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# Galangin Suppresses the Proliferation of β-Catenin Response Transcription-Positive Cancer Cells by Promoting Adenomatous Polyposis Coli/Axin/Glycogen Synthase Kinase-3β-Independent β-Catenin Degradation

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## ABSTRACT

Galangin is a naturally occurring bioflavonoid with anticancer activity against certain human cancers, yet little is known about its mechanism of action. Here, we used a chemical biology approach to reveal that galangin suppresses β-catenin response transcription (CRT), which is aberrantly up-regulated in colorectal and liver cancers, by promoting the degradation of intracellular β-catenin. Inhibition of glycogen synthase kinase-3β (GSK-3β) activity or mutation of the GSK-3β-targeted sequence from β-catenin was unable to abrogate the galangin-mediated degradation of β-catenin. In addition, galangin down-regulated the intracellular β-catenin levels in cancer cells with inactivating mutations of adenomatous polyposis coli (APC) or Axin, which are components of the β-catenin destruction complex. Galangin repressed the expression of β-catenin/T-cell factor-dependent genes, such as cyclin D1 and c-myc, and thus inhibited the proliferation of CRT-positive cancer cells. Structure-activity data indicated that the major structural requirements for galangin-mediated β-catenin degradation are hydroxyl groups at positions 3, 5, and 7. Our findings suggest that galangin exerts its anticancer activity by promoting APC/Axin/GSK-3β-independent proteasomal degradation of β-catenin.

## Introduction

β-Catenin is involved in regulating cell-cell adhesion as a part of the E-cadherin/catenin adherence complex and controlling the Wnt/β-catenin pathway, which plays important roles in cellular proliferation, morphology, motility, fate, axis formation, and organ development (Wodarz and Nusse, 1998; Peifer and Polakis, 2000). The level of intracellular β-catenin is regulated tightly by two major proteasomal degradation pathways involving adenomatous polyposis coli (APC) tumor suppressor protein. In one, β-catenin is phosphorylated by a destruction complex composed of APC, Axin, and casein kinase 1 (CK1) and glycogen synthase kinase-3β (GSK-3β) (Hart et al., 1998; Ikeda et al., 1998). Phosphorylated β-catenin is then recognized by F-box β-transducin repeat-containing protein (β-TrCP), a component of the ubiquitin ligase complex, leading to its ubiquitin-dependent proteasomal degradation (Aberle et al., 1997). In the second pathway, Siah-1, which is induced by p53, interacts with the carboxyl terminus of APC, recruits the ubiquitination complex, and promotes the degradation of β-catenin through a pathway independent of both GSK-3β and β-TrCP, an F-box

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**ABBREVIATIONS:** APC, adenomatous polyposis coli; CRT, β-catenin response transcription, GSK-3β, glycogen synthase kinase-3β; Wnt3a-CM, Wnt3a conditioned medium; β-TrCP, β-transducin repeat-containing protein; HEK, human embryonic kidney; hFz-1, human Frizzled-1; DMSO, dimethyl sulfoxide; MG-132, *N*-benzyoloxycarbonyl (*Z*)-Leu-Leu-leucinal; TCF, T-cell factor; CK1, casein kinase 1; FL, firefly luciferase; SEAP, secreted alkaline phosphatase; siRNA, small interfering RNA.

protein in the E3 ubiquitin ligase complex (Liu et al., 2001; Matsuzawa and Reed, 2001).

The aberrant accumulation of intracellular β-catenin is associated with the development and progression of certain human cancers (Giles et al., 2003). Oncogenic mutations in β-catenin or other components of the destruction complex (APC or Axin) are observed in colon cancer, hepatocellular carcinoma, and prostate cancer (Miyoshi et al., 1998; Morin, 1999; Fearnhead et al., 2001). These mutations lead to the excessive accumulation of β-catenin in the cytoplasm, and then β-catenin is translocated into the nucleus, where it complexes with T-cell factor/lymphocyte enhancer factor (TCF/LEF) family transcription factors activating the expression of Wnt/β-catenin-responsive genes, such as *c-myc*, *cyclin D1*, *metalloproteinase-7*, and peroxisome proliferator-activated receptor-δ, which play important roles in tumorigenesis and metastasis (He et al., 1998, 1999; Tetsu and McCormick, 1999; Takahashi et al., 2002). The accumulation of β-catenin is also observed in other types of cancer, such as ovarian cancer, melanoma, endometrial cancer, medulloblastoma, and pilomatricoma (Fearnhead et al., 2001; Karim et al., 2004). Thus, abnormally up-regulated intracellular β-catenin is a potential therapeutic target for chemoprevention and the treatment of various cancers.

Galangin (3,5,7-trihydroxyflavone), a member of the flavonol class of flavonoids, is present at high concentration in propolis, a natural material produced by honeybees, and in India root, *Alpinis officinarum*, which is a common spice and herbal medicine in Asia (Heo et al., 2001). Galangin inhibits the growth of human mammary tumor cells through the suppression of cyclin D1 (Murray et al., 2006) and promotes apoptosis in human leukemia cells (Bestwick and Milne, 2006). Among several biological activities, galangin suppresses the genotoxicity of chemicals (Heo et al., 2001), represses Cox-2 expression (O'Leary et al., 2004), inhibits viral replication (Amoros et al., 1992), and acts as an inhibitor of aryl hydrocarbon receptor (Quadri et al., 2000). In the present study, we identified galangin as an antagonist of the Wnt/β-catenin pathway using cell-based small-molecule screening. Galangin may inhibit the growth of CRT-positive cancer cells by promoting the degradation of intracellular β-catenin.

