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Murrayafoline A attenuates the Wnt/β-catenin pathway by promoting the degradation of intracellular β-catenin proteins

Hyuk Choi [a], Jungsug Gwak [a], Munju Cho [a], Min-Jung Ryu [a], Jee-Hyun Lee [b], Sang Kyum Kim [b], Young Ho Kim [b], Gye Won Lee [c], Mi-Young Yun [d], Nguyen Manh Cuong [e], Jae-Gook Shin [a], Gyu-Yong Song [b,*], Sangtaek Oh [a,*]

[a] PharmacoGenomics Research Center, Inje University, Busan 614-735, Republic of Korea
[b] College of Pharmacy, Chungnam National University, Daejeon 305-764, Republic of Korea
[c] Department of Pharmaceutical Engineering, Konyang University, Nonsan 320-711, Republic of Korea
[d] Department of Beauty Health Care, Daejeon University, Daejeon 305-764, Republic of Korea
[e] Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology, Hanoi, Viet Nam

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ABSTRACT

Molecular lesions in Wnt/β-catenin signaling and subsequent up-regulation of β-catenin response transcription (CRT) occur frequently during the development of colon cancer. To identify small molecules that suppress CRT, we screened natural compounds in a cell-based assay for detection of TOPFalsh reporter activity. Murrayafoline A, a carbazole alkaloid isolated from *Glycosmis stenocarpa*, antagonized CRT that was stimulated by Wnt3a-conditioned medium (Wnt3a-CM) or LiCl, an inhibitor of glycogen synthase kinase-3β (GSK-3β), and promoted the degradation of intracellular β-catenin without altering its N-terminal phosphorylation at the Ser33/37 residues, marking it for proteasomal degradation, or the expression of Siah-1, an E3 ubiquitin ligase. Murrayafoline A repressed the expression of *cyclin D1* and *c-myc*, which is known β-catenin/T cell factor (TCF)-dependent genes and thus inhibited the proliferation of various colon cancer cells. These findings indicate that murrayafoline A may be a potential chemotherapeutic agent for use in the treatment of colon cancer.

Introduction

β-catenin is an essential component of both cell–cell adherent junctions and the Wnt/β-catenin signaling, which play an important roles in cellular proliferation, morphology, motility, fate, axis formation, and organ development [1–3]. The level of intracellular β-catenin, which is a key control of Wnt/β-catenin pathway, is regulated by phosphorylation-dependent proteasomal degradation pathway. Casein kinase 1 (CK 1) and glycogen synthase kinase-3β (GSK-3β) sequentially catalyzed β-catenin phosphorylation at Ser45, Thr 41, Ser37, and Ser33 in a complex with adenomatous polyposis coli (APC) and Axin [4,5]. In addition, PKA/GSK-3β, cyclin-dependent kinase2 (CDK2)/cyclin E, and protein kinase Cα (PKCα) phosphorylate these N-terminal residues of β-catenin [6,8]. Phosphorylated β-catenin is then recognized by F-box β-transducin repeat-containing protein (β-TrCP), a component of ubiquitin ligase complex, leading to its ubiquitin-dependent proteasomal degradation [9,10]. Therefore, intracellular β-catenin usually maintains at a low level in a normal cells.

Colon cancer is the most common type of cancer and the third leading cause of cancer—related deaths in Western countries [11]. Besides surgical resection, which is rarely curative in advanced disease, current therapy for colon cancer relies on traditional cytotoxic agents with limited effect. This limitation urges us to develop new therapeutics for colon cancer based on the defined molecular lesion. Mutation that cause the development and progression of colon cancer is well characterized compared to other human tumors [12]. Mutations in the APC gene are identified in familial adenomatous polyposis coli (FAP) [13] and occur in majority of sporadic colorectal cancer. In addition N-terminal phosphorylation motif of the β-catenin is frequently mutated in colorectal cancers [14]. These mutations lead to the excessive accumulation of cytoplasmic β-catenin, which is translocated into the nucleus and then activates the expression of its target genes, such as cyclin D1, myc, matrix metalloproteinase-7, and PPAR-δ, which play important roles in colorectal tumorigenesis [15–18]. Thus, promotion of β-catenin degradation is a potential therapeutic strategy for chemoprevention and treatment of colon cancers.

