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Identification of *N*-[3-(3,4-Dihydroxyphenyl)-1-oxo-2-propenyl]-2-hydroxybenzamide (CGK-101) as a Small Molecule Inhibitor of the Wnt/β-catenin Pathway

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Colorectal cancer is the most prevalent type of cancer and the second leading cause of cancer-related mortalities in Western countries.[1] Current therapies for colorectal cancer rely on surgical resection, which is rarely curative in advanced disease, and traditional cytotoxic agents exhibit limited effects. Therefore, it is crucial to develop new therapeutic strategies that are based on defined molecular lesions. The development and progression of colorectal cancer are related to the accumulation of a series of genetic and epigenetic alterations.[2,3] Molecular lesions in the adenomatous polyposis coli (APC) gene are observed in most sporadic colorectal cancers as well as in familial adenomatous polyposis (FAP) and they appear early in the progression of this type of cancer.[4] In addition, the N-terminal phosphorylation motif of β-catenin is frequently mutated in colorectal cancer.[5] These alterations lead to the accumulation of β-catenin in the nucleus, where it forms a complex with T-cell factor/lymphocyte enhancer factor (TCF/LEF) family transcription factors. It then activates its target genes, such as *c*-myc, cyclin D1, and metalloproteinase-7, which play important roles in colorectal tumorigenesis and metastasis.[6-8] Thus, inhibition of the Wnt/β-catenin pathway, which is aberrantly up-regulated in colorectal cancer, is a potential therapeutic strategy for treating colorectal cancer.

In this study, we used cell-based chemical screening to identify *N*-[3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]-2-hydroxybenzamide (CGK-101) as an inhibitor of the Wnt/β-catenin pathway. It has been reported that CGK-101, unique polyphenol found in oatmeal, has the effect for the prevention and improvement of muscular disorders and for the improvement of muscle function.[9] Also this compound exhibited epidermal growth factor receptor (EGFR) tyrosin kinase inhibitory activity.[10] CGK-101 attenuated β-catenin response transcription (CRT) that was increased by Wnt3a-conditioned medium (Wnt3a-CM) and LiCl, which is an inhibitor of glycogen synthase kinase-3β (GSK-3β), by promoting intracellular β-catenin degradation. In addition, CGK-101 down-regulated the level of β-catenin, which resulted in the repression of the β-catenin/T cell factor-dependent gene *cyclin D1* in HCT116 colon cancer cells and thus inhibited the proliferation of these cells. These results indicate that CGK-101 provide a novel therapeutic strategy for colon cancer via its suppression of the Wnt/β-catenin pathway.

To screen for cell permeable compounds that inhibit Wnt/β-catenin signaling, we used HEK293-FL reporter cells that were stably transfected with TOPFlash reporter and the human Frizzled-1 (hFz-1) expression plasmid.[11] After the addition of Wnt3a-CM and compounds to a HEK293 reporter cell culture, the luciferase activity, which was governed by the β-catenin/TCF complex, was measured using a microplate reader (Fig. 1(a)). The results showed that CGK-101 is an inhibitor of Wnt/β-catenin signaling (Fig. 1(a) and (b)). As shown in Figure 2(a), CGK-101 inhibited CRT, which was activated by Wnt3a-CM, in a concentration-dependent