## Materials and Methods

**Cell Culture, Reporter Assays, and Chemicals.** HEK293, HCT116, SW480, SNU475, HCT-15, and Wnt3a-secreting L cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 120 μg/ml penicillin, and 200 μg/ml streptomycin. HEK293-FL reporter (TOPFlash), control (FOPFlash), and HEK293-secreted alkaline phosphatase (SEAP) reporter cells were established as described previously (Park et al., 2006; Ryu et al., 2008). Wnt3a-conditioned medium (Wnt3a-CM) was prepared as described previously (Park et al., 2006). Luciferase assay was performed using the Dual Luciferase Assay kit (Promega, Madison, WI), and secreted alkaline phosphatase assay was carried out using Phospha-Light Assay kit (Applied Biosystems, Foster City, CA). LiCl, cycloheximide, and *N*-benzyoloxycarbonyl (*Z*)-Leu-Leu-leucinal (MG-132) were purchased from Sigma-Aldrich (St. Louis, MO). Flavonoids were obtained from Tokyo Chemical Industry (Tokyo, Japan).

**Plasmid Constructs, siRNA, and Transfection.** Human Frizzled-1 (hFz-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 elements, by polymerase chain reaction and insertion into pRL-null vectors, yielding pCyclinD1-RL. The pTOPFlash and pFOPFlash reporter plasmids were obtained from Millipore Corp. (Billerica, MA). 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. siRNA specific for GSK-3β (5′-GUAAUCCACCUCUGGCUAC-3′) were synthesized by Invitrogen (Carlsbad, CA). Negative control siRNA (Silencer) was purchased from Ambion (Austin, TX). Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

**Screening for a Small-Molecule Inhibitor of Wnt/β-Catenin Signaling.** The HEK293 reporter cells were inoculated into 96-well plates at $15 \times 10^3$ cells/well in duplicate and grown for 24 h. Wnt3a-CM was added, and then compounds including coumarins, flavonoids, naphthoquinones, and terpenoids were added to the wells at a final concentration of 50 μM. 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-phospho-Ser9-GSK-3β (Cell Signaling Technology, Danvers, MA), anti-GSK-3β (Santa Cruz Biotechnology, Santa Cruz, CA), anti-cyclin D1 (Santa Cruz Biotechnology), anti-myc (Santa Cruz Biotechnology), and anti-actin antibodies (Cell Signaling Technology). The membranes were then incubated with horseradish-peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG and visualized using the ECL system (all from Santa Cruz Biotechnology).

**RNA Extraction and Semiquantitative Reverse Transcription-Polymerase Chain Reaction.** 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, Madison, WI) according to the manufacturer's instructions.

## Results

**Identification of Galangin as an Antagonist of the Wnt/β-Catenin Pathway.** We used HEK293-FL reporter cells stably transfected with a synthetic β-catenin/Tcf-dependent firefly luciferase (FL) reporter and hFz-1 expression plasmid to identify cell-permeable small molecule antagonists of the Wnt/β-catenin pathway. After the addition of Wnt3a-CM and each compound, we detected firefly luciferase activity using a microplate reader (Fig. 1A) and then identified galangin (3,5,7-trihydroxyflavone) as an inhibitor of the Wnt/β-catenin pathway (Fig. 1B). Treatment of HEK293-FL reporter cells with different concentrations of galangin produced a consistent dose-dependent decrease in β-catenin response transcription (CRT) induced by Wnt3a-CM without detectable cytotoxicity; a concentration of 80 μM galangin induced near-complete inhibition of CRT relative to the control treatment (Fig. 1C). In contrast, galangin and Wnt3a-CM did

not affect the activity of FOPFlash, a negative control reporter with mutated β-catenin/Tcf binding elements, in HEK293 control cells (Fig. 1C). In HEK293-SEAP reporter cells, which stably harbored a synthetic β-catenin/Tcf-dependent SEAP reporter and hFz-1 expression plasmid, galangin consistently attenuated Wnt3a-stimulated SEAP activity in a concentration-dependent manner (Fig. 1D), suggesting that galangin is a specific antagonist of the Wnt/β-catenin pathway.

**Galangin Down-Regulates the Level of Intracellular β-Catenin through a β-TrCP-Dependent Proteasomal Degradation Pathway.** Because CRT is regulated primarily by the level of intracellular β-catenin, we examined whether galangin affects the intracellular level of β-catenin by Western blot analysis. As shown in Fig. 2A, galangin decreased the level of cytosolic β-catenin that accumulated by Wnt3a-CM in HEK293-FL reporter cells, consistent with its effect on CRT. However, the mRNA level of β-catenin was unchanged by any of the concentrations of galangin used (Fig. 2B), suggesting that galangin inhibits the Wnt/β-catenin pathway by down-regulating β-catenin protein levels rather than repressing β-catenin gene expression. We next determined the effect of galangin on the half-life of β-catenin by the treatment of HEK293-FL reporter cells with cycloheximide, an inhibitor of protein translation. As shown in Fig. 2C, galangin dramatically decreased the half-life of β-catenin in the presence of Wnt3a-CM. Previous studies have demonstrated that the intracellular β-catenin level is regulated by a proteasomal degradation pathway (Aberle et al., 1997). We used MG-132, a proteasome inhibitor, to examine the involvement of the proteasome in galangin-mediated β-catenin down-regulation. As shown in Fig. 2D, the treatment of HEK293-FL reporter cells with galangin consistently led to a decrease in the cytosolic β-catenin level; however, the addition of MG-132 abolished the effect of galangin on the reduction in β-catenin, indicating that galangin induces proteasome-mediated degradation of intracellular β-catenin. Next, we investigated the mechanism by which galangin-mediated β-catenin degradation might be achieved. It has been reported that the association of β-catenin with β-TrCP leads to its subsequent proteasomal degradation. To test whether β-TrCP is necessary for β-catenin degradation induced by galangin, we ectopically expressed a dominant-negative form of β-TrCP (Δβ-TrCP), which had been observed to interact with β-catenin but is unable to form a $SCF^{\beta-TrCP}$ ubiquitin ligase complex (Hart et al., 1999), in the presence of galangin. As depicted in Fig. 2E, the overexpression of Δβ-TrCP abrogated the galangin-induced degradation of β-catenin, suggesting that galangin promotes the degradation of β-catenin via a β-TrCP-dependent mechanism.

