Anti-Proliferative Activity of Nodosin, a Diterpenoid from Isodon serra, via Regulation of Wnt/β-Catenin Signaling Pathways in Human Colon Cancer Cells

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Abstract

Colorectal cancer (CRC) is one of the most malignant types of cancers and its incidence is steadily increasing, due to life style factors that include western diet. Abnormal activation of canonical Wnt/β-catenin signaling pathway plays an important role in colorectal carcinogenesis. Therefore, targeting Wnt/β-catenin signaling has been considered a crucial strategy in the discovery of small molecules for CRC. In the present study, we found that Nodosin, an ent-kaurene diterpenoid isolated from Isodon serra, effectively inhibits the proliferation of human colon cancer HCT116 cells. Mechanistically, Nodosin effectively inhibited the over-activated transcriptional activity of β-catenin/T-cell factor (TCF) determined by Wnt/β-catenin reporter gene assay in HEK293 and HCT116 cells. The expression of Wnt/β-catenin target genes such as Axin2, cyclin D1, and survivin were also suppressed by Nodosin in HCT116 cells. Further study revealed that a longer exposure of Nodosin induced the G2/M phase cell cycle arrest and subsequently apoptosis in HCT116 cells. These findings suggest that the anti-proliferative activity of Nodosin in colorectal cancer cells might in part be associated with the regulation of Wnt/β-catenin signaling pathway.

Key Words: Nodosin, Isodon serra, Wnt/β-catenin signaling pathway, Anti-proliferative activity, Apoptosis, Colon cancer cells

INTRODUCTION

During the last decades, the incidence and mortality of colorectal cancer (CRC) have gradually increased and among all cancers worldwide, CRC is now ranked third (Maharian et al., 2019; Siegel et al., 2019). However, current chemotherapies for CRCs, such as 5-fluorouracil and irinotecan have shown the limitation of a relative lack of selectivity against cancer cells compared to normal cells, and the occurrence of acquired drug resistance after repeated exposures of the drug to patients (Yao et al., 2017). Therefore, there is still the urgently necessity to discover novel small molecules for CRCs.

The aberrant activation of canonical Wnt/β-catenin signaling pathways is frequently found in CRC patients with a relatively poorer clinical outcome (Fodde et al., 2001; Yoshida et al., 2015). The Wnt/β-catenin signaling is an evolutionarily conserved pathway that regulates critical biological processes such as cell fate determination, migration, polarity, and organogenesis (Reya and Clevers, 2005; Komiya and Habas, 2008). In the absence of the Wnt ligand, Axin (Lee et al., 2003), glycogen synthase 3β (GSK-3β), and adenomatous polyposis coli (APC) form destruction complex to induce phosphorylation of β-catenin (Behrens et al., 1998) and β-catenin undergoes proteasomal degradation by the β-TRCP and E3 ubiquitin ligase subunit (He et al., 2004). This event subsequently hinders the translocation of β-catenin into nucleus and further its binding with T cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins which blocks the capacity of the transcriptional activity (Tolwinski and Wieschaus, 2004; MacDonald et al., 2009). However, when Wnt proteins bind to a receptor complex consisting of a member of the Frizzled family, and the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) (Hua et al., 2018), this results in the subsequent recruitment and phosphorylation of the Dishevelled protein (DVL). This in turn induces the translocation of β-catenin into nucleus and initiates the transcription of its downstream tar-
get genes (Kobayashi et al., 2000; Komiya and Habas, 2008). Defects of the Wnt signaling components such as mutations of APC, Axin, or β-catenin itself result in β-catenin stabilization (Yang et al., 2016), and thus these events are known to contribute to carcinogenesis of many types of cancers, especially colorectal cancer (Pai et al., 2017; Schatoff et al., 2017). In view of this, the discovery of compounds that specifically target the Wnt signaling pathway is considered to be a strategy to treat colorectal cancers.

