Decursin Suppresses Human Androgen-Independent PC3 Prostate Cancer Cell Proliferation by Promoting the Degradation of β-Catenin

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ABSTRACT

Alterations in the Wnt/β-catenin pathway are associated with the development and progression of human prostate cancer. Decursin, a pyranocoumarin isolated from the Korean Angelica gigas root, inhibits the growth of androgen-independent human prostate cancer cells, but little is known about its mechanism of action. Using a cell-based screen, we found that decursin attenuates the Wnt/β-catenin pathway. Decursin antagonized β-catenin response transcription (CRT), which was induced with Wnt3a-conditioned medium and LiCl, by promoting the degradation of β-catenin. Furthermore, decursin suppressed the expression of cyclin D1 and c-myc, which are downstream target genes of β-catenin and thus inhibited the growth of PC3 prostate cancer cells. In contrast, decursinol, in which the (CH3)2–C=CH–COO– side chain of decursin is replaced with –OH, had no effect on CRT, the level of intracellular β-catenin, or PC3 cell proliferation. Our findings suggest that decursin exerts its anticancer activity in prostate cancer cells via inhibition of the Wnt/β-catenin pathway.

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Materials and Methods

Preparation of Decursin and Decursinol. (+)-Decursinol was prepared from the roots of *Angelica gigas* Nakai, which has been traditionally used to treat anemia and other diseases (Chi and Kim, 1970). Decursin has a cytoxic effect on leukemia cells through a mechanism involving protein kinase C and reactive oxygen species, and it has antitumor activity in mice inoculated with sarcoma cells (Lee et al., 2003b; Kim et al., 2005). It has been reported that decursin suppresses growth and promotes apoptosis in human prostate carcinoma cells (Yim et al., 2005; Jiang et al., 2006). Moreover, the (CH3)2–C=CH–COO– side chain of decursin, which is replaced with an OH group in decursinol, seems to be necessary for its anticancer activity (Yim et al., 2005; Guo et al., 2007). In the present study, we identified decursin as an inhibitor of the Wnt/β-catenin pathway using cell-based small-molecule screening. Decursin may inhibit the growth of prostate cancer cells by promoting the degradation of intracellular β-catenin.

Plasmid Constructs. Human Frizzled-1 cDNA was cloned as described previously (Cho et al., 2005). Reporter plasmids containing the cyclin D1 promoters were prepared by amplifying the promoter regions, which harbored TCF-4 response genes, by polymerase chain reaction and insertion into pRL-null vectors, yielding pcyclinD1-RL. The pTOPFlash and pFOPFlash reporter plasmids were obtained from Upstate Biotechnology (Lake Placid, NY). The dominant-negative β-TrCP (Δβ-TrCP) expression plasmid was a gift from M. Davis (Hebrew University-Hadassah Medical School, Jerusalem, Israel). pCMV-RL and pSV-FL plasmids were purchased from Promega.

Screening for a Small-Molecule Inhibitor of Wnt/β-Catenin Signaling. The HEK293 reporter and control cell line was established as described previously (Park et al., 2006). The cells were inoculated into 96-well plates at 15,000 cells/well in duplicate and grown for 24 h. Wnt3a CM was added, and then chemicals including coumarins, flavonoids, naphthoquinones, and terpenoids were added to the wells at a final concentration of 30 μg/ml. After 15 h, the plates were assayed for firefly luciferase activity and cell viability.

Western Blotting. The cytosolic fraction was prepared as described previously (Dignam et al., 1983). Proteins were separated by SDS-polyacrylamide gel electrophoresis in a 4 to 12% gradient gel (Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked with 5% nonfat milk and probed with anti-β-catenin (BD Transduction Laboratories, Lexington, KY), anti-cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-myc (Santa Cruz Biotechnology), and anti-actin antibodies (Cell Signaling Technology, Danvers, MA). The membranes were then incubated with horseradish-peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology) and visualized using the enhanced chemiluminescence system (Santa Cruz Biotechnology).

RNA Extraction and Semiquantitative RT-PCR. Total RNA was isolated with TRIzol reagent (Invitrogen) in accordance with the manufacturer’s instructions. cDNA synthesis, reverse transcription, and polymerase chain reaction were performed as described previously (Park et al., 2006). The amplified DNA was separated on 2% agarose gels and stained with ethidium bromide.

Cell Viability Assay. Cells were inoculated into 96-well plates and treated with decursin for 48 h. The cell viability from each treated sample was measured in triplicate using CellTiter-Glo assay kit (Promega) according to the manufacturer’s instructions.