**Galangin Promotes β-Catenin Degradation by a CK1/GSK-3β-Independent Mechanism.** In the Wnt/β-catenin pathway, the N-terminal phosphorylation of β-catenin at Ser45 and Ser33/37/Thr41 catalyzed by CK1 and GSK-3β is a key control of ubiquitin-dependent β-catenin degradation (Liu et al., 2001). To further gain insight into the mechanism of galangin-mediated β-catenin degradation, we examined whether β-catenin degradation by galangin requires CK1/GSK-3β activity. HCT116 colon cancer cells, which contain a Ser45 (CK1 phosphorylation site) deletion mutation in β-catenin (Ilyas et al., 1997), were transfected with TOP-

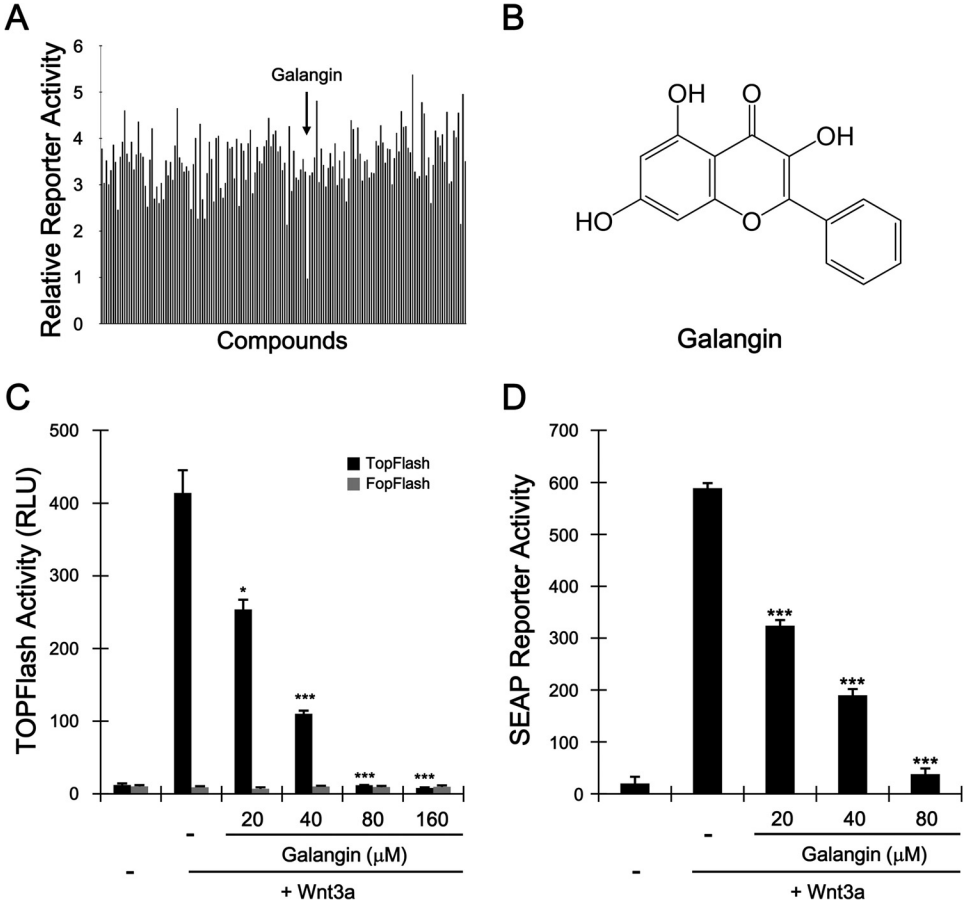


**Fig. 1.** Identification of galangin as a small-molecule inhibitor of Wnt/β-catenin pathway. A, screening of compounds that inhibit the Wnt/β-catenin pathway. Compounds modulating TOPFlash reporter activity were screened using the HEK293-FL reporter cells. The controls were assayed in the presence or absence of Wnt3a-CM. B, chemical structure of galangin. C and D, dose-dependent inhibition of CRT. HEK293-FL, HEK293-SEAP reporter, and control cells were incubated with indicated concentrations of galangin in the presence of Wnt3a-CM. After 15 h, luciferase activity (C) or SEAP activity (D) was determined. The results are the average of three experiments, and the bars indicate standard deviations. *, $P < 0.05$; ***, $P < 0.005$, compared with the Wnt3a-CM-treated control group.

Flash and then incubated with galangin. As illustrated in Fig. 3A, treatment with galangin resulted in a reduction of CRT. The intracellular β-catenin level was consistently down-regulated in galangin-treated HCT116 cells (Fig. 3B), suggesting that CK1 is not involved in galangin-mediated β-catenin degradation.

Next, we tested the effect of GSK-3β activity on β-catenin degradation induced by galangin. In the presence of LiCl, an inhibitor of GSK-3β (Klein and Melton, 1996), galangin still suppressed CRT in HEK293-FL cells (Fig. 3C). Western blot analysis showed that galangin consistently led to a reduction of the cytosolic β-catenin level that accumulated with LiCl treatment, without affecting the phosphorylation of GSK-3β at Ser9 (Fig. 3D). In addition, when we depleted the endogenous GSK-3β using siRNA, galangin was able to down-regulate the level of intracellular β-catenin in HEK293-FL cells (Fig. 3E). Furthermore, we found that S37A-β-catenin, which has the mutation of serine to alanine at the GSK-3β phosphorylation site, was down-regulated in response to galangin (Fig. 3F). Together, these results indicate that β-catenin degradation by galangin is independent of CK1/GSK-3β.

**Galangin-Mediated β-Catenin Degradation Is Independent of Axin and APC.** We then evaluated the effects of Axin and APC, components of the destruction complex, on galangin-induced β-catenin degradation. To address this, the TOPFlash plasmid was transfected into SNU475 hepatoma and SW480 colon cancer cells, which harbor inactivating mutations of Axin and APC, respectively (Ilyas et al., 1997), followed by treatment with increasing concentrations of galangin. As presented in Fig. 4A, galangin repressed CRT consistently in SW480 and SNU475 cells. In parallel with this experiment, we determined the effect of galangin on the levels of cytosolic β-catenin in these cancer cells by Western blot analysis. As expected, the addition of galangin to SW480, HCT15 (APC mutation), and SNU475 cells led to the down-regulation of intracellular β-catenin levels in a concentration-dependent manner (Fig. 4B). These results suggest that Axin and APC are not involved in galangin-mediated β-catenin degradation.