*Isodon serra* (Labiatae) has been known as a traditional Chinese medicine for the treatment of toxification and inflammation (Sun et al., 2006). *Isodon serra* has also been used for diseases such as cholecystitis and the protection of liver disorder (Wang et al., 2012; Chen et al., 2014). Phytochemical study revealed that Nodosin is one of the major components in the aerial parts of *I. serra* (Yan et al., 2008). Previous studies for the biological activities of Nodosin exhibited anti-inflammatory (Li et al., 2010), anti-melanogenesis (Satooka et al., 2012), and immunosuppressive effects (Zhang et al., 2005). Nodosin was also reported with the perfusion protection of liver by induction of the cytoprotective enzyme heme oxygenase-1 in *vivo* (Wang et al., 2012).

In our continuous study to explore the anticancer agents from natural products, the extract of *Isodon serra* was found to exhibit a potential growth-inhibitory activity against human cancer cell lines. Although the extract and its isolated compounds have previously exerted several pharmacological effects including anti-inflammatory activity, the anti-proliferation and its underlying mechanisms of actions in cancer cells have not been reported yet. In the present study, we found that Nodosin, a major component of the plant, exhibited a potent anti-proliferative activity in a panel of cancer cell lines. In particular, the human colorectal cancer cell HCT116 was the most sensitive to Nodosin among the tested cell lines. Since the aberrant activation of canonical Wnt/β-catenin signaling pathway was highly associated with colorectal cancer cells, therefore, we employed the HCT116 colon cancer cells (β-catenin mutated cell line) for elucidation of the anti-proliferative activity and its underlying molecular mechanisms of actions with Nodosin in the anti-proliferative activity of the cancer cells.

**MATERIALS AND METHODS**

**Reagents and chemicals**

Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, antibiotic/antimycotic solution, TRI reagent, Lipofectamine 2000 and trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), sulfurhodamine B (SRB), trichloroacetic acid (TCA), bovine serum albumin (BSA), isopropanol and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The luciferase reporter plasmids (TOPflash and FOPflash), Renilla luciferase reporter vectors, and pcDNA β-catenin- and TCF4-expression vectors were obtained from Upstate Biotechnology (Lake Placid, NY, USA). The Reverse Transcription Kit was purchased from Toyobo (Osaka, Japan). The Dual Luciferase Reporter Assay System was purchased from Promega (Madison, WI, USA). The IQ SYBR Green Supermix was obtained from Bio-Rad Laboratories (Hercules, CA, USA). For real-time PCR, the gene-specific primers were designed by Roche (Basel, Switzerland) and synthesized by Bioneer (Daejeon, Korea). The goat anti-rabbit IgG-HRP, anti-mouse IgG-HRP, anti-β actin, anti-TCF4, anti-cyclin B1, anti-cyclin A, anti-CDK2, anti-CDC2, anti-PARP, anti-p21 and anti-p27 were purchased from Santa Cruz Biotechnology, Inc (Dallas, TX, USA). Anti-β-catenin, anti-survivin, anti-Axin1, anti-Axin2, anti-c-Myc, anti-cyclin D, anti-GSK-3β, anti-cleaved caspase3 and anti-cleaved caspase9 were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-cleaved poly (ADP-ribose) polymerase (PARP) and Annexin V-Fluoscein isothiocyanate (Annexin V-FITC) Apoptosis Detection Kit I were purchased from BD Bioscience (Franklin Lakes, NJ, USA).

**Isolation of Nodosin from *Isodon serra***

Dried whole plant materials (1,150 g) were pulverized and then extracted with MeOH (twice 10 L each) in room temperature (RT), yielding a crude extract of 123.2 g. The crude extract (119.8 g) was dissolved in deionized water (500 mL) and then partitioned with EtOAc (500 mL×2), furnishing 35 g of EtOAc soluble extract. A portion of the EtOAc-soluble extract (32 g) was subjected to silica gel column chromatography (620 g) eluted with a gradient mixture of chloroform and MeOH (100:0 to 1:100) and gave 11 subfractions (JaE1-JaE11). JaE3 (10.4 g) was subjected to silica gel column chromatography (200 g), eluting with a gradient mixture of chloroform and MeOH (40:1 to 0:1), and furnished 16 subfractions (JaE3-1 to JaE3-16). From JaE3-14, Nodosin (23.2 mg, Fig. 1A) was precipitated. The structure of Nodosin was confirmed by comparing the measured values with the published values (Yan et al., 2008).

