGF-D cells (Fig. 3C and D). As shown above, rapamycin treatment led to enhanced PI3K/Akt activation via a negative feedback regulation by the mTOR pathway, suggesting that the activation of the PI3K/Akt pathway by a mTOR inhibitor could in fact make the tumor more aggressive. Therefore, we sought to examine whether B-DIM induces activation of Akt. To this end, we tested the effects of B-DIM on phosphorylation of Akt. B-DIM dramatically reduced phosphorylation of 4E-BP1 and S6K in PC3 PDGF-D cells concomitant with inactivation of Akt (Fig. 3C and D). Moreover, B-DIM also inhibited the phosphorylation of mTOR at Ser<sup>2448</sup> similar to those observed by rapamycin (Fig. 3D).



**Figure 3.** B-DIM and rapamycin inhibits mTOR pathway, and rapamycin but not B-DIM results in activation of Akt in PC3 PDGF-D cells. PC3 Neo and PC3 PDGF-D cells were seeded in 100-mm plates in 5% FBS. After 24 h, cells were treated with 10 nmol/L rapamycin for 0.5 to 48 h (A) or treated with 0, 10, and 100 nmol/L rapamycin for 8 h (B). Cells were seeded as above and treated with 10 μmol/L B-DIM for 0.5 to 48 h (C) or treated with 0, 10, and 25 μmol/L B-DIM for 16 h (D). Cell lysates were prepared and equal amounts of proteins were subjected to gel electrophoresis. Western blot analysis was performed using antibody against mTOR, phospho-mTOR (Ser<sup>2448</sup>), p70S6K, phospho-p70S6K (Thr<sup>389</sup>), 4E-BP1, phospho-4E-BP1 (Thr<sup>37</sup>/Thr<sup>46</sup>), Akt, and phospho-Akt (Ser<sup>473</sup>). β-Actin protein was used as loading control.

**Figure 4.** B-DIM is more effective than rapamycin in inhibiting cell proliferation, and B-DIM reduces full-length and active forms of PDGF-D. PC3 Neo and PC3 PDGF-D cells were seeded in 96-well plates. After 24 h, the cells were treated with 0, 1, 10, and 100 nmol/L rapamycin (A), or treated with 0, 10, and 25  $\mu\text{mol/L}$  B-DIM (B) for 72 h. After treatment, the cells were incubated with cell proliferation reagent WST-1 in medium for 4 h at 37°C and 5% CO<sub>2</sub>. The spectrophotometric absorbance was determined by using Ultra Multifunctional Microplate Reader at 450/595 nm. Viable cells were calculated relative to the PC3 Neo control with assigned value of 100% ( $n = 6$ ; #,  $P < 0.05$  and \*,  $P < 0.01$  compared with PC3 Neo or PC3 PDGF-D control). C and D, PC3 Neo and PC3 PDGF-D cells that were seeded in 100-mm plates in 5% FBS. After 24 h, cells were treated with 0, 10, and 25  $\mu\text{mol/L}$  B-DIM for 48 h in 0.5% FBS. CM was collected and cell lysates were prepared. CM and equal amounts of proteins were subjected to gel electrophoresis, and Western blot analysis was performed using antibody against PDGF-D.



**B-DIM inhibits cell growth partly via decreasing the full-length and active forms of PDGF-D.** Rapamycin has been shown to induce cell cycle arrest and inhibit cell proliferation. PDGF-D overexpression resulted in an increase in cell proliferation of PC3 cells through up-regulation of the mTOR pathway. Therefore, we speculated that rapamycin could be more effective in inhibiting cell proliferation of PC3 PDGF-D compared with PC3 Neo cells. We have determined the growth rate of PC3 Neo and PC3 PDGF-D cells in the presence of rapamycin and found that rapamycin significantly inhibited PC3 PDGF-D cell proliferation (Fig. 4A). On the other hand, B-DIM not only inhibited mTOR pathway activity but also repressed Akt activation in PDGF-D-overexpressing PC3 cells, which was consistent with the inhibition of cell growth showing that PC3 PDGF-D cells could be more sensitive to B-DIM treatment without activating Akt compared with rapamycin (Fig. 4B).