**Galangin Represses the Expression of β-Catenin-Dependent Genes.** Given that galangin down-regulates the levels of intracellular β-catenin in CRT-positive cancer cells, such as HCT116, SNU475, and SW480 cells, we then investigated whether galangin affects the expression of β-catenin-dependent genes. To this end, a reporter construct containing the *cyclin D1* promoter, which contains a β-catenin/TCF-4 responsive region, was transfected into HCT116, SNU475,

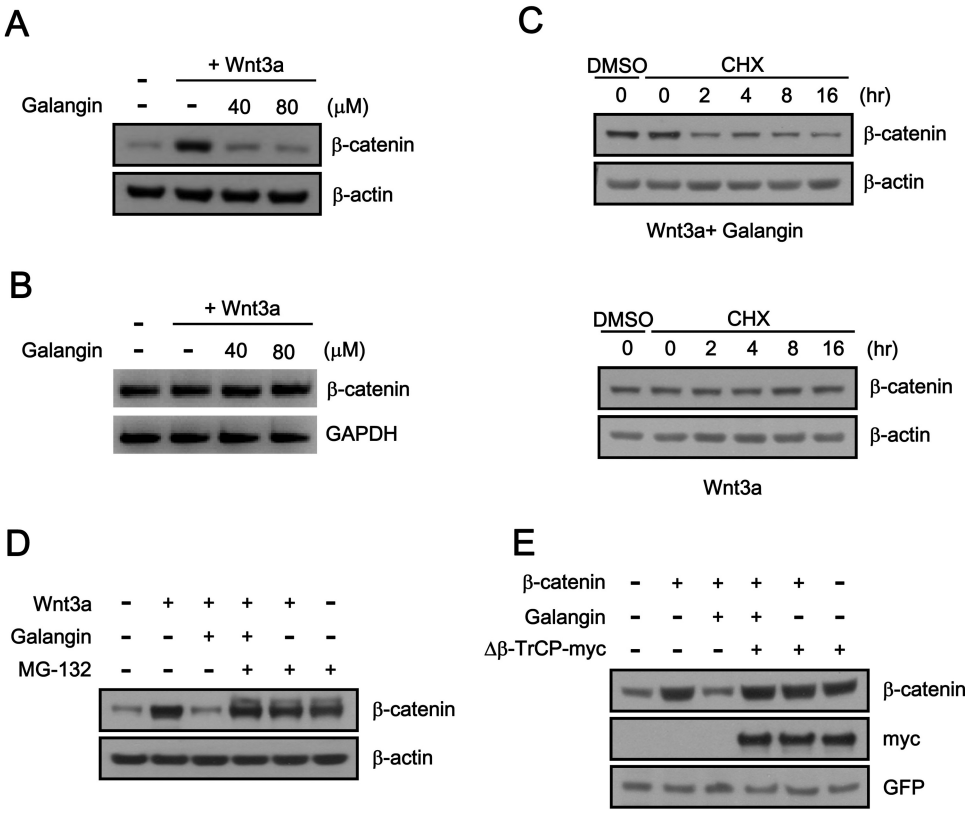


**Fig. 2.** Galangin promotes the degradation of β-catenin through a β-TrCP-dependent proteasomal degradation pathway. A, cytosolic proteins were prepared from HEK293-FL reporter cells treated with either vehicle (DMSO) or indicated concentrations of galangin in the presence of Wnt3a-CM for 15 h and then subjected to Western blotting with anti-β-catenin antibody. B, semiquantitative reverse transcription-polymerase chain reaction for β-catenin and glyceraldehyde-3-phosphate dehydrogenase was performed with total RNA prepared from HEK293-FL reporter cells either vehicle (DMSO) or indicated concentrations of galangin in the presence of Wnt3a-CM for 15 h. C, cytosolic proteins prepared from HEK293-FL reporter cells, which were incubated with vehicle (DMSO) or galangin (40 μM) in the presence of Wnt3a-CM, exposed to cycloheximide (CHX, 10 μg/ml) for indicated periods of time, were subjected to Western blotting with anti-β-catenin antibody. D, cytosolic proteins prepared from HEK293-FL reporter cells, which were incubated with vehicle (DMSO) or galangin (40 μM) in the presence of Wnt3a-CM, exposed to MG-132 (10 μM) for 8 h, were subjected to Western blotting with anti-β-catenin antibody. E, HEK293 cells were cotransfected with Δβ-TrCP expression plasmid and then incubated with either the vehicle (DMSO) or galangin (40 μM) in the presence of Wnt3a-CM for 15 h. Cytosolic proteins were subjected to Western blotting with anti-β-catenin or anti-myc antibodies. In A, C, D, and E, to confirm equal loading, the blot was reprobed with anti-actin antibody.