**Cell culture**

Human embryonic kidney cells (HEK293), human normal lung fibroblast cell (MRC-5), human colorectal cancer cells (HCT116), human breast cancer cells (MDA-MB-231), human lung cancer cells (A549), human liver cancer cells (SK-HEP-1), and human gastric cancer cells (SNU-638) were purchased from the American Type Culture Collection (Manassas, VA, USA). HEK293, MRC5, MDA-MB-231 and SK-HEP-1 were maintained in DMEM, while HCT116, A549 and SNU-638 were cultured in RPMI-1640 media supplemented with 10% FBS and antibiotics-antimycotics (PSF: 100 units/mL sodium penicillin G, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B) (Thermo-Scientific, MA, USA) in a humidified incubator containing 5% CO₂ at 37°C.

**Cell proliferation assay**

Cells were seeded in 96-well plates and then treated with samples for 24, 48, or 72 h. After incubation, the cells were fixed with 10% TCA for 30 min, dried overnight, and stained with 0.4% SRB in 1% acetic acid for 2 h. The unbound dye was washed out using 1% acetic acid, after which the stained cells were dried and then were resuspended in 10 mM Tris (pH 10.0) (Kang et al., 2016). The absorbance value was measured at 515 nm, and the cell proliferation rates were determined using the following equation: cell proliferation (%)=(average absorbancecontrol–average absorbance day zero)×100 (Kim et al., 2018). The IC₅₀ values were calculated using non-linear regression analysis using TableCurve 2D v5.01 software (Systat Software Inc., San Jose, CA, USA).
Transfection and luciferase reporter gene assays

Transient transfections were conducted using Lipofectamine 2000 (Invitrogen). HEK293 and HCT116 cells were seeded in 48-well plates, and then the cells were transfected with 0.1 µg of a luciferase reporter plasmid (TOPflash or FOPflash) and 0.005 µg of the Renilla luciferase vector for normalization. HEK293 cells were also co-transfected with 0.02 µg of the pcDNA β-catenin expression vector and 0.004 µg of the TCF4 expression vector to activate the Wnt pathway (Kang et al., 2012; Kim et al., 2017). After 24 h transfection, the cells were treated with a test compound. After incubation for an additional 24 h, the cells were lysed and were subjected to a dual luciferase activity assay using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. pTOP/FOPflash and Renilla reporter plasmids were obtained from Upstate Biotechnology. TCF4 and pc-β-catenin expression plasmids were gifted by Dr. Meeldijk of the University Medical Center (Kim et al., 2017). The levels of luciferase activity were measured and calculated relative to that of the vehicle control.

RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA was extracted from cells using TRI reagent (Invitrogen). RNA extracts were reverse-transcribed using the Toyobo Reverse Transcription System (Toyobo). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer’s instructions. The primer sequences were as follows: CCND1 (sense: 5′-GAA GAT CGT CGC CAC CTG-3′/antisense: 5′-GAC CTC CTC GCA CT CT CT CT-3′), Axin2 (sense: 5′-CAA CAC CAG GCG GAA CGA A-3′/antisense: 5′-GCC CAA TAA GGA GTG TAA GGA CT-3′), BIRC5 (sense: 5′-CCG ACG TTG CCC CCT GC-3′/ antisense: 5′-TCG ATG GCA CGG CGC AC-3′), CTNNB1 (sense: 5′-GTC TTA CCT GTA CTC TTC TCT GCA ATG AG-3′), CTNB1 (sense: 5′-GTC TTA CCT GTA CTC TTC TCT GCA ATG AG-3′), ATCB (sense: 5′-CCA ACC GGC AGA TGA-3′/ antisense: 5′-CCA GAG GGC TAC AG GAT AG-3′).