In this study, we identified murrayafoline A as an antagonist of Wnt/β-catenin signaling using cell-based natural compound screening. Murrayafoline A may inhibit the proliferation of colon cancer cells by promoting the degradation of intracellular β-catenin.

* Corresponding authors. Fax: +82 42 823 6566 (G.-Y. Song); fax: +82 51 893 1232 (S. Oh).
E-mail addresses: gysong@cnu.ac.kr (G.-Y. Song), ohsa@inje.ac.kr (S. Oh).

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Materials and methods

Cell cultures, reporter assays, and chemicals. HEK293, HCT-116, SW480, DLD-1, LS174T, and Wnt3a-secreting L cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 120 μg/ml penicillin, and 200 μg/ml streptomycin. HEK 293 reporter (TOPFlash), control (FOPFlash), and HEK293 reporter (SEAP) cells were established as previously described [19,20]. Wnt3a-conditioned medium (Wnt3a-CM) was prepared as previously described [21]. Luciferase assay was performed using the Dual Luciferase Assay Kit (Promega) and secreted alkaline phosphatase assay was carried out using Phospha-Light™ Assay kit (Applied Biosystems). LiCl and MG-132 were purchased from Sigma–Aldrich.

Isolation of murrayafoline A. Dried powdered roots (1.1 kg) of *Glycosmis stenocarpa* were extracted with MeOH, filtered and evaporated in vacuo. The suspension of the methanol residue in MeOH/H_2O (1:1) was successively extracted with *n*-hexane, chloroform, and butanol to give hexane (30 g), chloroform (17 g), and butanol extracts. The hexane extract (30 g) was chromatographed on silica gel (250 g, Merck silica 80–120 mesh) using a gradient of hexane and EtOAc as eluent to give 19 fractions. Fraction 4 was further rechromatographed on flash silica gel (hexane/EtOAc 10/1 as eluent) to yield murrayafoline A (3.3 g, 3%). Murrayafoline A: Brown oil, $C_{14}H_{13}NO$, *Rf*: 0.25 (hexane/EtOAc, 10:0.5), EI-MS *m/z*: 211 (100%) (M), 196 $(M\text{-}CH_3)^+$, 167, 139, 115, 101, 77. UV l max ($CHCl_3$) nm: 209, 222, 243, 291, 327, 340; 1H- and 13C NMR ($CDCl_3$) data were as reported [22].

Screening for natural compound inhibitor of Wnt/β-catenin signaling. The 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 then the natural compounds were added to the wells. After 15 h, the plates were assayed for firefly luciferase activity and cell viability.

Western blot and antibodies. The cytosolic fraction was prepared as previously described [23]. Proteins were separated using 4–12% gradient SDS–PAGE (Invitrogen) and transferred to PVDF membranes (Amersham Bioscience) by wet blotting. The membranes were blocked with 5% nonfat milk in TBS–T (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) and probed with primary antibodies (1:1000). The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology, 1:2500) and visualized using the ECL chemiluminescence (Santa Cruz Biotechnology). The antibody against β-catenin was purchased from BD Transduction Laboratories. β-Actin and phospho-β-catenin antibodies (Ser33/Ser37/Thr41) were purchased from Cell Signaling Technology.

RNA extraction and semi-quantitative RT-PCR. Total RNA was isolated with TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions, cDNA synthesis, reverse transcription, and PCR were performed as described previously [24]. The amplified DNA was separated on 1.5% agarose gels and stained with ethidium bromide.