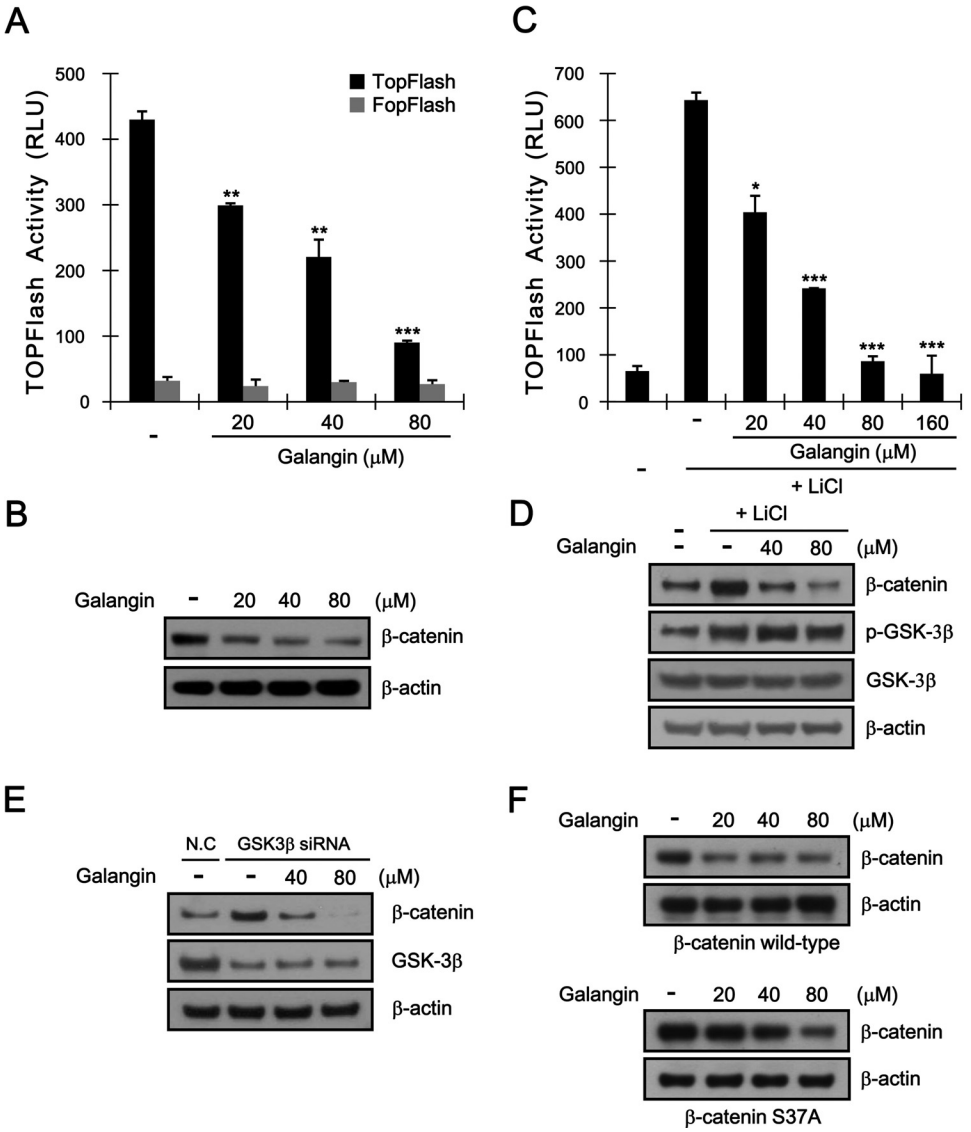


**Fig. 3.** Galangin induces β-catenin degradation by a mechanism independent of CK1/GSK-3β. A, HCT116 cells were cotransfected with TOPFlash and pCMV-RL plasmids and incubated with galangin for 15 h. Luciferase activities were measured 39 h after transfection. TOPFlash activity is reported as relative light unit (RLU) normalized to *Renilla reniformis* luciferase activity. Results are the average of three experiments, and the bars indicate S.D. **, $P < 0.01$; and ***, $P < 0.005$, compared with the vehicle control group. B. cytosolic proteins were prepared from HCT116 cells treated with the vehicle (DMSO) or galangin for 15 h and then subjected to Western blotting with anti-β-catenin antibody. C, HEK293-FL reporter cells were incubated with galangin (20, 40, and 80 μM) in the presence of 20 mM LiCl. After 15 h, luciferase activity was determined. Results are the average of three experiments, and the bars indicate S.D. *, $P < 0.05$, and ***, $P < 0.005$, compared with the LiCl-treated control group. D, cytosolic proteins were prepared from HEK293-FL reporter cells treated with either vehicle (DMSO) or galangin (40 and 80 μM) in the presence of 20 mM LiCl for 15 h and then subjected to Western blotting with anti-β-catenin, anti-phosho-Ser9-GSK-3β, or GSK-3β antibodies. E, HEK293-reporter cells were transfected with negative control siRNA (NC, 40 nM) and GSK3-β siRNA (40 nM) for 36 h and then incubated with galangin (40 and 80 μM) for 12 h. Cell lysates were subjected to Western blot analysis with anti-GSK-3β and anti-β-catenin antibodies. F, HEK293 cells were transfected with wild-type β-catenin or β-catenin S37A plasmids, incubated with galangin (20, 40, and 80 μM) for 15 h, and then cytosolic proteins were immunoblotted with anti-β-catenin antibody. In B, D, E, and F, the blots were reprobed with anti-actin antibody as a loading control.