Western blotting analysis

Total cell lysates were mixed with lysis buffer, and boiled for 10-20 min at 100°C. The protein concentrations of the cell lysates were determined by the BCA protein assay (Byun et al., 2019). Samples containing equal amounts of protein
were subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were electrically transferred to PVDF membranes (Millipore, Darmstadt, Germany) activated with 100% methanol. The membranes were blocked using 5% bovine serum albumin (BSA, Sigma-Aldrich) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 30 min at least at room temperature. The desired primary antibodies were diluted in 2.5% BSA (1:500-1:10,000) overnight at 4°C. The membranes were washed three times with PBST and were then incubated with the appropriate secondary antibodies diluted in TBST for 2 h at room temperature. The membranes were washed three times with PBST, and then were visualized using an enhanced chemiluminescence detection kit (Intron Biotechnology, Sungnam, Korea). The membranes were analyzed using an ImageQuant LAS 4000 imager (Fujifilm Corp., Tokyo, Japan).

Flow cytometry analysis of cell cycle distribution

The cells were seeded at a density of 2×10⁶ cells in 100 mm cell culture dish. After 24 h incubation, the cells were treated with Nodosin and washed with PBS, and then the cell pellets were fixed with 70% ethanol overnight at −20°C. Fixed cells were pelleted and washed with PBS. The cells were resuspended in 100 µg/mL RNase A and 50 µg/mL of propidium iodide (PI) in the dark for 30 min at room temperature. The DNA content of the fluorescence binding cells was analyzed using a flow cytometry FACScalibur flowcytometer (BD Bioscience, Franklin Lakes, NJ, USA). The distribution of cell contents was measured with 5,000 cells in each group and the results were represented as histograms of the DNA content (Jung et al., 2015; Byun et al., 2018).

Annexin V-FITC/propidium iodide (PI) double staining

The cells were treated with Nodosin for 48 h and then stained with Annexin V-FITC and PI using an annexin V-FITC apoptosis detection kit (BD Bioscience) according to the manufacturer’s instruction. Briefly, the incubated cells were harvested and resuspended with 1× binding buffer. Annexin V-FITC and PI (5 µL) were added to cell suspensions and further incubated in the dark for 15 min at room temperature. Stained cells were immediately analyzed with 1× binding buffer using a flow cytometer (Kim et al., 2016).

Statistical analysis

Data are presented as the mean values ± standard deviation for the indicated number of independently performed experiments. All data are representative of the results of at least three independent experiments. Statistical significance (*p<0.05, **p<0.01, ***p<0.001) was assessed using Student’s t-test, or one-way analysis of variance coupled with Dunnett’s t-test.

**Table 1.** Anti-proliferative activity of Nodosin in a panel of human cancer cell lines

|          | SNU638 | SK-HEP-1 | A549  | HCT116 | MDA-MB-231 | MRC-5 |
|----------|--------|----------|-------|--------|------------|-------|
| Nodosin  | 7.53   | 5.33     | 13.73 | 4.05   | 7.12       | >20   |
| Etoposide| 0.39   | 0.77     | 0.25  | 0.21   | 7.1        | >20   |

* indicates: (1) SNU638 (stomach), SK-HEP-1 (liver), A549 (lung), HCT116 (colon), MDA-MB-231 (breast), MRC-5 (normal lung epithelial cell); (2) Etoposide was used as a positive control.

**RESULTS**

Effect of Nodosin on cancer cell proliferation

To evaluate whether Nodosin inhibits the growth of human cancer cells, a cell proliferation assay was conducted in a panel of cancer cell lines. Nodosin effectively inhibited the growth of various solid cancer cell lines. Among the cancer cell lines tested, the HCT116 colon cancer cells were shown to be the most sensitive in the growth-inhibitory activity of Nodosin with the IC₅₀ values of 4.05 µM after 72 h incubation (Table 1). In addition, Nodosin did not significantly inhibit the growth of MRC5, a normal lung fibroblast cell line (IC₅₀ value >20 µM), suggesting that Nodosin exhibits a relatively selective growth inhibition against cancer cells compared to a normal cell. We further extended to determine the growth inhibitory activity of Nodosin in the HCT116 cells after incubation of 24 h and 48 h treatment. Nodosin showed growth inhibition of the cells in a time- and concentration-dependent manner (Fig. 1B). The morphological change of the cancer cells treated with Nodosin was also observed under phase-contrast microscopy after 24 h treatment. The treatment of Nodosin (30 µM) evoked morphological changes with rounded shapes and the cells were floated in culture media (Fig. 1C). Moreover, the effects of Nodosin on colony formation were evaluated in the HCT116 cells. The cells were treated with Nodosin for 72 h, and then media were replaced two or three times per week for approximately 2 weeks. As shown in Fig. 1D, Nodosin significantly suppressed colony formation in the HCT116 cells. These data suggest that Nodosin effectively inhibits cancer cell proliferation and colony formation in human colorectal cancer cells.