Cell viability. SW480, DLD-1, LS174T, and HCT-116 colon cancer cells were inoculated into 96-well plates at 5000 cells and treated with murrayafoline A 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 and discussion

Identification of murrayafoline A as an antagonist of the Wnt/β-catenin pathway

To identify cell-permeable natural compound antagonists of the Wnt/β-catenin signaling, we used a cell-based screening system. HEK reporter cells that are stably harbored TOPFlash, a synthetic

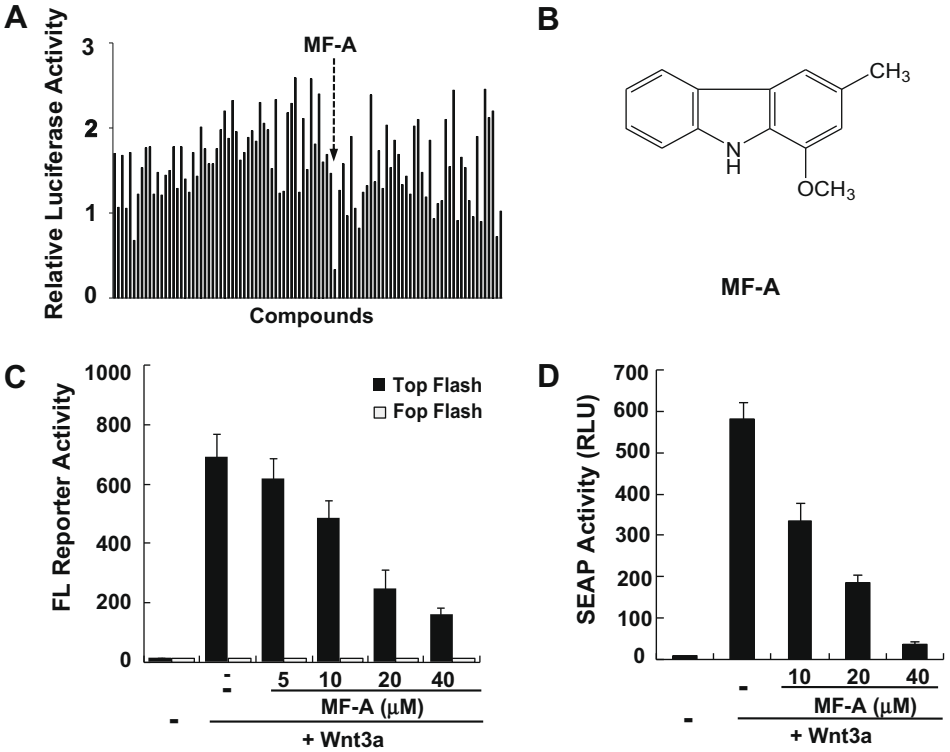

Fig. 1. Identification of murrayafoline A as a small molecule inhibitor of Wnt/β-catenin pathway. (A) Screening of natural compounds that inhibit Wnt/β-catenin pathway. Compounds modulating TOPFlash reporter activity were screened using the HEK293 reporter cells and TOPFlash activities were normalized with Celltiter-Glo (Promega) activity. (B) Chemical structure of murrayafoline A. (C, D) HEK293 reporter (TOPFlash and SEAP) and control cells were incubated with indicated concentrations of murrayafoline A 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.

β-catenin/Tcf-dependent luciferase reporter, and the human Frizzled-1 (hFz-1) expression plasmids were inoculated into 96-well plate. After the addition Wnt3a-conditioned medium (Wnt3a-CM) and each compound, we detected TOPFlash reporter activity using a microplate reader (Fig. 1A). One of the compounds from this screen was murrayafoline A (Fig. 1B). As shown in Fig. 1C, incubation of HEK293 reporter (TOPFlash) cells with murrayafoline A produced a dose-dependent decrease in Wnt3a-CM induced β-catenin response transcription (CRT). In contrast, activity of FOPFlash, a negative control reporter with mutated β-catenin/TCF binding sites, was unchanged by treatment with murrayafoline A and Wnt3a-CM in HEK293 control cells (Fig. 1C). To further confirm the inhibitory effect of murrayafoline A on the Wnt/β-catenin signaling, we used other HEK293 reporter cells, which stably transfected with a synthetic β-catenin/Tcf-dependent secreted alkaline phosphatase (SEAP) reporter and hFz-1 expression plasmids. Consistent with results from the luciferase reporter assay, murrayafoline A inhibited SEAP activity that was upregulated by Wnt3a-CM in a dose-dependent manner (Fig. 1D). Taken together, these results suggest that murrayafoline A is a specific antagonist of Wnt/β-catenin signaling.

