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Synthesis and evaluation of (+)-decursin derivatives as inhibitors of the Wnt/β-catenin pathway

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ABSTRACT

We synthesized (+)-decursin derivatives substituted with cinnamoyl- and phenyl propionyl groups originating from (+)-CGK062 and screened them using a cell-based assay to detect relative luciferase reporter activity. Of this series, compound **8b**, in which a 3-acetoxy cinnamoyl group was introduced, most potently inhibited (97.0%) the Wnt/β-catenin pathway. Specifically, compound **8b** dose-dependently inhibited Wnt3a-induced expression of the β-catenin response transcription (CRT) and increased β-catenin degradation in HEK293 reporter cells. Furthermore, compound **8b** suppressed expression of the downstream β-catenin target genes cyclin D1 and c-myc and suppressed PC3 cell growth in a concentration-dependent manner.

The Wnt/β-catenin signaling pathway is associated with embryonic development and regulates cell proliferation and differentiation. Abnormal activity of this pathway and increased β-catenin-dependent transcription induces tumor development including colon and prostate cancer.[1–3]

6,7-Dihydroxycoumarin (**1**, Fig. 1), also called esculetin, was recently identified as a potential Wnt/β-catenin pathway inhibitor. This compound suppresses growth of human colon cancer cells by disrupting formation of the β-catenin-Tcf complex.[4] We also found that (+)-decursin (**2**, Fig. 1) and (+)-CGK062 (**3**, Fig. 1), which have a coumarin-ring skeleton similar to esculetin, inhibit Wnt/β-catenin pathway activity.[5–7] In particular, (+)-CGK062, in which a 3,4-dihydroxy cinnamoyl group was introduced, potently inhibits (99.5%) the Wnt/β-catenin pathway and dose-dependently antagonizes Wnt3a-induced β-catenin response transcription (CRT). In addition, 100 mg/kg (+)-CGK062 inhibits PC3 tumor growth and 50 mg/kg (+)-CGK062 inhibits >80% of subcutaneously established PC3 xenograft tumor growth in athymic nude mice. This compound induces no observable signs of toxicity in mice.

In this study, we synthesized (+)-decursin derivatives with various cinnamoyl and phenyl propionyl groups using (+)-CGK062 as the lead compound. These compounds were screened using a cell-based assay to detect relative luciferase reporter activity.

Semi-synthesis of the (+)-decursin derivatives is outlined in Scheme 1. (+)-Decursinol (**4**) was the starting material obtained by hydrolyzing an ethanol extract of *Angelica gigas* root with alkaline solvent.[8] Esterification by EDC-mediated coupling provided non-substituted cinnamoyl-, halo-substituted cinnamoyl-, nitro-substituted cinnamoyl- and methoxy-cinnamoyl-decursin derivatives **5a–d** and **6a–i**. Demethylation of $(OCH_3)_n$-cinnamoyl decursin derivatives **6a–d** and **6h** was accomplished using 1 M BBr_3 solution, leading to **7a–e**. The $(OH)_n$-cinnamoyl decursin derivatives **3** (CGK062) and **7a–e** were acetylated using acetyl chloride to obtain good yields of the $(OAc)_n$-cinnamoyl decursin derivatives **8a–f**.[9–16] We synthesized (+)-decursin derivatives by introducing various substituted phenyl propionyl groups. The reaction conditions for these compounds were identical to the conditions used for compounds **6–8**.

We used a cell-based screening system to identify decursin derivatives that inhibit the Wnt/β-catenin pathway. HEK293 reporter cells were stably transfected with TOPFlash and the hFz-1 expression plasmid. TOPFlash reporter activity was monitored using a microplate reader after adding Wnt3a-conditioned

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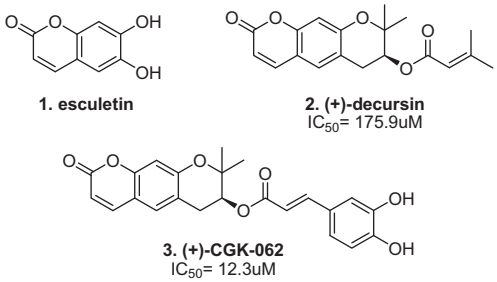


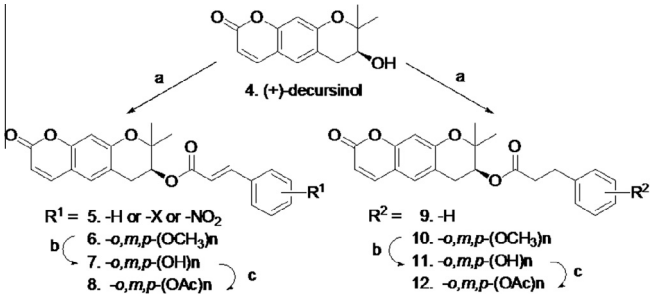
Figure 1. Inhibitors of the Wnt/β-catenin pathway.

medium (Wnt3a-CM) to each decursin derivative.[17–22] The controls were assayed in the presence or absence of Wnt3a-CM.