Suppressive effect of Nodosin on the Wnt signaling pathways in HCT116 cells

To further confirm whether the growth-inhibitory activity of Nodosin against HCT116 cells is associated with the suppression of the Wnt signaling pathways, the effect of Nodosin on Wnt/β-catenin-mediated transcriptional activity was primarily evaluated. Using the T-cell factor (TCF) reporter gene (TOPflash), luciferase reporter gene assay was conducted in HCT116 cells and HEK293 cells. Nodosin significantly inhibited TOPflash activity in the TCF/β-catenin-stimulated luciferase activity in HEK293 cells (Fig. 2A). In addition, Nodosin also effectively suppressed overactivated TOPflash activity in the intrinsic β-catenin-mutated HCT116 colon cancer cells (Fig. 2B). We further detected the effect of Nodosin on the nuclear localization of β-catenin in HCT116 cells. As shown in Fig. 2C, immunocytochemical analysis revealed that overexpression of β-catenin in the nuclear fractions was significantly suppressed by the treatment of Nodosin (30 µM) in HCT116 cells. Real-time PCR analysis also suggested that the suppression of Wnt/β-catenin signaling by Nodosin is involved in the down-regulation of mRNA expression of Wnt target genes.
such as CCND1 (cyclin D1), AXIN2, BIRC5 (survivin), and CTNNB1 (β-catenin), which are key regulators of cancer cell proliferation, survival, and progression (Fig. 2D). Furthermore, Nodosin also significantly suppressed Wnt/β-catenin target protein expressions including β-catenin, p-GSK-3β, c-Myc, and Survivin in HCT116 cells (Fig. 2E). Collectively, these data indicated that the growth-inhibitory activity of Nodosin against colorectal cancer cells may be in part associated with the effective suppression of endogenously activated Wnt signaling pathways in HCT116 colorectal cancer cells.

Induction of G2/M cell cycle arrest by Nodosin in HCT116 cells

The Wnt signaling pathway plays a pivotal role in cell proliferation and survival. To further clarify the involvement of anti-proliferative activity of Nodosin with the modulation of the Wnt signaling pathway, we primarily determined cell cycle distribution with the treatment of Nodosin in HCT116 cells. Flow cytometry analysis revealed that the treatment of Nodosin for 24 h in cancer cells significantly induced G2/M phase cell cycle arrest in a concentration-dependent manner. In particular, the cell populations of G2/M phase were manifestly increased from 13.87% in the vehicle-treated control cells, to 45.88% in the Nodosin (30 μM)-treated cells (Fig. 3A). The cell cycle is finely controlled by the regulation of cell cycle checkpoint proteins.

In the G2/M phase, the SCF E3 ubiquitin ligase consisting of S phase kinase-associated protein 1 (Skp1) and cullin-1 (Cul-1), and a member of the F-box protein, are actively involved in the cell cycle progression (Willems et al., 2004). Therefore, we determined whether Nodosin affected Skp2 in cancer cells. Nodosin effectively suppressed the expression of Skp2 protein levels in HCT116 cells. Further study revealed that additional checkpoint protein expressions in the G2/M phase were also modulated by Nodosin: the cyclin-dependent kinase inhibitors p21 and p27 protein expressions were up-regulated, while the expressions of checkpoint proteins cyclin B1 and cdc25C were down-regulated (Fig. 3B). These data suggest that the anti-proliferative activity of Nodosin in cancer cells may in part be associated with cell cycle arrest followed by inhibition of the Wnt signaling pathway in colon cancer cells.