Murrayafoline A promotes proteasome-mediated β-catenin degradation

In the Wnt/β-catenin signaling, β-catenin response transcription is largely dependent on the level of intracellular β-catenin, which is regulated by ubiquitin-dependent proteasomal degradation pathway [25,26]. To investigate whether murrayafoline A affects the intracellular β-catenin level, we used Western blot analysis with an anti-β-catenin antibody to determine of the amount of cytosolic β-catenin in murrayafoline A-treated HEK293 reporter (TOPFlash) cells. Consistent with results from reporter assays, murrayafoline A down-regulated the level of cytosolic β-catenin that was accumulated by Wnt3a-CM (Fig. 2A), In contrast to the protein level of β-catenin, mRNA expression of β-catenin was not altered by any of the concentrations of murrayafoline A used (Fig. 2B), suggesting that murrayafoline A suppresses Wnt/β-catenin signaling through reducing cytosolic β-catenin protein level rather than repressing β-catenin gene expression. Next, to examine whether murrayafoline A-induced β-catenin down-regulation is mediated by proteasome, we used MG-132, an inhibitor of proteasome. As shown in Fig. 2C, murrayafoline A-induced consistent decrease in the level of cytosolic β-catenin in HEK293 reporter (TOPFlash) cells; however the effect of murrayafoline A on the reduction of β-catenin was abrogated by the addition of MG-132. These results indicated that murrayafoline A attenuates Wnt/β-catenin signaling via proteasome-mediated β-catenin degradation.

Murrayafoline A induces β-catenin degradation through a mechanism independent of its N-terminal phosphorylation and Siah-1

Since GSK-3β activity is essential in intracellular β-catenin degradation [27], we examined involvement of GSK-3β in murrayafoline A-mediated β-catenin degradation. Consistent with previous results [28], incubation HEK293 reporter (TOPFlash) cells with LiCl, an inhibitor of GSK-3β, led to stimulation of CRT (Fig. 3A). It is interesting that murrayafoline A abolished LiCl-mediated CRT activation (Fig. 3A). Furthermore, Western blot analysis showed that the amount of intracellular β-catenin induced by LiCl was decreased by treatment with murrayafoline A (Fig. 3B), suggesting that murrayafoline A-induced β-catenin degradation is not require β-catenin activity.

It has been reported that phosphorylation of by several kinases, such as protein kinase C and cyclin-dependent kinase 2/cyclin E