Results are shown in Tables 1 and 2. The (+)-decursin derivatives with a cinnamoyl group were more potent inhibitors of the Wnt/β-catenin pathway than (+)-decursin derivatives with a phenyl propionyl group. This result indicates that the double bond between the ester and phenyl groups is very important for Wnt/β-catenin pathway inhibitory activity. The position of the cinnamoyl group substituent on the decursin derivative was a significant factor for inhibiting the Wnt/β-catenin pathway. *Ortho*- or *meta*-substituted cinnamoyl decursin derivatives **5b**, **5c**, **6a**, **6b**, **7a**, **7b**, **7d**, **8a**, **8b**, **8d**, **8e**, and **11d** were potent inhibitors of the Wnt/β-catenin pathway.

In particular, (+)-decursin derivative **8b**, with a 3-acetoxy cinnamoyl group, exhibited strong inhibitory activity, similar to that of CGK062 which had two phenolic OH group. To our best knowledge, because the proportion of poly phenolic groups of CGK-062 excreted in the free state was very low due to conjugation phenol groups with glucuronic acid and sulfuric acid in vivo, compound **8b** with acetyl group instead of phenolic groups had the advantage compared to CGK-062. (+)-Decursin derivatives with a $(OH)_n$-cinnamoyl group (**3**, **7a**–**e**) or a $(OAc)_n$-cinnamoyl (**8a**–**f**) group strongly inhibited the Wnt/β-catenin pathway.

We further characterized the effects of compound **8b**, 3-(3-acetoxy-phenyl)-acrylic acid, and 8,8-dimethyl-2-oxo-6,7-dihydro-*2H,8H*-pyrano[3,2-*g*]chromen-7yl-ester on the Wnt/β-catenin pathway. Treating HEK293 reporter cells with varying concentrations of compound **8b** dose-dependently decreased CRT induced by Wnt3a (IC_{50} = 9.85 μM) (Fig. 2A). A western blot analysis with an anti-β-catenin antibody was performed to examine whether compound **8b** affected intracellular β-catenin levels in HEK293 cells, as CRT is regulated by intracellular β-catenin.[23] β-Catenin level increased consistently upon treatment with Wnt3a-CM, which was downregulated by compound **8b** (Fig. 2B), consistent with its inhibitory effect on Wnt3a-stimulated CRT, and suggesting



Scheme 1. Reagents and conditions: (a) cinnamic acid or phenyl propionic acid, EDC, 4-DMAP, dry MC, (b) 1 M BBr3 solution, dry MC, (c) acetyl chloride, pyridine, dry MC.

Table 1
Inhibitory percentage of the Wnt/β-catenin pathway for (+)-decursin derivatives, introduced cinnamoyl group

Compd No.	R^1	% of inhibition[a]
2	(+)-Decursin	79.4
3	(+)-CGK062	99.5
5a	–H	81.1
5b	–3-F	93.6
5c	–3-Br	84.8
5d	–4-Br	40.1
5e	$-4\text{-}NO_2$	34.3
6a	$-2\text{-}OCH_3$	88.3
6b	$-3\text{-}OCH_3$	87.5
6c	$-4\text{-}OCH_3$	54.8
6d	$-2,3\text{-}(OCH_3)_2$	57.8
6e	$-2,4\text{-}(OCH_3)_2$	52.8
6f	$-2,5\text{-}(OCH_3)_2$	65.3
6g	$-3,4\text{-}(OCH_3)_2$	42.3
6h	$-3,4,5\text{-}(OCH_3)_3$	46.9
6i	$-2,4,5\text{-}(OCH_3)_3$	59.6
7a	–2-OH	94.2
7b	–3-OH	93.3
7c	–4-OH	78.1
7d	$-2,3\text{-}(OH)_2$	95.7
7e	$-3,4,5\text{-}(OH)_3$	75.3
8a	–2-OAc	95.9
8b	–3-OAc	97.0
8c	–4-OAc	79.6
8d	$-2,3\text{-}(OAc)_2$	89.7
8e	$-3,4\text{-}(OAc)_2$	94.0
8f	$-3,4,5\text{-}(OAc)_3$	67.4

[a] These compounds were tested in concentration of 20 μM.