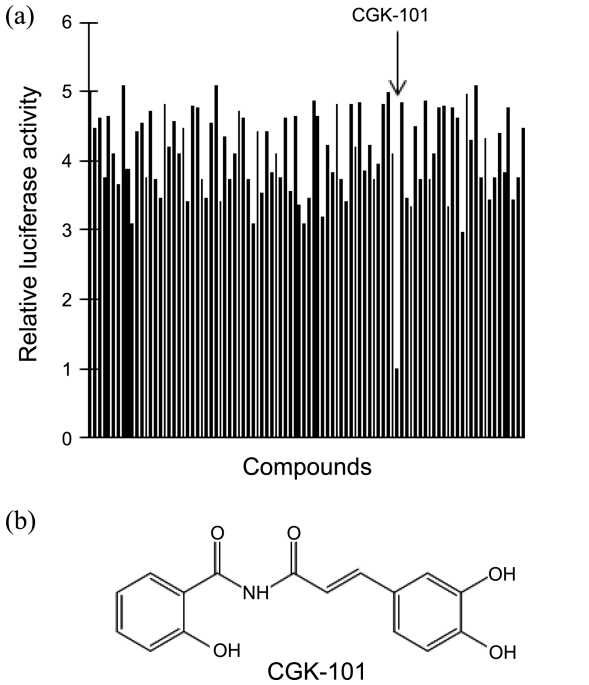

Figure 1. Identification of CGK-101 as an inhibitor of the Wnt/β-catenin pathway. (a) Screening of compounds that inhibit the Wnt/β-catenin pathway. Compounds modulating TOPFlash reporter activity were screened using the HEK293-FL reporter cells. (b) Chemical structure of CGK-101.

[a]These authors contributed equally to this work.

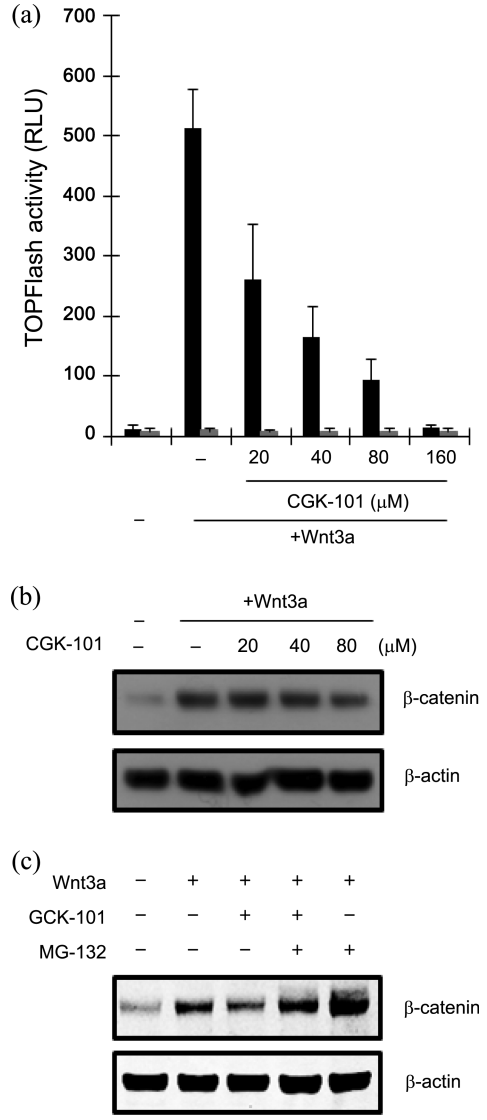

Figure 2. CGK-101 inhibits the Wnt/β-catenin pathway by promoting the degradation of β-catenin. (a) HEK293-FL reporter cells (black bars) and control cells (gray bars) were incubated with indicated concentrations of CGK-101 in the presence of Wnt3a-CM. After 15 h, their luciferase activity was determined. The results are the average values of 3 experiments, and the bars indicate standard deviations. (b) Cytosolic proteins were prepared from HEK293-FL reporter cells that were treated with either vehicle (DMSO) or CGK-101 in the presence of Wnt3a-CM for 15 h and then subjected to western blotting with β-catenin antibody. (c) Cytosolic proteins that were prepared from HEK293-FL reporter cells that were incubated with vehicle (DMSO) or CGK-101 (80 μM) in the presence of Wnt3a-CM and exposed to MG-132 (10 μM) for 8 h were subjected to Western blotting with *anti*-β-catenin antibody. In (b) and (c), the blot was reprobed with an *anti*-actin antibody to confirm equal loading.

manner without detectable cytotoxicity toward the HEK293-FL reporter cells. In contrast, the FOPFlash reporter activity, which was controlled by a mutated β-catenin/TCF-binding element, was unchanged by treatment with CGK-101 and Wnt3a-CM in HEK293 control cells (Fig. 2(a)), which indicates that CGK-101 is a specific inhibitor of Wnt/β-catenin signaling.