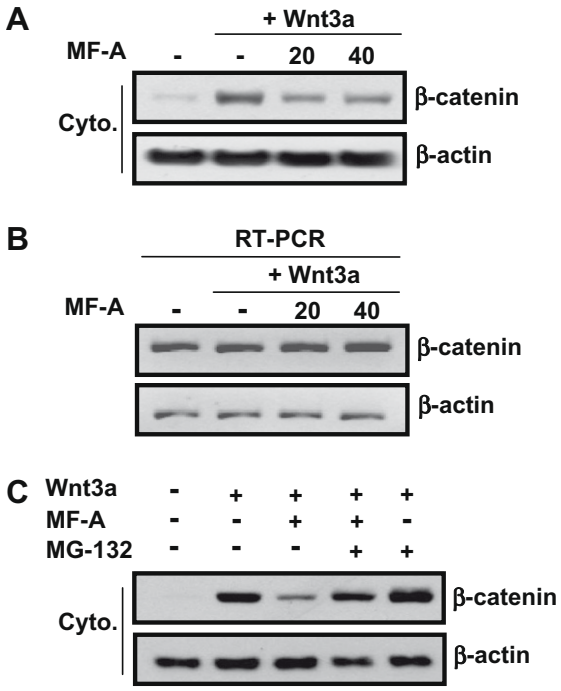

Fig. 2. Murrayafoline A promotes the degradation of β-catenin via a proteasome. (A) Cytosolic proteins were prepared from HEK293 reporter (TOPFlash) cells treated with either vehicle (DMSO) or murrayafoline A in the presence of Wnt3a-CM for 15 h and then subjected to Western blotting with β-catenin antibody. (B) Semi-quantitative RT-PCR for β-catenin, and GAPDH was performed with total RNA prepared from HEK293 reporter (TOPFlash) cells either vehicle (DMSO) or murrayafoline A in the presence of Wnt3a-CM for 15 h. (C) Cytosolic proteins prepared from HEK293 reporter (TOPFlash) cells, which were incubated with vehicle (DMSO) or murrayafoline A (20 μM) in the presence or absence of Wnt3a-CM, exposed to MG-132 (10 μM) for 8 h, were subjected to Western blotting with anti-β-catenin antibody, In (A) and (C), to confirm equal loading, the blots were re-probed with anti-actin antibody.

and its subsequent association with β-TrCP lead to β-catenin degradation [7,8]. To test whether treatment of murrayafoline A induces β-catenin phosphorylation at Ser33/37/41 residues, we used Western blot analysis with anti-phospho-p33/37/41-β-catenin. As shown in Fig. 3C, Wnt3a-CM led to the inhibition of Ser33/37/41 phosphorylation of β-catenin, in agreement with previous reports [4]; however, murrayafoline A was not able to abrogated this inhibition, indicating that β-catenin degradation by murrayafoline A is independent of N-terminal phosphorylation of β-catenin.

As Siah-1 recruits the ubiquitination complex in a complex with adenomatous polyposis coli (APC) to β-catenin, thereby promoting its degradation [29,30], we then determined whether Siah-1 is necessary for murrayafoline A-induced β-catenin degradation using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Consistent with previous report [31], the expression of Siah-1 was increased in the presence of hexachlorophene in HEK293 reporter (TOPFlash) cells (Fig. 3D). In contrast, incubation of HEK293 reporter (TOPFlash) cells with increasing amounts of murrayafoline A did not affect Siah-1 expression (Fig. 3D), suggesting that murrayafoline A promotes the degradation of β-catenin through a Siah-independent mechanism.

Murrayafoline A inhibits proliferation of various colon cancer cells

We next tested whether murrayafoline A also down-regulates intracellular β-catenin in colon cancer cells, which frequently occur mutations that lead to the accumulation of β-catenin, using