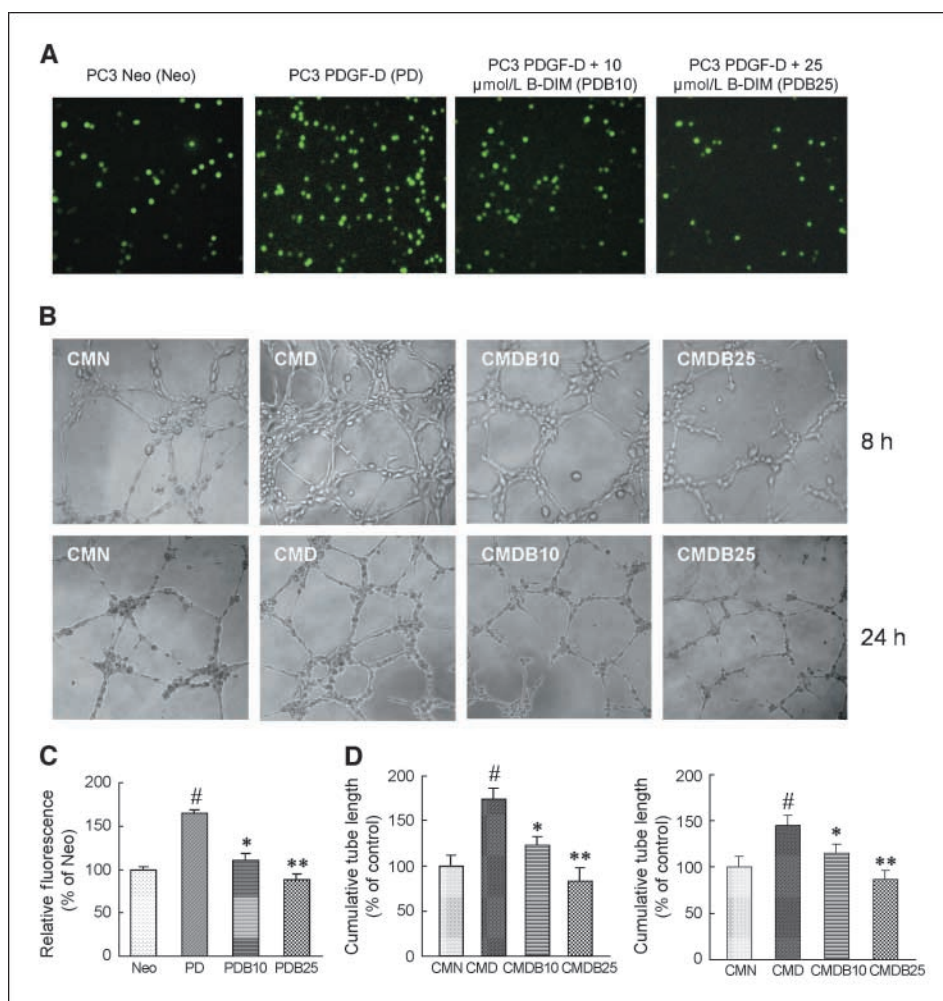
To determine whether the inhibition of cell proliferation of PC3 PDGF-D cells by B-DIM was partly due to inhibition in the expression and activation of PDGF-D, we have investigated to find the full-length and activated forms of PDGF-D in CM and cell lysates by Western blot analysis. We found that both 10 and 25  $\mu\text{mol/L}$  B-DIM markedly decreased the activated and the full-length forms of PDGF-D in the CM and in cell lysates (Fig. 4C and D). These results suggest that B-DIM-induced inhibition in cell proliferation of PC3 PDGF-D cells is partly mediated by reduced expression of PDGF-D as well as the activated form of PDGF-D.