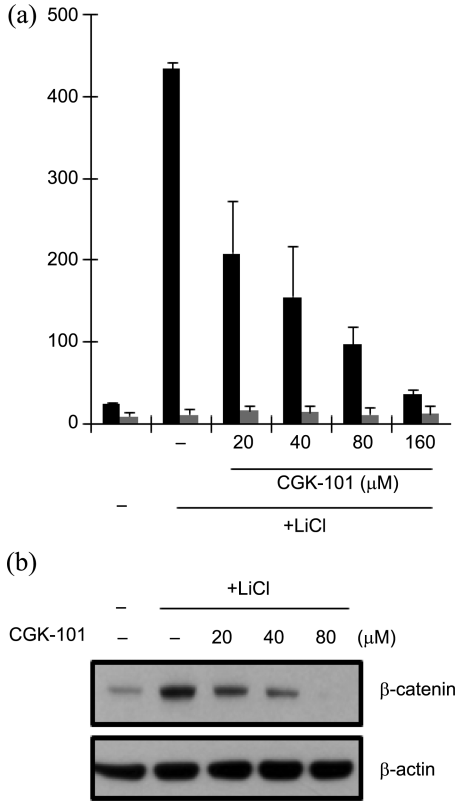

Figure 3. CGK-101 induces β-catenin degradation by a mechanism independent of GSK-3β. (a) HEK293-FL reporter cells (black bars) and control cells (gray bars) cells were incubated with the indicated concentrations of CGK-101 in the presence of 20 mM LiCl. After 15 h, their luciferase activity was determined. The results represent the average of 3 experiments, and the bars indicate the standard deviation. (b) Cytosolic proteins were prepared from HEK293-FL reporter cells that were treated with vehicle (DMSO) or the indicated concentrations of CGK-101 in the presence of 20 mM LiCl for 15 h and then subjected to western blotting with an *anti*-β-catenin *anti*-body. The blot was reprobed with an *anti*-actin antibody to confirm equal loading.

Since CGK-101 suppressed the Wnt/β-catenin pathway, we examined whether CGK-101 down-regulates the intracellular level of β-catenin by western blot analysis with *anti*-β-catenin antibody. As shown in Figure 2(b), incubation of the HEK293-FL reporter cells with different concentrations of CGK-101 produced a dose-dependent decrease with respect to the cytosolic β-catenin that had accumulated in response to Wnt3a-CM. We next examined the involvement of the proteasome in the CGK-101-induced down-regulation of cytosolic β-catenin. Treatment of CGK-101 consistently resulted in decreases in cytosolic β-catenin levels in HEK293-FL reporter cells (Fig. 2(c)). However, the addition of MG-132, which is a well-known inhibitor of the proteasome, abrogated CGK-101-mediated β-catenin down-regulation (Fig. 2(c)). These results suggest that CGK-101 inhibits the Wnt/β-catenin pathway via proteasome-dependent β-catenin degradation.

In Wnt/β-catenin signaling, GSK-3β-mediated β-catenin phosphorylation at residues Ser33/37 is a prerequisite for proteasome-dependent β-catenin degradation.[12] Therefore,

we tested whether GSK-3β activity is required for CGK-101-mediated β-catenin degradation. Incubation of HEK293-FL reporter cells with LiCl, which is an inhibitor of GSK-3β, resulted in increased CRT (Fig. 3(a)). Interestingly, CGK-101 still abrogated LiCl-mediated CRT activation (Fig. 3(a)). In addition, western blot analysis consistently showed that the amount of cytosolic β-catenin, which accumulated with LiCl treatment, was reduced by CGK-101 in the HEK293-FL reporter cells (Fig. 3(b)). These results indicate that CGK-101 induced the degradation of β-catenin through a mechanism that was independent of GSK-3β.