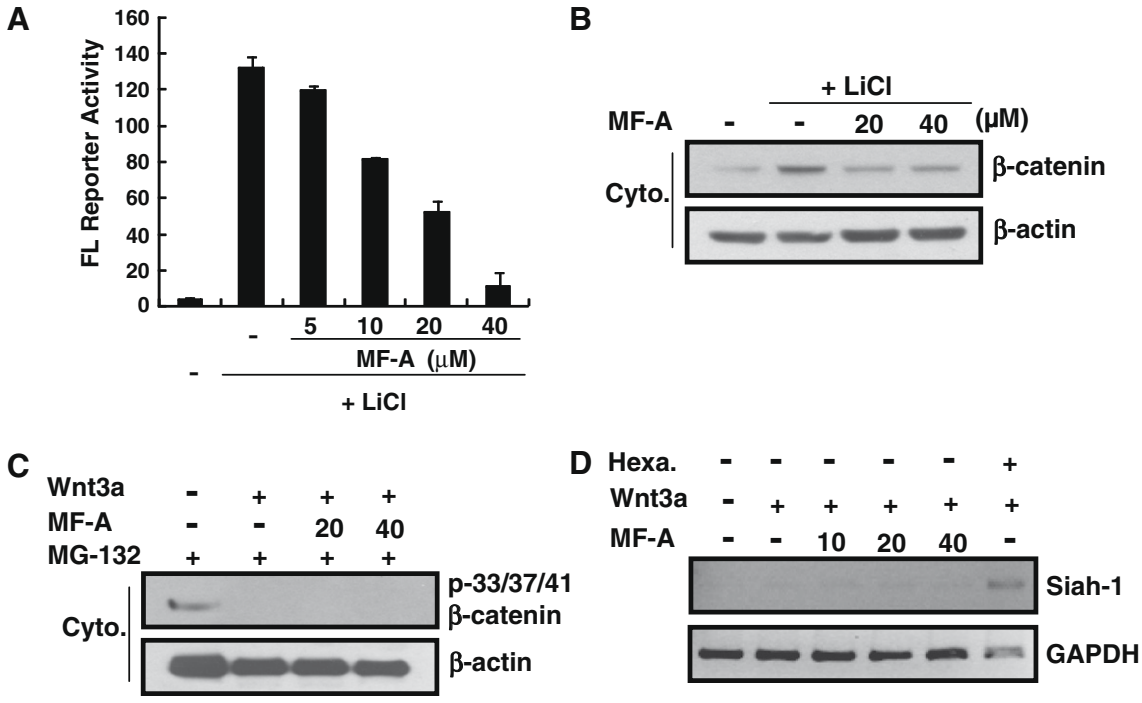

Fig. 3. Murrayafoline A induces β-catenin degradation through a mechanism independent of β-catenin phosphorylation and Siah-1. (A) HEK293 reporter (TOPFlash) cells were incubated with murrayafoline A in the presence of 20 mM LiCl. After 15 h, luciferase activity was determined. The results are the average of three experiments, and the bars indicate standard deviations. (B) Cytosolic proteins were prepared from HEK293 reporter (TOPFlash) cells treated with either vehicle (DMSO) or murrayafoline A in the presence of 20 mM LiCl for 15 h and were then subjected to Western blotting with anti-β-catenin antibody. (C) HEK293 reporter (TOPFlash) cells were incubated with vehicle (DMSO) or murrayafoline A and MG-132 (10 μM) for 8 h. Cytosolic fractions were prepared and subjected to Western blot analysis with anti-phospho-Ser33/37-β-catenin and anti-β-catenin antibody. (D) Semi-quantitative RT-PCR for Siah-1 and GAPDH was performed with total RNA prepared from HEK293 cells treated with the vehicle (DMSO), murrayafoline A or hexachlorophene (20 μM) for 15 h.

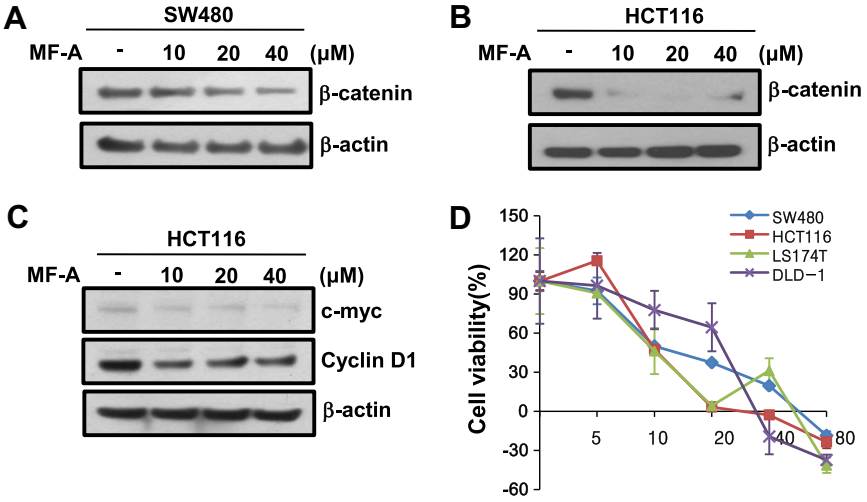

Fig. 4. The effects of murrayafoline A on colon cancer cells. (A, B) Cytosolic proteins were prepared from SW480 (A) and HCT-116 (B) cells treated with the vehicle (DMSO) or murrayafoline A for 15 h and then analyzed by Western blotting with anti-β-catenin antibody. (C) HCT-116 cells were incubated with the vehicle (DMSO) or murrayafoline A for 15 h. Cell extracts was prepared for Western blotting with anti-cyclin D1 and anti-myc antibodies. In (A–C), to confirm equal loading, the blots were re-probed with anti-actin antibody. (D) The effect of murrayafoline A on colon cancer cell growth. Colon cancer cells were incubated, in the indicated concentrations of murrayafoline A, for 48 h in 96-well plates. Cell viability was examined using the celltiter-Glo assay (Promega). To calculate the inhibition of growth, the value at time 0 was subtracted.