Table 2
Inhibitory percentage of the Wnt/β-catenin pathway for (+)-decursin derivatives, introduced phenyl propionyl group

Compd No.	R^2	% of inhibition[a]
2	(+)-Decursin	79.4
3	(+)-CGK062	99.5
9	–H	10.6
10a	$-2\text{-}OCH_3$	50.4
10b	$-3\text{-}OCH_3$	49.9
10c	$-4\text{-}OCH_3$	41.5
10d	$-2,3\text{-}(OCH_3)_2$	13.5
10e	$-2,4\text{-}(OCH_3)_2$	18.8
10f	$-2,5\text{-}(OCH_3)_2$	19.9
10g	$-3,4\text{-}(OCH_3)_2$	25.1
11a	–2-OH	10.0
11b	–3-OH	42.0
11c	–4-OH	32.8
11d	$-2,3\text{-}(OH)_2$	81.4
11e	$-3,4\text{-}(OH)_2$	68.0
12a	–2-OAc	23.1
12b	–3-OAc	27.5
12c	–4-OAc	12.0
12d	$-2,3\text{-}(OAc)_2$	77.7
12e	$-3,4\text{-}(OAc)_2$	46.4

[a] These compounds were tested in concentration of 20 μM.

that compound **8b** attenuates the Wnt/β-catenin pathway by lowering β-catenin protein levels.[24]

The Wnt/β-catenin pathway is activated by aberrant upregulation of intracellular β-catenin in PC3 prostate cancer cells. Thus, we carried out a western blot analysis to determine cytoplasmic β-catenin levels in compound **8b**-treated PC3 cells.[25] As shown in Figure 3A, compound **8b** dose-dependently downregulated cytoplasmic β-catenin. Next, we examined its effect on the expression of β-catenin-dependent genes in PC3 cells. After incubating with increasing concentrations of compound **8b**, expression of cyclin D1 and c-myc, established β-catenin target genes, was quantified by western blot.[24,26] As expected, we found a significant reduction in cyclin D1 and c-myc protein levels (Fig. 3B).

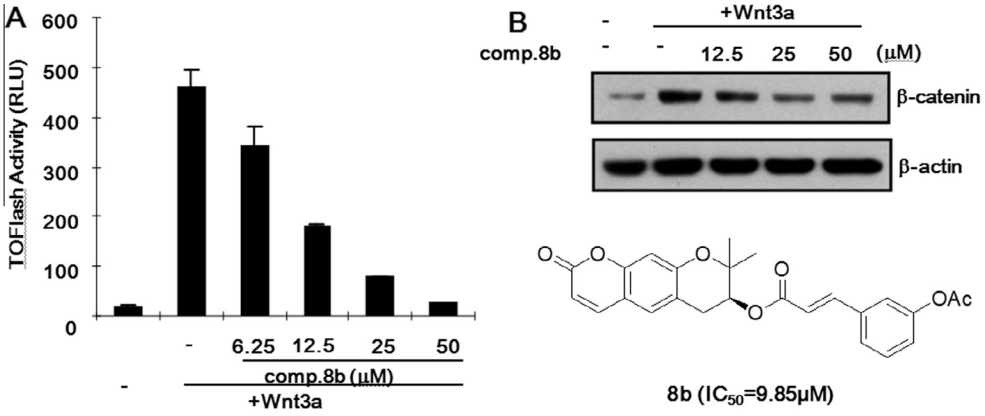


Figure 2. (A) Dose-dependent inhibition of the β-catenin transcription response. HEK293 reporter cells were incubated with indicated concentrations of compound **8b** in the presence of Wnt3a-CM. After 15 h, luciferase activities were determined and reported as relative light unit (RLU) normalized to cell titer. The results are the average of three experiments, and the bars indicate standard deviations. (B) Downregulation of the β-catenin protein by compound **8b**. Cytosolic proteins were prepared from HEK293 reporter cells treated with either vehicle (DMSO) or indicated concentrations of compound **8b** in the presence of Wnt3a-CM for 15 h and then subjected to western blotting with β-catenin antibody. The blots were reprobed with anti-actin antibody as fraction controls.

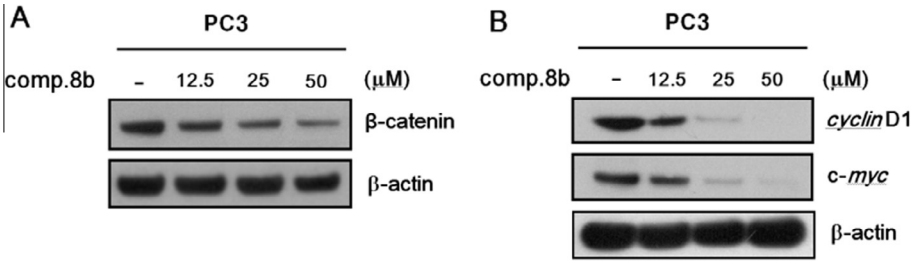


Figure 3. Effects of compound **8b** on prostate cancer cells. (A) Cytosolic fractions were prepared from PC3 cells treated with vehicle (DMSO) or compound **8b** for western blotting with β-catenin antibody. (B) PC3 cells were incubated with the vehicle (DMSO) or compound **8b** for 15 h and then cell extracts were prepared for western blotting with anti-*cyclin D1* and anti-*myc* antibodies. The blots were reprobed with anti-actin antibody as fraction controls.