Results

Identification of Decursin as an Inhibitor of the Wnt/β-Catenin Pathway. To identify cell-permeable small molecules capable of inhibiting the Wnt/β-catenin pathway, we used a cell-based screening system. HEK293 reporter cells that are stably transfected with TOPFlash, a synthetic β-catenin/Tcf-dependent luciferase reporter, and human Frizzled-1 expression plasmids were dispensed into a 96-well plate. After the addition of Wnt3a CM and each compound, we monitored TOPFlash reporter activity using a microplate reader (Fig. 1A). One of the compounds identified from this screen was decursin (Fig. 1B). As shown in Fig. 1C, decursin produced a dose-dependent decrease in Wnt3a CM-induced β-catenin response transcription (CRT); approximately 80% of CRT was blocked at a concentration of 200 μM decursin. In contrast, activity of FOPFlash, a negative control reporter with mutated β-catenin/Tcf binding elements, was not altered by incubation with decursin and Wnt3a CM (Fig. 1C). Under these conditions, no cytotoxic effects of decursin were observed (data not shown). These results suggest that decursin specifically inhibits the Wnt/β-catenin pathway.

Decursin Promoted Proteasome-Mediated β-Catenin Degradation. In the Wnt/β-catenin pathway, CRT is largely controlled by the level of intracellular β-catenin, which regulates the expression of several target genes. To determine whether decursin affects the intracellular level of β-catenin, we used Western blot analysis with anti-β-catenin antibody to determine the amount of cytosolic β-catenin in decursin-
treated HEK293 reporter cells. As shown in Fig. 2A, incubation with decursin resulted in down-regulation of the β-catenin that had accumulated in the presence of Wnt3a. We next examined whether decursin reduces the mRNA expression of β-catenin in HEK293 reporter cells using semiquantitative RT-PCR. In contrast to the protein level of β-catenin, mRNA expression of β-catenin was unchanged by any of the concentrations of decursin used, suggesting that decursin inhibits Wnt/β-catenin signaling by reducing β-catenin protein level without affecting β-catenin mRNA expression (Fig. 2B). It was demonstrated previously that the intracellular level of β-catenin is regulated by ubiquitin-dependent proteolysis (Aberle et al., 1997). To explore whether the decursin-induced down-regulation of β-catenin is mediated by the proteasome, we used MG-132 to inhibit proteasome-mediated protein degradation. Decursin induced a consistent decrease in the level of β-catenin in HEK293 reporter cells (Fig. 2C); however, the effect of decursin was nullified by the addition of MG-132 (Fig. 2C). Taken together, these results indicate that decursin inhibits the Wnt/β-catenin pathway via proteasome-mediated degradation of β-catenin.

β-TrCP but Not GSK-3β Was Necessary for Decursin-Mediated Degradation of β-Catenin. Because the phosphorylation of β-catenin by GSK-3β and its subsequent association with β-TrCP leads to β-catenin degradation (Winston et al., 1999), we examined whether decursin-mediated inhibition of the Wnt/β-catenin pathway requires GSK-3β activity. To this end, HEK293 reporter cells were incubated with LiCl, an inhibitor of GSK-3β (Klein and Melton, 1996), resulting in increased CRT (Fig. 3A). It is interesting that decursin suppressed LiCl-induced CRT (Fig. 3A). Furthermore, Western blot analysis showed that decursin reduced the amount of β-catenin induced by LiCl (Fig. 3B), indicating that decursin-mediated inhibition of the Wnt/β-catenin pathway is independent of GSK-3β. We also tested whether β-TrCP is required for decursin-induced degradation of β-catenin. As shown in Fig. 3C, ectopic expression of a dominant-negative form of β-TrCP (β-TrCP), which inter-

![Fig. 1. Identification of decursin as a small-molecule inhibitor of Wnt/β-catenin pathway. A, screening of compounds that inhibit Wnt/β-catenin pathway. Compounds modulating TOPFlash reporter activity were screened using the HEK293 reporter cells. The controls were assayed in the presence or absence of Wnt3a CM. B, chemical structure of decursin. C, dose-dependent inhibition of CRT. HEK293 reporter and control cells were incubated with indicated concentrations of decursin in the presence of Wnt3a CM. After 15 h, luciferase activity was determined. TOPFlash activity is reported as relative light unit (RLU) normalized to CellTiter-Glo (Promega) activity. The results are the average of three experiments, and the bars indicate standard deviations. *P < 0.05, and **P < 0.01, compared with the vehicle control group.](molpharm.aspetjournals.org)