Western blot analysis. In HCT-116 and SW480, which display elevated β-catenin expression due to APC-mutation and Ser45 deletion mutation in β-catenin, respectively, the levels of cytosolic β-catenin were decreased by treatment with murrayafoline A (Fig. 4A and B). We also evaluated the effects of murrayafoline A on the expression of cyclin D1 and c-myc, established β-catenin-dependent genes. As shown in Fig. 4C, the protein expression of cyclin D1 and c-myc were significantly suppressed in response to murrayafoline A. Several studies have reported that the disruption of β-catenin function specifically reduced the cell growth of human colon cancer cells [32–35]. Given that murrayafoline A promotes β-catenin degradation, we examined whether murrayafoline A sup-

press the proliferation of colon cancer cells. Various colon cancer cells, such as SW480, DLD-1, HCT-116, and LS174T, were incubated with increasing concentrations of murrayafoline A and cell growth was measured. As shown in Fig. 4D, murrayafoline A efficiently inhibited the growth of colon cancer cells in a concentration-dependent manner.

Murrayafoline A, a carbazole alkaloid isolated from *Glycosmis stenocarpa*, exhibits cytotoxic effects against various cancer cells including colon cancer cells [36], but little is known about its mechanism of action. In this reports, we used a cell-based chemical screening to reveal that murrayafoline A inhibits the growth of colon cancer cells by promoting the degradation of intracellular β-catenin. It has been reported that the degradation of intracellular β-catenin is mediated by multiple pathways. In a phosphorylation-dependent pathway, β-catenin is phosphorylated at Ser33/37 residues by several kinases, such as GSK-3β, Cdk2/cyclin E, and PKCα [6–8]. This phosphorylation marks the β-catenin protein for degradation by an ubiquitin-dependent pathway [25,26]. In Siah-1 dependent pathway, Siah-1 associates with β-catenin through the carboxyl terminus of APC and promotes the degradation [29,30].

Several lines of evidences in this study suggest that a novel degradation pathway, other than above-described pathways, may be involved in the murrayafoline A-mediated β-catenin degradation. First, murrayafoline A was able to promote the degradation of β-catenin in the presence of GSK-3β inhibitor, indicating that GSK-3β activity is not required for β-catenin down-regulation by murrayafoline A. Second, incubation with murrayafoline A does not induce β-catenin phosphorylation at Ser33/37 residues, suggesting that murrayafoline A-mediated β-catenin degradation is β-catenin phosphorylation-independent. Finally, the expression of Siah-1 was not upregulated in response to murrayafoline A. In addition, in SW480 wherein APC-dependent pathways were impaired because of loss-of-function mutations in APC, murrayafoline A was still able to decrease intracellular β-catenin level, suggesting that Siah-1/APC pathway is not responsible mechanism for murrayafoline A-mediated β-catenin degradation. Similar to murrayafoline A-mediated β-catenin degradation, retinoid X receptor (RXR) agonists have been reported to induce RXR-mediated degradation of β-catenin by a mechanism independent of β-catenin N-terminus and APC [37]. We plan to investigate the mechanism of murrayafoline A-induced β-catenin degradation in the future.

In conclusion, we uncovered the anti-proliferative effect of murrayafoline A on colon cancer cells using cell-based small molecule screening. Murrayafoline A suppressed Wnt/β-catenin signaling by promoting the degradation of β-catenin via a mechanism independent of β-catenin phosphorylation and APC. Therefore, murrayafoline A can be developed into therapeutic agents against various cancers that contain abnormally upregulated β-catenin activity.

Acknowledgments

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