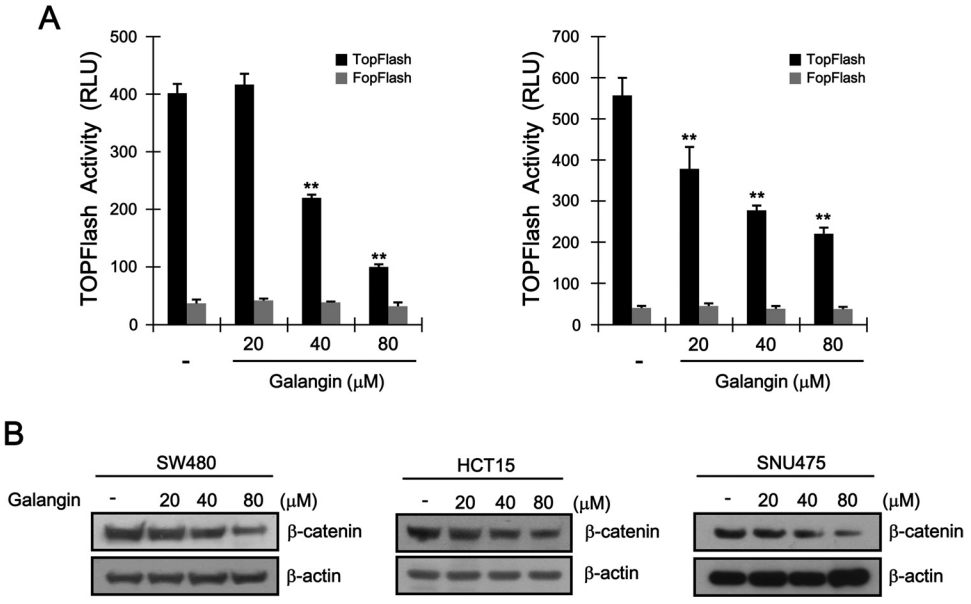


**Fig. 4.** Axin and APC are not involved in galangin-mediated β-catenin degradation. A, SW480 and SNU475 cells were cotransfected with TOPFlash and pCMV-RL plasmids and incubated with galangin for 15 h. Luciferase activities were measured 39 h after transfection. TOPFlash activity is reported as relative light unit (RLU) normalized to *R. reniformis* luciferase activity. Results are the average of three experiments, and the bars indicate S.D. **, $P < 0.01$, compared with the vehicle control group. B, cytosolic proteins were prepared from SW480, HCT15, and SNU475 cells treated with the vehicle (DMSO) or galangin for 15 h and then subjected to Western blotting with anti-β-catenin antibody. The blots were reprobed with anti-actin antibody as a loading control.

and SW480 cells, followed by incubation with various concentrations of galangin. As shown in Fig. 5A, cyclin D1 promoter activity was inhibited by galangin in these CRT-positive cancer cells. We also measured the protein expression of cyclin D1 in galangin-treated HCT116, SNU475, and SW480 cells. Consistent with our results for the cyclin D1 promoter, a dose-dependent decrease in cyclin D1 protein expression was observed in response to galangin (Fig. 5B). In addition, the expression of c-myc, an established downstream target of β-catenin (He et al., 1998), was significantly lower in these CRT-positive cancer cells after incubation with galangin (Fig. 5B).

The specific reduction of β-catenin by antisense oligonucleotides or small interference RNA has been shown to inhibit the proliferation of cancer cells in vitro and tumor growth in a xenograft mouse model (Green et al., 2001; Roh et al., 2001; Verma et al., 2003). Because galangin induced β-catenin degradation, we evaluated the effect of galangin on the growth of CRT-positive cancer cells. HCT116, SNU475, and SW480 cells were incubated with varying concentrations of galangin, and cell growth was monitored. As depicted in Fig. 5C, galangin efficiently suppressed the growth of HCT116, SNU475, and SW480 cells with $IC_{50}$ values of 24.6, 28.0, and 20.4 μM, respectively.

**3-, 5-, and 7-Hydroxyl Residues Are Essential for Galangin-Mediated β-Catenin Degradation.** Galangin is a flavonol derivative that has no hydroxyl groups in the B-ring but has a 2,3-double bond with a 3-hydroxyl group in the C-ring and 5,7-dihydroxyl groups in the A-ring (Fig. 1B). To determine the structural features of galangin, we examined the effects of chemically related flavonoids (chrysin, flavone, flavonol) on the Wnt/β-catenin pathway. In comparison with galangin, chrysin, which has a 2,3-double bond in the C-ring and 5,7-dihydroxyl groups in the A-ring (Fig. 6A), had a lesser effects on inhibiting Wnt3a-induced CRT in HEK293-FL reporter cells (Fig. 6B). In addition, when HEK293-FL reporter cells were incubated with flavone, which contains a 2,3-double bond in the C-ring (Fig. 6A), and flavonol, which contains a 2,3-double bond and 3-hydroxyl group in the C-ring (Fig. 6A), no significant inhibition of Wnt3a-induced CRT was observed (Fig. 6B). Consistent with this result, chrysin, flavone, and flavonol did not decrease the amount of cytosolic β-catenin in HEK293-FL reporter cells or in HCT116 cells at any of the concentrations tested (Fig. 6C), suggesting that these flavonoids are unable to induce the degradation of β-catenin. As expected, chrysin, flavone, and flavonol inhibit the proliferation of HCT116 cancer cells less compared with galangin (Fig. 6D).

## Discussion

Mutations in the Wnt/β-catenin pathway often lead to elevated levels of β-catenin, which is involved in the progression of certain human cancers at an early stage, during the formation of the primary lesion, and at the advanced stage