![Fig. 2. Decursin promotes the degradation of β-catenin via a proteasome. A, cytosolic proteins were prepared from HEK293 reporter cells treated with either vehicle (DMSO) or indicated concentrations of decursin in the presence of Wnt3a CM for 15 h and then subjected to Western blotting with β-catenin antibody. B, semiquantitative RT-PCR for β-catenin, and glyceraldehydes-3-phosphate dehydrogenase was performed with total RNA prepared from HEK293 reporter cells either vehicle (DMSO) or indicated concentrations of decursin in the presence of Wnt3a CM for 15 h. C, cytosolic proteins prepared from HEK293 reporter cells, which were incubated with vehicle (DMSO) or decursin (100 μM) in the presence or absence of Wnt3a CM, exposed to MG-132 (20 μM) for 8 h, were subjected to Western blotting with anti-β-catenin antibody. In A and C, to confirm equal loading, the blot was reprobed with anti-actin antibody.](molpharm.aspetjournals.org)
acts with phosphorylated β-catenin but is unable to form a SCFβ-TrCP ubiquitin ligase complex (Hart et al., 1999), abolished the decursin-mediated degradation of β-catenin. These results suggest that decursin promotes the degradation of β-catenin via a β-TrCP-dependent mechanism.

**Decursin Attenuated the Wnt/β-Catenin Pathway in Prostate Cancer Cells.** Because decursin was identified recently as a chemopreventive agent against prostate carcinoma cells (Yim et al., 2005; Jiang et al., 2006), we tested whether decursin also suppresses the Wnt/β-catenin pathway in prostate cancer cells, which frequently show abnormally high levels of intracellular β-catenin (Barker and Clevers, 2000). Androgen-independent PC3 prostate cancer cells were transiently transfected with TOPFlash followed by incubation with increasing amounts of decursin. As seen in the HEK293 reporter cells, decursin repressed CRT in PC3 cells (Fig. 4A). In conjunction with this experiment, we evaluated the level of cytosolic β-catenin in decursin-treated PC3 cells. Consistent with our results in HEK293 cells, Western blot analysis showed that β-catenin was down-regulated in a concentration-dependent manner (Fig. 4B). Furthermore, MG-132 abolished the decursin-induced down-regulation of β-catenin in PC3 cells (Fig. 4C). These results indicate that decursin promotes β-catenin degradation in prostate cancer cells.

**Decursin Repressed the Expression of β-Catenin-Dependent Genes and Inhibited the Proliferation of Prostate Cancer Cells.** We next examined whether decursin affects the expression of β-catenin-dependent genes. To this end, PC3 cells were transfected with a reporter construct containing the cyclin D1 promoter, which contains a β-catenin/TCF-4-responsive region, and then incubated with different concentrations of decursin. As shown in Fig. 5A, cyclin D1 promoter activity was repressed by decursin. We also measured the mRNA and protein expression of cyclin D1 in decursin-treated PC3 cells. Consistent with our result for the cyclin D1 promoter, a dose-dependent decrease in cyclin D1

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**Fig. 3.** Decursin induces β-catenin degradation through a mechanism independent of GSK-3β and dependent of β-TrCP. A. HEK293 reporter cells were incubated with decursin in the presence of 20 mM LiCl. After 15 h, luciferase activity was determined. TOPFlash activity is reported as relative light unit (RLU) normalized to CellTiter-Glo (Promega) activity. The results are the average of three experiments, and the bars indicate standard deviations. *, *P < 0.05, and **, *P < 0.01, compared with the vehicle control group. B. Cytosolic proteins were prepared from HEK293 reporter cells treated with either vehicle (DMSO) or increasing amounts of decursin in the presence of 20 mM LiCl for 15 h and then subjected to Western blotting with β-catenin antibody. C. HEK293 cells were transfected with the Δβ-TrCP expression plasmid and then incubated with either the vehicle (DMSO) or decursin (100 μM) in the presence of Wnt3a CM for 15 h. Cytosolic proteins were subjected to Western blotting with β-catenin antibody. In B and C, the blots were reprobed with anti-actin antibody as a loading control.

**Fig. 4.** Decursin promotes the degradation of β-catenin in PC3 prostate cancer cells. A. PC3 cells were cotransfected with TOPFlash and pCMV-RL plasmids and incubated with decursin for 15 h. Luciferase activities were measured 39 h after transfection. TOPFlash activity is reported as relative light unit (RLU) normalized to Renilla reniformis luciferase activity. Results are the average of three experiments, and the bars indicate standard deviations. **, *P < 0.01 compared with the vehicle control group. B. Cytosolic proteins were prepared from PC3 cells treated with the vehicle (DMSO) or decursin for 15 h and then subjected to Western blotting with β-catenin antibody. C. Cytosolic proteins prepared from PC3 cells were incubated with vehicle (DMSO) or decursin (100 μM), exposed to MG-132 (20 μM) for 8 h, and then subjected to Western blotting with anti-β-catenin antibody. In B and C, to confirm equal loading, the blot was reprobed with anti-actin antibody.
mRNA and protein expression was observed in response to decursin (Fig. 5, B and C). In addition, the expression of c-myc, an established downstream target of β-catenin (He et al., 1998), was also significantly reduced in PC3 cells after incubation with decursin (Fig. 5D). Recently, inhibition of the Wnt/β-catenin pathway by expression of Frzb/secreted Frizzled-related protein, a secreted Wnt antagonist, suppressed the proliferation of androgen-dependent and -independent prostate cancer cells (Zi et al., 2005; Horvath et al., 2007). Moreover, cyclin D1, which forms a complex with cyclin-dependent kinase 4/6, mediates growth factor-dependent G1 phase progression (Sherr, 1996). Given that decursin inhibits the Wnt/β-catenin pathway and cyclin D1 expression, we examined whether decursin reduces PC3 cell growth. PC3 cells were incubated with varying concentrations of decursin, and cell growth was monitored. As shown in Fig. 5E, decursin efficiently suppressed PC3 cell growth in a concentration-dependent manner.