**B-DIM inhibits invasion of PC3 PDGF-D cells.** The mTOR pathway not only controls cell growth and proliferation but also regulates cell invasion. Recent studies have shown that expression of constitutively active form of S6K is sufficient to induce invasion and migration in ovarian cancer cells (31). In this study, we found that overexpression of PDGF-D induced activation of mTOR pathway, resulting in an increase in S6K phosphorylation. Because

we found that B-DIM treatment inhibits active PDGF-D and phosphorylation of S6K, we speculated that B-DIM might inhibit cell invasion of PC3 PDGF-D cells. As expected, PDGF-D-overexpressing PC3 cells exhibited an increased invasion, whereas B-DIM treatment significantly inhibited invasion of PC3 PDGF-D cells (Fig. 5A and C).

**Reduced tube formation of HUVECs induced by CM from PC3 PDGF-D cells treated with B-DIM.** mTOR activation increases vascular endothelial growth factor expression that mediates angiogenesis (32), and rapamycin repressed tumor growth by antiangiogenesis (33). In this study, we found that B-DIM inhibited mTOR activation in PC3 PDGF-D-transfected cells. Therefore, we hypothesized that B-DIM might inhibit angiogenesis induced by mTOR activation. To test this hypothesis, we performed tube formation assay by incubating HUVECs with CM from PC3 Neo and PC3 PDGF-D cells treated with B-DIM or DMSO. CM from PC3 PDGF-D cells significantly increased tube formation of HUVECs compared with PC3 Neo. Importantly, CM from PC3 PDGF-D cells treated with 10 and 25  $\mu\text{mol/L}$  B-DIM reduced tube formation of HUVECs (Fig. 5B and D).

**Decreased invasion and angiogenesis of PC3 PDGF-D cells by knocking down of mTOR and raptor.** To confirm whether the mTOR pathway is responsible for increased invasion and angiogenesis of PC3 PDGF-D cells, we knocked down mTOR and raptor expression by siRNA with specific oligonucleotides (Santa Cruz Biotechnology). Knockdown of mTOR and raptor markedly repressed the invasion of PC3 PDGF-D cells (Fig. 6A and C, left). Moreover, CM from PC3 PDGF-D cells with knockdown of mTOR or raptor reduced tube formation of HUVECs (Fig. 6B and C, right). Figure 6D showed that transfection of PC3 PDGF-D cells with mTOR or raptor siRNA led to a significant decrease in mTOR and raptor expression, respectively, with concomitant reduction in the



**Figure 5.** B-DIM inhibits invasion of PC3 PDGF-D cells and CM from PC3 PDGF-D cells treated with B-DIM reduces tube formation of HUVECs. *A*, the effects of B-DIM treatment on cell invasion as determined by using BD BioCoat Tumor Invasion Assay System. Invasive cells were stained with 4  $\mu\text{g}/\text{mL}$  Calcein AM. B-DIM (10 and 25  $\mu\text{mol}/\text{L}$ ) significantly decreased invasion of PC3 PDGF-D cells. *B*, PC3 Neo and PC3 PDGF-D cells cultured in medium containing 0.5% FBS were treated with B-DIM or DMSO for 48 h. CM was collected for angiogenesis assay (tube formation by HUVECs). HUVECs were seeded in each well of the eight-well chamber coated with growth factor-reduced Matrigel. The chamber was incubated for 8 or 24 h. Each well was photographed using an inverted microscope with digital camera. Image analysis of tubule/capillary length was carried out using the software image analysis program Scion Image downloaded from the NIH Web site. *C*, value of relative fluorescence from the invaded cells. The value represents the comparative amount of invaded cells. *D*, image analysis of tubule/capillary length was carried out using software image analysis program Scion Image. Quantification of cumulative tube length of endothelial cells incubated for 8 h (*left*) and 24 h (*right*). *Columns*, mean; *bars*, SE;  $n = 4$ . #,  $P < 0.01$  compared with CMN. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared with CMN. CM, CM from PC3 Neo cells; CMD, CM from PC3 PDGF-D cells; CMDB10, CM from PC3 PDGF-D cells treated with 10  $\mu\text{mol}/\text{L}$  B-DIM; CMDB25, CM from PC3 PDGF-D cells treated with 25  $\mu\text{mol}/\text{L}$  B-DIM).

levels of p-S6K and p-4E-BP1. These results suggest that mTOR activation contributes to PC3 PDGF-D cell invasion and angiogenesis as determined by tube formation of HUVECs using CM from PC3 PDGF-D cells.