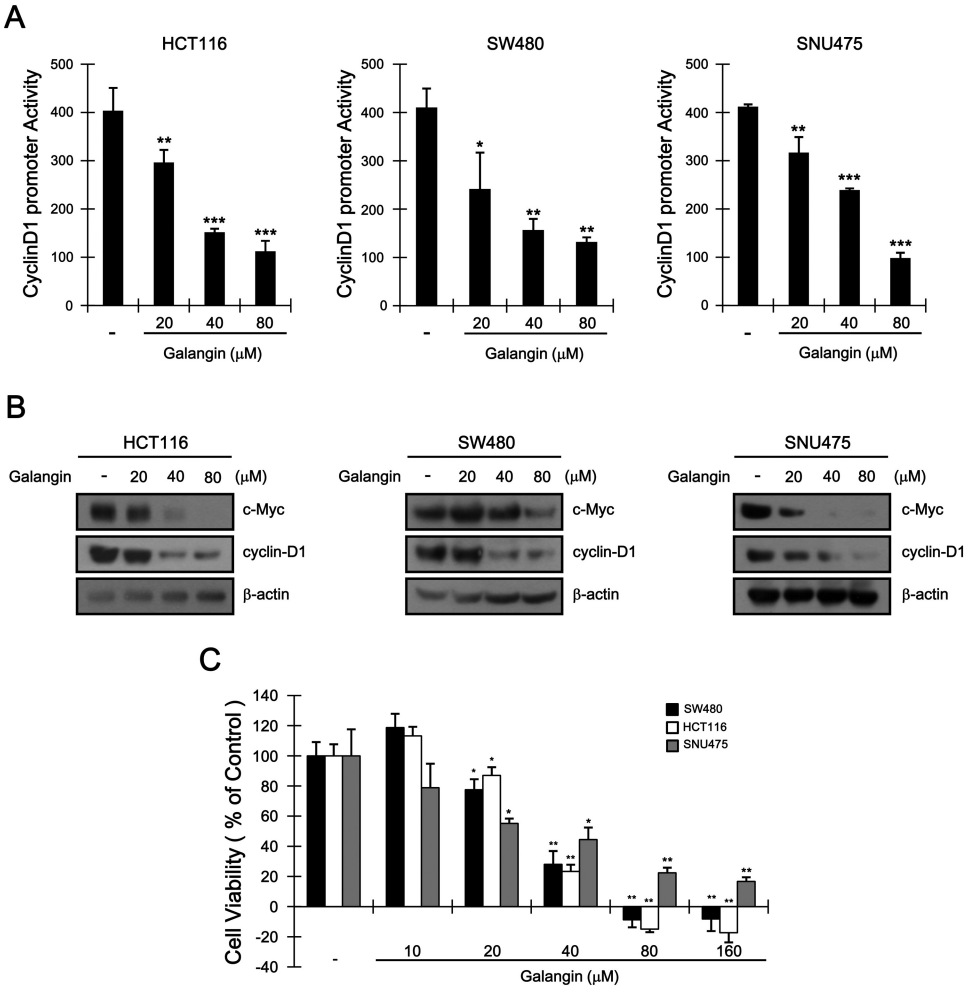


**Fig. 5.** Galangin inhibits the expression of the β-catenin-dependent genes. A, HCT116, SW480, and SNU475 cells were cotransfected with cyclin D1-RL and pSV40-FL and then incubated with the indicated amounts of galangin for 15 h. Luciferase activities were measured 39 h after transfection. Cyclin D1 promoter activity is reported as relative light unit (RLU) normalized to firefly luciferase activity. The results are the average of three experiments, and the bars indicate S.D. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$, compared with the vehicle control group. B, HCT116, SW480, and SNU475 cells were incubated with the vehicle (DMSO) or galangin for 15 h and then cell extracts were prepared for Western blotting with anti-cyclin D1 and anti-c-myc antibodies. To confirm equal loading, the blots were reprobed with anti-actin antibody. C, the effect of galangin on cell growth. Cells were incubated, in the indicated concentrations of galangin, for 48 h in 96-well plates. Cell viability was examined using the CellTiter-Glo assay (Promega). The $IC_{50}$ values were calculated with WinNonlin Professional (Pharsight Corporation, Sunnyvale, CA). The results are the average of three experiments, and the bars indicate S.D. *, $P < 0.05$, **; and $P < 0.01$, compared with the vehicle control group.

(Barker and Clevers, 2000). In this study, we used natural compound screening to reveal that galangin inhibits Wnt3a-induced CRT by promoting the degradation of intracellular β-catenin. The scaffolding protein Axin interacts with APC, GSK-3β, CK1, and β-catenin through separate domains to form the destruction complex (Hart et al., 1998) and coordinates the sequential phosphorylation of β-catenin at Ser45 by CK1 and then at Ser33/37/Thr41 by GSK-3β, leading to the degradation of β-catenin through a ubiquitin-dependent mechanism (Aberle et al., 1997). Several lines of evidence in this study suggest that galangin-mediated β-catenin degradation is distinct from the APC/Axin/GSK-3β-dependent pathway. We demonstrated that mutant β-catenin proteins, which have mutations at the CK1 phosphorylation site (Ser45) or GSK-3β phosphorylation site (Ser37), were still degraded by treatment with galangin. Furthermore, although the inhibition of GSK-3β by LiCl or siRNA increased the intracellular β-catenin level, it did not abrogate the galangin action. In addition, galangin promoted the degradation of β-catenin in SNU475 (Axin mutation), SW480 (APC mutation), and HCT15 (APC mutation) cells. Likewise, peroxisome proliferator-activated receptor γ and retinoid X receptor have been reported to mediate β-catenin degradation by a mechanism independent of GSK-3β and APC (Xiao et al.,