**Decursinol Did Not Inhibit the Wnt/β-Catenin Pathway.** Decursinol is natural analog of decursin that lacks the \((\text{CH}_3)_2\text{C}=\text{CH}–\text{COO}–\) side chain (Fig. 6A). A recent study reported that decursinol has lower anticancer activity compared with decursin, suggesting that the missing side chain may be responsible for the decursin-mediated growth inhibition of prostate cancer cells (Yim et al., 2005). To further analyze the relationship between structure and function in these compounds, we determined the effect of decursinol on the Wnt/β-catenin pathway. When HEK293 reporter cells were incubated with decursinol, no significant inhibition of Wnt3a-induced CRT was observed, in contrast to our results with decursin (Fig. 6B). Consistent with this result, decursinol did not decrease the amount of cytosolic β-catenin in HEK293 reporter cells or in PC3 cells at any of the concentrations tested (Fig. 6, C and D), suggesting that decursinol was unable to induce the degradation of β-catenin. As expected, decursinol did not repress the expression of cyclin D1 in PC3 cells (Fig. 6E), and it did not affect their growth at concentrations of 50 and 100 μM, although it decreased their growth by approximately 20% at a concentration of 200 μM (Fig. 6F). Taken together, these results indicate that the \((\text{CH}_3)_2\text{C}=\text{CH}–\text{COO}–\) side chain of decursin is crucial for...
inhibition of the Wnt/β-catenin pathway and the anticancer activity of the compound in PC3 cells.

Discussion

Aberrant accumulation of intracellular β-catenin is involved in the progression of prostate cancer at an early stage, during the formation of the primary lesion and at the advanced, hormone-refractory stage (Barker and Clevers, 2000). Using a cell-based screen, we showed that decursin suppresses the Wnt/β-catenin pathway by promoting the degradation of β-catenin. The level of intracellular β-catenin is predominantly regulated by two pathways, a GSK3-dependent pathway, and an Siah-dependent pathway. In the GSK3-dependent pathway, the N-terminal residue of β-catenin is phosphorylated by a multiprotein complex composed of APC, Axin, and GSK-3β (Hart et al., 1998), leading to the degradation of β-catenin through a ubiquitin-dependent mechanism (Aberle et al., 1997). In the Siah-dependent pathway, Siah-1 interacts with the carboxyl terminus of APC, which recruits the ubiquitination complex and promotes the degradation of β-catenin via a GSK-3β- and β-TrCP-independent pathway (Liu et al., 2001). In this study, decursin was able to induce the degradation of intracellular β-catenin in the presence of Wnt3a and LiCl, which are well known GSK-3β inhibitors, suggesting that GSK3β is not necessary for decursin-mediated β-catenin degradation. In addition, overexpression of Δβ-TrCP abolished β-catenin degradation by decursin, indicating that β-TrCP is required for decursin-mediated β-catenin degradation. These data suggest that decursin promotes the degradation of β-catenin through a mechanism other than those described above. We plan to investigate the mechanism of decursin-induced β-catenin degradation in the future.

Intracellular β-catenin promotes the development of prostate cancer through the β-catenin/TCF and β-catenin/androgen receptor (AR) pathways (Chesire and Issac, 2002). Activation of β-catenin/TCF pathway promotes proliferation and suppresses apoptosis in prostate cancer cells (de la Taille et al., 2003). In β-catenin/AR pathway, β-catenin interacts with AR, resulting in the up-regulation of AR activity in a ligand-dependent manner, which supports the growth of prostate cancer cells (Mulholland et al., 2003). Recently, it was shown that decursin inhibits growth and promotes cell death in both androgen-dependent and -independent prostate cancer cells (Yim et al., 2005). Jiang et al. (2006) suggested that decursin inhibits the proliferation of androgen-dependent LNCaP cells.
Decursin-Induced β-Catenin Degradation

1605


Park MJ, et al. (2006) Decursin and a Decursin-Induced β-Catenin Degradation 1605...


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