## Discussion

PDGF-D has been shown to be expressed in many tumor cell lines (4). Recent studies have shown that PDGF-D is expressed in prostate tumor tissues (27), suggesting that PDGF-D might play an important role in the development and progression of prostate cancer. However, the biological and mechanistic role of PDGF-D in tumorigenesis remains elusive. To examine whether PDGF-D plays any role in prostate cancer progression, PC3 cells were stably transfected with human full-length PDGF-D cDNA. We found that PC3 cells transfected with PDGF-D (PC3 PDGF-D cells) exhibit a rapid growth rate and increased invasion *in vitro*, which were associated with a high level of mTOR activity and increased Bcl-2 expression, but reduced activity of Akt. Moreover, CM from PDGF-D-overexpressing PC3 cells induced tube formation of HUVECs. However, the mechanism by which PDGF-D induced angiogenesis is unclear. Recent studies have shown that CM from Bcl-2-transfected human dermal microvascular endothelial cells is sufficient to induce neovascularization in the rat corneal assay and also found that the expression of proangiogenic chemokines interleukin-8 (CXCL8)

and growth-related oncogene- $\alpha$  (CXCL1) is significantly higher in Bcl-2-transfected cells than that in control (34). Anai et al. (35) found that knockdown of Bcl-2 by antisense oligodeoxynucleotides inhibited angiogenesis in human PC3 prostate tumor xenografts. In our study, Bcl-2 expression was significantly higher in PDGF-D-overexpressing PC3 cells compared with that in PC3 Neo cells. Thus, Bcl-2-induced proangiogenic factors could be responsible for HUVEC tube formation induced by CM from PC3 PDGF-D cells.

We also observed that PDGF-D existed in both full-length and active forms in the culture medium and cellular lysates of PC3 PDGF-D cells, suggesting that proteases capable of proteolytic activation of PDGF-D are expressed in PC3 cells. Taken together, these results suggested that activated PDGF-D could promote cell proliferation and invasion by activating mTOR pathway. Growth factor-mediated PI3K/Akt/mTOR pathway has been shown to control cell growth, proliferation, invasion, and angiogenesis by phosphorylating mTOR downstream targets S6K and 4E-BP1 (36). In this study, we found that PDGF-D-overexpressing PC3 cells exhibited growth advantage by activating mTOR downstream targets such as S6K and 4E-BP1 with concomitant inactivation of Akt. These results are consistent with recent studies showing that constitutively activated mTOR could result in reduced activation of PI3K/Akt through a negative feedback inhibition (8–13).