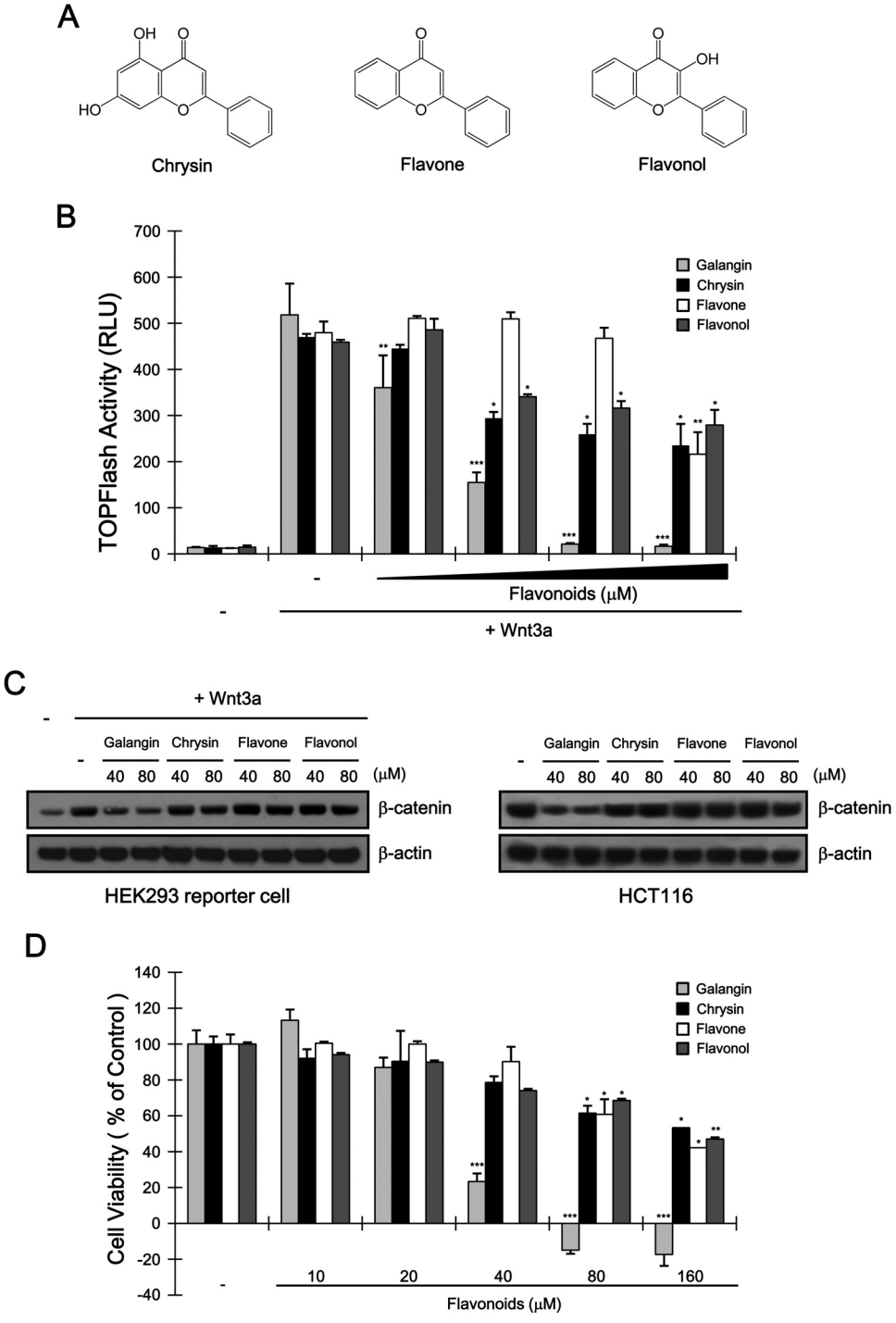


**Fig. 6.** 3-, 5-, and 7-hydroxyl residues are necessary for galangin-mediated β-catenin degradation. A, chemical structures of flavonoids. B, HEK29-FL reporter cells were incubated with indicated concentrations of flavonoids in the presence of Wnt3a-CM. After 15 h, luciferase activity was determined. 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 S.D. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$, compared with the Wnt3a-CM-treated control group. C, cytosolic proteins were prepared from HEK293-FL reporter cells (left) or HCT116 cells (right) treated with either vehicle (DMSO) or indicated concentrations of flavonoids in the presence of Wnt3a-CM (left) for 15 h and then subjected to Western blotting with β-catenin antibody. To confirm equal loading, the blots were reprobed with anti-actin antibody. D, HCT116 cells were incubated with increasing amounts of flavonoids and cell viability was determined by CellTiter-Glo assay (Promega). *, $P < 0.05$; and **, $P < 0.01$ compared with the vehicle control group. The results shown are the average of three experiments, and the bars indicate S.D. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$, compared with the vehicle control group.

2003; Sharma et al., 2004). We plan to investigate the mechanism of galangin-induced β-catenin degradation further.

A slow turnover of β-catenin as a result of mutations in the N-terminal CK1/GSK-3β phosphorylation sites of β-catenin or in other subunits of the destruction complex (APC or Axin) are major pathological events in cancers, such as prostate cancer, hepatoma, and colorectal cancer (Miyoshi et al., 1998; Morin, 1999; Fearnhead et al., 2001). These events lead to the escape of β-catenin from cellular surveillance and, thereafter, to the pathogenic activation of genes involved in tumorigenesis and metastasis. Small molecules that down-regulate the level of β-catenin have been identified by high-throughput screening. We reported previously that hexachlorophene promoted β-catenin degradation by up-regulating Siah-1 expression in HCT116 and LS174T colon cancer cells, which have β-catenin, a mutation at the CK1/GSK-3β phosphorylation sites, but not in SW480 and DLD-1 colon cancer cells, which carry a mutation in APC (Park et al., 2006). On the other hand, the small molecules IWR-3 and XAV939 were shown to stimulate the degradation of β-catenin by stabilizing Axin, thereby inhibiting the proliferation of DLD-1 colon cancer cells (Chen et al., 2009; Huang et al., 2009). In this study, galangin was able to reduce the intracellular β-catenin level by an APC/Axin/GSK-3β-independent mechanism and suppressed the growth of both β-catenin mutant colon cancer cells (HCT116) and APC-mutant colon cancer cells (SW480 and HCT-15). In addition, galangin promoted β-catenin degradation in SNU475 cells, which have an Axin mutation; thus, it may be applicable to cancer therapeutics against most CRT-positive cancer cells.

Galangin is the most lipophilic compound among similar compounds, such as kaempferol, quercetin, morin, and myricetin (Imamura et al., 2000). It has been suggested previously that the 2,3-double bond and 3,5,7-hydroxyl groups of flavonoids may be essential to producing the anticlastogenic effect (Heo et al., 1992). Galangin with the 3,5,7-trihydroxyl groups enhances its inhibitory effects on CYP1A2 activity (Zhai et al., 1998). In the present study, the hydroxyl groups at positions 3, 5, and 7 of galangin were revealed to be essential for both promotion of the degradation of intracellular β-catenin and antiproliferative activity of the compound in HCT116 cells, suggesting that inhibition of the Wnt/β-catenin pathway is a central regulatory mechanism of galangin-mediated inhibition of CRT-positive cancer cell proliferation.

In conclusion, we investigated the anticancer effect of galangin on CRT-positive cancer cells using cell-based screening. Galangin promoted the degradation of β-catenin through the APC/Axin/GSK-3β-independent pathway. We also identified the required chemical structure in galangin-mediated β-catenin degradation and inhibition of CRT-positive cancer cell proliferation. Although galangin exhibited anticancer activity against CRT-positive cancer cells at high concentrations, our structure-activity data may facilitate the development of effective cancer-preventive agents and antineoplastic therapeutics for CRT-positive cancer.

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**Authorship Contributions**

*Participated in research design:* Gwak, J. Oh, and S. Oh.
*Conducted experiments:* Gwak, J. Oh, and Cho.
*Contributed new reagents or analytic tools:* Liu, Jeong, and Chung.
*Performed data analysis:* Bae and Song.
*Wrote or contributed to the writing of the manuscript:* Kim and S. Oh.
*Other:* S. Oh acquired funding for the research.

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