Induction of apoptosis by Nodosin in HCT116 cells

To further evaluate whether Nodosin is able to induce apoptosis for prolonged exposure in HCT116 cells, the cells were treated with Nodosin for 48 h. Flow cytometric analysis was performed after double staining with Annexin V-FITC/PI. As shown in Fig. 4A, the populations of apoptotic cells including early and late apoptosis were induced in a concentration-dependent manner. In particular, Nodosin significantly exhibited the population of apoptotic cell death with up to 61.3%
A body of evidence suggests that the constitutive activation of Wnt signaling is essential for the growth and survival of colorectal cancer cells (Zhan et al., 2017). Therefore, the
dependent pathway in HCT116 cells.

DISCUSSION

A body of evidence suggests that the constitutive activation of Wnt signaling is essential for the growth and survival of colorectal cancer cells (Zhan et al., 2017). Therefore, the
Wnt signaling pathway is considered an attractive target in the development of novel anticancer agents for treating colon cancers. In the present study, we explored whether the growth-inhibitory activity of Nodosin against colon cancer HCT116 cells is associated with the suppression of canonical Wnt/β-catenin signaling pathways in the cancer cells. Nodosin is an ent-kaurene diterpenoid isolated from the herb of Isoodon serra, and a few of its biological activities such as anti-inflammatory (Li et al., 2010), anti-melanogenesis (Satooka et al., 2012), and immune-suppressive activity were reported (Zhang et al., 2005). The present study showed that Nodosin effectively inhibits the proliferation and colony formation of HCT116 colon cancer cells without affecting the growth-inhibitory activity against normal human lung fibroblast MRC-5 cells, indicating the selective anti-proliferative activity of Nodosin for cancer cells, compared to normal cells. Based on these findings, further study was designed to elucidate the involved mechanisms of actions for the growth-inhibitory activity of Nodosin in HCT116 colon cancer cells, a colon cancer cell line expressing β-catenin-mutation. Primarily, a luciferase reporter gene assay revealed that Nodosin significantly suppressed TCF/β-catenin-responsive transcriptional activity in HEK293 cells. In addition, the treatment of Nodosin also effectively inhibited the intrinsic overactivated transcriptional activity with a β-catenin mutation in HCT116 cells. The down-regulation of transcriptional activity by Nodosin subsequently suppressed the expressions of Wnt signaling components, such as β-catenin, axin, and GSK-3β. In particular, Nodosin significantly suppressed the expression of Axin2 mRNA and protein levels in a concentration-dependent manner. Although the function of Axin2 on regulation of Wnt/β-catenin signaling pathways is multifactorial, it is also involved in the regulation of cell cycle. Since Nodosin modulates Axin2, we extended investigation to evaluate the effect of Nodosin on cell cycle distribution in the cancer cells. Nodisin exhibited G2/M phase cell cycle arrest and this event was in part correlated with the regulation of checkpoint proteins including Skp2, p21, and p27, in HCT116 cells. Further study revealed that the longer exposure of Nodosin in the cancer cells is able to induce apoptotic cell death, which is determined by the up-regulation of cleaved caspase-9 and cleaved PARP levels, which is the mitochondrial-mediated apoptosis pathway.

In summary, the present study for the first time demonstrates that Nodosin, a natural diterpenoid from Isoodon serra, has the potential to inhibit the proliferation of colon cancer cells through effective suppression of the Wnt/β-catenin signaling pathways. Therefore, Nodosin might serve as a Wnt inhibitor for the development of anticancer agents targeting colon cancers with modulation of the Wnt/β-catenin signaling pathways.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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REFERENCES

Behrens, J., Jerchow, B. A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D. and Birringer, W. (1998) Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science 280, 596-599.

Byun, W. S., Kim, W. K., Han, H. J., Chung, H. J., Jang, K., Kim, H. S., Kim, S., Kim, D., Bae, E. S., Park, S., Lee, J., Park, H. G. and Lee, S. K. (2019) Targeting histone methyltransferase DOT1L by a novel psammaplin A analog inhibits growth and metastasis of triple-negative breast cancer. Mol. Ther. Oncolytics. 15, 140-152.