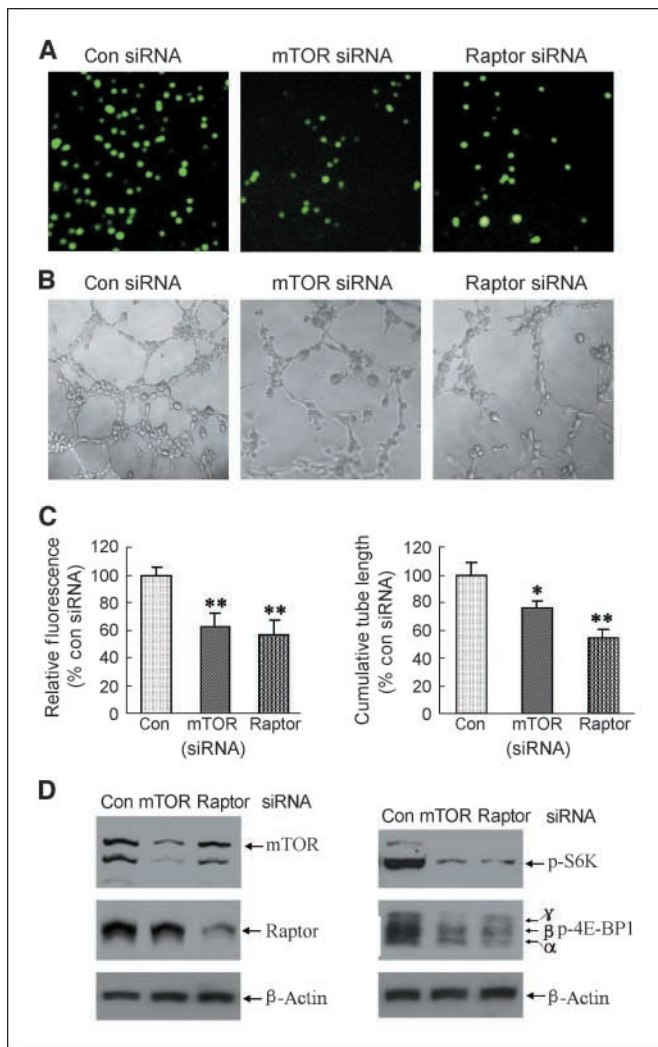
Akt is an upstream effector of mTOR pathway and activates mTOR by phosphorylating tuberous sclerosis complex 2 (TSC2), a

mTOR inhibitor. TSC1 and TSC2 form heterodimers with GTPase activity that inhibits the active Rheb, a small GTPase required for mTOR activation (37). A recent study has shown that activation of Akt mediated by serum or growth factors is inhibited when mTOR is activated in TSC1- or TSC2-deficient cells via a negative feedback regulation loop (12). This feedback inhibitory loop has been attributed to the inhibitory effect of S6K on IRS-1, which mediates PI3K/Akt activation by insulin-like growth factor-I and insulin (11, 12, 21, 23). However, little is known whether growth factors, except for insulin, could activate mTOR pathway that may be linked to inactivation of Akt through a negative feedback mechanism. In this report, we found that prolonged exposure of PC3 cells to PDGF-D led to hyperactivation of mTOR and consequently inactivated Akt. We also found that LY294002, a PI3K inhibitor,

prevented Akt activation induced by rapamycin, suggesting that PI3K activity is required for the negative feedback inhibition of Akt mediated by hyperactive mTOR pathway. Taken together, these results suggest that cancer cells secrete PDGF-D, which, in turn, activate mTOR pathway to promote tumor cell proliferation, invasion, and induce angiogenesis, and concomitantly desensitize cells to stimulation by these growth factors through inactivation of PI3K/Akt pathway. These results further suggest that the growth of tumors with higher levels of PDGF-D could be inhibited by inhibiting mTOR and its downstream targets.

Rapamycin, the bacterially derived macrolide ester, is clinically approved as an immunosuppressant and has been shown to be a promising antitumor agent. In this study, we found that rapamycin treatment significantly inhibited PC3 PDGF-D cell proliferation, whereas less inhibition of proliferation was observed in PC3 Neo cells. Concomitantly, rapamycin treatment dramatically reduced phosphorylation of S6K and 4E-BP1 but activated Akt during 24 hours of treatment. These results suggest that hyperactivation of the mTOR pathway was responsible for growth advantage and, in turn, Akt was inactivated through a negative feedback regulation in PC3 PDGF-D cells. Interestingly, rapamycin treatment not only repressed mTOR downstream targets S6K and 4E-BP1 phosphorylation but also suppressed mTOR phosphorylation at Ser<sup>2448</sup>. Recent studies have shown that mTOR is one of the substrates for S6K, and phosphorylation of mTOR at Ser<sup>2448</sup> is mediated by S6K (29, 30). Thus, S6K can regulate mTOR activity by a positive feedback regulation. It is known that phosphorylation site Ser<sup>2448</sup> in mTOR lies within a COOH-terminal regulatory region, which upon deletion results in elevated mTOR activity *in vitro* and in cells. Thus, this COOH-terminal regulatory region is known as a "repressor domain" and, as such, phosphorylation of Ser<sup>2448</sup> in mTOR results in enhanced mTOR activity. In the present study, rapamycin treatment led to the inactivation of S6K, which, in turn, resulted in the decreased phosphorylation of mTOR at Ser<sup>2448</sup>. These results strongly suggest that rapamycin could serve as a promising antitumor drug in many tumors with hyperactivated mTOR pathway. However, S6K is not only a positive regulator for mTOR but is also a negative factor for the PI3K/Akt pathway. Thus, inhibition of S6K by rapamycin could lead to the activation of Akt, resulting from abrogating feedback inhibition mediated by activated mTOR pathway, which is likely to attenuate the overall therapeutic effects of mTOR inhibitors, and further suggest that development of newer and novel agents are urgently needed.