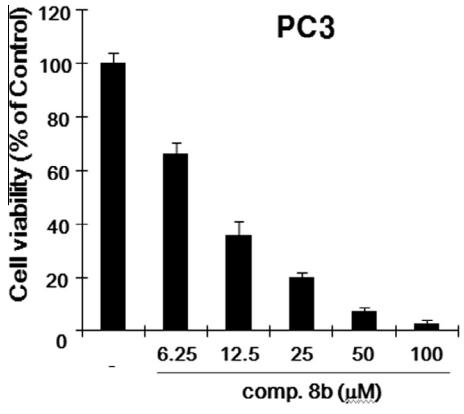


Figure 4. The effect of compound **8b** on prostate cancer cell growth. Cells were incubated, in the indicated concentrations of compound **8b**, for 24 h in 96-well plates. Cell viability was examined using the CellTiter-Glo assay (Promega). The results are the average of three experiments, and the bars indicate standard deviations.

Previous studies have demonstrated that specifically inhibiting the Wnt/β-catenin pathway by expressing Frzb/secreted Frizzled-related protein, which is a secreted Wnt antagonist, suppresses growth of androgen-dependent and -independent prostate cancer.[27–29] In addition, *cyclin D1* regulates growth factor-induced G1 phase cell cycle progression.[30] Because compound **8b** inhibited both the Wnt/β-catenin pathway and downstream targets of *cyclin D1*, we hypothesized that compound **8b** would suppress prostate cancer cell growth. To explore this hypothesis, androgen-independent PC3 cells were incubated with varying concentrations of compound **8b**, and cell proliferation was measured using the CellTiter-Glo assay. As presented in Figure 4, compound **8b** efficiently inhibited PC3 cell proliferation with an IC_{50} value 8.97 μM.[31]

In summary, our results show that the double bond included in the cinnamoyl group of decursin derivatives is necessary for inhibition of the Wnt/β-catenin pathway. Substituting the functional group in the *ortho*- or *meta*-position on the benzene ring of the cinnamoyl group was most effective. Compound **8b** was the most potent at inhibiting Wnt/β-catenin pathway activity. Compound **8b** dose-dependently inhibited CRT induced by Wnt3a (IC50 = 9.85 μM) in HEK293 reporter cells and decreased intracellular levels of β-catenin protein in the cytosol of PC3 prostate cancer cells. In addition, compound **8b** promoted β-catenin degradation and suppressed the expression of cyclin D1 and c-myc, downstream β-catenin target genes. Compound **8b** may lead to a candidate anti-tumor agent for prostate cancer.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.06.029.

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20. *Cell culture, transfection, and luciferase assay.* HEK293, PC-3, and Wnt3a-secreting L cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 120 μg/ml penicillin, and 200 μg/ml streptomycin. Wnt3a conditioned medium (Wnt3a CM) was prepared as described previously. In brief, Wnt3a-secreting L cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum for 4 days. The medium was harvested and sterilized using a 0.22-μm filter. Fresh medium was added, the cells were cultured for another 3 days, and the medium was collected and combined with the previous medium. Transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The luciferase assay was performed using a Dual Luciferase Assay kit (Promega, Madison, WI, USA).
21. *Plasmid constructs and transfection.* The pTOPFlash reporter plasmid was obtained from Upstate Biotechnology (Lake Placid NY, USA). The pCMV-RL and pSV-FL plasmids were purchased from Promega. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.
22. *Screening for a Wnt/β-catenin signaling inhibitor.* HEK293 reporter (TOPFlash) cells were inoculated into 96-well plates at 15,000 cells per well in duplicate and grown for 24 h. Next, Wnt3a-CM was added, and the compounds were added to the wells. After 15 h, the plates were assayed for firefly luciferase activity and cell viability.
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24. *Western blotting.* The cytosolic fraction was prepared as described previously. Proteins were separated by SDS–polyacrylamide gel electrophoresis in a 4–12% gradient gel (Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% nonfat milk and probed with anti-β-catenin (BD Transduction Laboratories, Lexington, KY, USA), anti-*cyclin* D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-*myc* (Santa Cruz Biotechnology), and anti-actin antibodies (Cell Signaling Technology, Danvers, MA, USA). The membranes were 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). The β-catenin antibody was purchased from BD Transduction Laboratories (San Diego, CA, USA). β-Actin was purchased from Cell Signaling Technology.
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31. *Cell viability assay.* Cells were inoculated into 96-well plates and treated with decursin for 48 h. Cell viability from each treated sample was measured in triplicate using the CellTiter-Glo assay kit (Promega) according to the manufacturer's instructions.