Byun, W. S., Jin, M., Yu, J., Kim, W. K., Song, J., Chung, H. J., Jeong, L. S. and Lee, S. K. (2018) A novel selenonucleoside suppresses tumor growth by targeting Skp2 degradation in paclitaxel-resistant prostate cancer. Biochem. Pharmacol. 158, 84-94.

Chen, C., Chen, Y., Zhu, H., Xiao, Y., Zhang, X., Zhao, J. and Chen, Y. (2014) Effective compoundscreening from Rabdosia serra (Maxim) HaraagainstHBV and tumor in vitro. Int. J. Clin. Exp. Med. 7, 384-392.

Fodde, R., Smits, R. and Clevers, H. (2001) APC, signal transduction and genetic instability in colorectal cancer. Nat. Rev. Cancer. 1, 55-67.

He, X., Semenov, M., Tamai, K. and Zeng, X. (2004) LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. Development 131, 1663-1677.

Hua, Y., Yang, Y., Li, Q., He, X., Zhu, W., Wang, J. and Gan, X. (2018) Oligomerization of Frizzled and LRPs/5/6 protein initiates intracellular signaling for the canonical Wnt/β-catenin pathway. J. Biol. Chem. 51, 19710-19724.

Jung, C., Hong, J., Bae, S. Y., Kang, S. S., Park, H. J. and Lee, S. K. (2015) Antitumor activity of americanin A isolated from the seeds of Phytolacca americana by regulating the ATM/ATR signaling pathway and the Skp2-p27 axis in human colon cancer cells. J. Nat. Prod. 78, 2983-2993.

Kang, J. Y., Hong, J. Y., Choi, J. S. and Lee, S. K. (2016) Columbianadin inhibits cell proliferation by inducing apoptosis and necroptosis in HCT116 colon cancer cells. Biomol. Ther. (Seou) 24, 320-327.

Kang, Y. P., Park, H. J., Chung, H. J., Min, H. Y., Park, E. J., Lee, M. A., Shin, Y. and Lee, S. K. (2012) Wnt/β-catenin signaling mediates the antitumor activity of magnolol in colorectal cancer cells. Mol. Pharmacol. 82, 168-177.

Kim, W. K., Bach, D. H., Ryu, H. W., Oh, J., Park, H. J., Hong, J. Y., Song, H. H., Eum, S., Bach, T. T. and Lee, S. K. (2017) Cytotoxic activities of Telectadium dongnaiense and its constituents by inhibition of the Wnt/β-catenin signaling pathway. Phytotherapy 34, 136-142.

Kim, W. K., Byun, W. S., Chung, H. J., Oh, J., Park, H. J., Choi, J. S. and Lee, S. K. (2018) Esculetin suppresses tumor growth and metastasis by targeting Axin2/E-cadherin axis in colorectal cancer. Biochem. Pharmacol. 152, 71-83.

Kim, W. K., Pyee, Y., Chung, H. J., Park, H. J., Hong, J. Y., son, K. H. and Lee, S. K. (2016) Antitumor activity of spicatoside A by modulation of autophagy and apoptosis in human colorectal cancer cells. J. Nat. Prod. 79, 1097-1104.

Kobayashi, M., Honma, T., Matsuda, Y., Suzuki, Y., Narisawa, R., Ajo, Y. and Asakura, H. (2000) Nuclear translocation of beta-catenin in colorectal cancer. Br. J. Cancer. 82, 1689-1693.

Komiya, Y. and Habas, R. (2006) Wnt signal transduction pathways. Organogenesis 4, 68-75.

Lee, E., Salic, A., Krüger, R., Heinrich, R. and Kirschner, M. W. (2003) The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. PloS Biol. 1, e10.

Li, J., Du, J., Sun, L., Liu, J. and Quan, Z. (2010) Anti-inflammatory function of Nodosin via inhibition of IL-2. Am. J. Chin. Med. 38, 127-142.