DIM and B-DIM (a formulated DIM with higher bioavailability) have been shown to inhibit the cell proliferation, invasion, angiogenesis, and growth of tumors in human prostate cancer (24, 25, 28). In this study, we found that B-DIM significantly inhibited the proliferation and invasion of PC3 PDGF-D cells, which were associated with a remarkable inhibition of mTOR, S6K, and 4E-BP1 phosphorylation and reduction of PDGF-D expression and reduced active form of PDGF-D. Moreover, CM from PC3 PDGF-D cells treated with B-DIM inhibited angiogenesis (tube formation of HUVECs). More importantly, inhibition of mTOR pathway by B-DIM in PDGF-D-overexpressing PC3 cells did not result in activation of Akt. Recent studies have shown that prolonged rapamycin treatment could inhibit mTORC2 assembly and therefore Akt activation (38, 39). In this study, we found that rapamycin treatment resulted in the activation of Akt. However, B-DIM not only inhibited mTOR but also inhibited Akt, which could be due to perturbation of mTORC2 assembly.



**Figure 6.** Knockdown of mTOR and raptor reduces invasion of PC3 PDGF-D cells, and CM from PC3 PDGF-D with knockdown of mTOR or raptor decreases the tube formation of HUVECs. *A*, PC3 PDGF-D cells were transfected with mTOR, raptor, or control (Con) siRNA. After 48 h of transfection, the cells were collected for invasion assay. *B*, cells were treated as above. CM was collected for tube formation assay. *C*, values of relative fluorescence from the invaded cells represents the comparative amount of invaded cells (left). Quantification of cumulative tube length of endothelial cells incubated for 8 h (right). Columns, mean ( $n = 4$ ); bars, SE. \*,  $P < 0.05$ , \*\*,  $P < 0.01$  compared with control. *D*, Western blot analysis showed expression levels of mTOR, raptor, p-S6K, and p-4E-BP1 in PDGF-D cells transfected with mTOR, raptor, or control siRNA.  $\beta$ -Actin protein was used as loading control.

In summary, we found that PDGF-D induced cell proliferation and invasion of PC3 cells, and that the CM from PC3 PDGF-D cells significantly increased angiogenesis (tube formation of HUVECs), which are likely mediated by hyperactivation of mTOR. Rapamycin inhibited the proliferation of PC3 PDGF-D cells and repressed the activity of the mTOR pathway but induced activation of Akt. In contrast, B-DIM strongly suppressed the proliferation and invasion of PC3 PDGF-D cells and inhibited mTOR activity without activation of Akt in PDGF-D-overexpressing PC3 cells, which was partly due to reduced levels of the full-length form as well as the active form of PDGF-D. Moreover, CM from PC3 PDGF-D cells treated with B-DIM decreased angiogenesis (tube formation of

HUVECs), suggesting that B-DIM could be a better promising agent that could be useful for the prevention and/or treatment of prostate cancer due to B-DIM-mediated down-regulation of PDGF-D signaling and inhibition of mTOR and Akt activity.

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## Mammalian Target of Rapamycin Repression by 3,3'-Diindolylmethane Inhibits Invasion and Angiogenesis in Platelet-Derived Growth Factor-D–Overexpressing PC3 Cells

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