MacDonald, B. T., Tamai, K. and He, X. (2009) Wnt/β-catenin signaling: components, mechanisms, and diseases. Dev. Cell 17, 9-26.
Maharjan, S., Park, B. K., Lee, S. I., Lim, Y., Lee, K., Lee, Y. and Kwon, H. J. (2019) Gomisin G suppresses the growth of colon cancer cells by attenuation of AKT phosphorylation and arrest of cell cycle progression. *Biomol. Ther. (Seoul)* **27**, 210-215.

Pai, S. G., Carneiro, B. A., Mota, J. M., Costa, R., Leite, C. A., Barros-Sousa, R., Kaplan, J. B., Chae, Y. K. and Giles, F. J. (2017) Wnt/β-catenin pathway: modulating anticancer immunoresponse. *J. Hematol. Oncol.* **10**, 101-112.

Reya, T. and Clevers, H. (2005) Wnt signalling in stem cells and cancer. *Nature* **434**, 843-850.

Satooka, H., Isobe, T., Nitoda, T. and Kubo, I. (2012) Melanogenesis inhibitors from Rabdosia japonica. *Phytotherapy Research* **19**, 1016-1023.

Schatoff, E. M., Leach, B. I. and Dow, L. E. (2017) Wnt signaling and colorectal cancer. *Curr. Colorectal. Cancer Rep.* **13**, 101-110.

Siegel, R. L., Miller, K. D. and Jemal, A. (2019) Cancer statistics, 2019. *CA Cancer J. Clin.* **69**, 7-34.

Sun, H. D., Hsuang, S. X. and Han, Q. B. (2006) Diterpenoids from *Isodon* species and their biological activities. *Nat. Prod. Rep.* **23**, 673-698.

Tolwinski, N. S. and Wieschaus, E. A. (2004) A nuclear function for armadillo-beta-catenin. *PLoS Biol.* **2**, e95.

Wang, C. F., Wang, Z. Y., Tao, S. F., Ding, J., Sun, L. J., Li, J. Y. and Quan, Z. W. (2012) Preconditioning donor liver with Nodosin perfusion lessens rat ischemia reperfusion injury via heme oxygenase-1 upregulation. *J. Gastroenterol. Hepatol.* **27**, 832-840.

Willems, A. R., Schwab, M. and Tyers, M. (2004) A hitchhiker’s guide to the cullin ubiquitin ligases: SCF and its kin. *Biochim. Biophys. Acta* **1695**, 133-170.

Yan, F. L., Guo, L. Q., Bai, S. P. and Sun, H. D. (2008) Two new diterpenoids and other constituents from Isodon nervosus. *J. Chin. Chem. Soc.* **55**, 933-936.

Yang, K., Wang, X., Zhang, H., Wang, Z., Nan, G., Li, Y., Zhang, F., Mohammed, M. K., Haydon, R. C., Liu, H. H., Bi, Y. and He, T. C. (2016) The evolving roles of canonical WNT signaling in stem cells and tumorigenesis: implications in targeted cancer therapies. *Lab. Invest.* **96**, 116-136.

Yao, C. W., Kang, K. A., Piao, M. J., Ryu, Y. S., Fernando, P. M. D. J., Oh, M. C., Park, J. E., Shihinikova, K., Na, S. Y., Jeong, S. U., Boo, S. J. and Hyun, J. W. (2017) Reduced autophagy in 5-fluorouracil resistant colon cancer cells. *Biomol. Ther. (Seoul)* **25**, 315-320.

Yoshida, N., Kinugasa, T., Ohshima, K., Yuge, K., Ohchi, T., Fujino, S., Shiraizawa, S., Katagiri, M. and Akagi, Y. (2015) Analysis of Wnt and β-catenin expression in advanced colorectal cancer. *Anticancer Res.* **35**, 4403-4410.

Zhan, T., Rindtorff, N. and Boutros, M. (2017) Wnt signaling in cancer. *Oncogene* **36**, 1461-1473.

Zhang, Y., Liu, J., Jia, W., Zhao, A. and Li, T. (2005) Distinct immunosuppressive effect by *Isodon serra* extracts. *Int. Immunopharmacol.* **5**, 1957-1965.