Since aberrant activation of CRT frequently occurs in colon cancer cells, we next examined whether CGK-101 could attenuate CRT in HCT116 colon cancer cells, which exhibit elevated CRT due to a Ser45 deletion mutation in β-catenin.[13] To this end, HCT116 cells were transfected with TOPFlash reporter plasmid, followed by treatment with CGK-101. As shown in Figure 4(a), the TOPFlash reporter activity was decreased by CGK-101 in a dose-dependent manner. In addition, western blot analysis consistently showed that CGK-101 dramatically down-regulated the levels of cytosolic β-catenin in the HCT116 cells (Fig. 4(b)). The

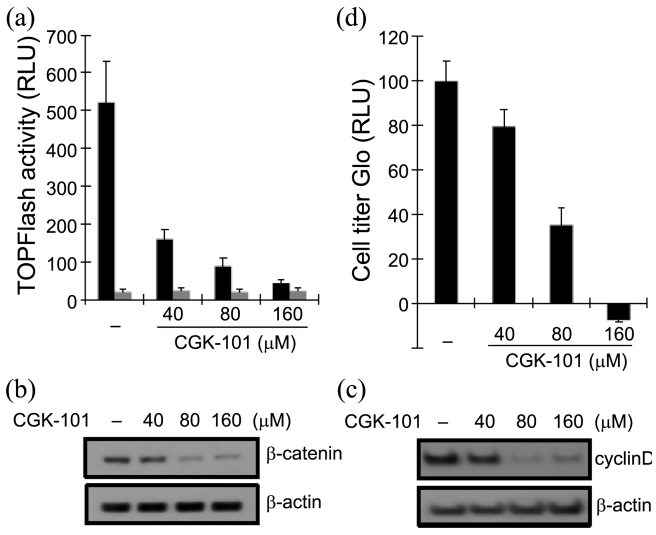

Figure 4. Effect of CGK-101 on HCT116 colon cancer cells. (a) HCT116 cells were cotransfected with TOPFlash (black bars) or FOPFlash (gray bars) and pCMV-RL plasmids and incubated with the indicated concentrations of CGK-101 for 15 h. Their luciferase activity was measured 39 h after transfection. TOPFlash activity is reported as relative light units (RLUs) normalized against *Renilla* luciferase activity. The results represent the average of 3 experiments, and the bars indicate the standard deviation. (b) Cytosolic proteins were prepared from HCT116 cells that were treated with the vehicle (DMSO) or the indicated concentrations of CGK-101 for 15 h and then subjected to western blotting with an *anti*-β-catenin antibody. (c) HCT116 cells were incubated with vehicle (DMSO) or the indicated concentrations of CGK-101 for 15 h, and the cell extracts were prepared for western blotting with *anti*-cyclin D1 antibody. In (b) and (c), the blots were reprobed with an *anti*-actin antibody as a loading control. (d) HCT116 cells were incubated with the indicated concentrations of CGK-101 for 48 h in 96-well plates. Cell viability was examined using a CellTiter-Glo assay (Promega). The results represent the average of 3 experiments, and the bars indicate the standard deviation.

expression of cyclin D1, which plays important roles in cell proliferation, is regulated by β-catenin.[14] We observed a concentration-dependent decrease in the protein expression of cyclin D1 in response to CGK-101 (Fig. 4(c)). Several studies have reported that specific reduction of β-catenin suppresses the growth of colon cancer cells.[15-17] Given that CGK-101 promotes the proteasomal degradation of β-catenin, we evaluated the effect of CGK-101 on the proliferation of HCT116 cells. As shown in Figure 4(d), the growth of the HCT116 cells was inhibited by treatment with CGK-101 at relatively high concentration. Since colon cancer cells have multiple mechanisms that induce or sustains cell proliferation, combination with other target therapeutics may be a good strategy for treatment of colon cancer.

In this study, we used chemical screening to reveal that CGK-101 inhibits Wnt3a-induced CRT by promoting the degradation of intracellular β-catenin. The N-terminal phosphorylation of β-catenin by CK1/GSK-3β are prerequisite events for the degradation of β-catenin through a proteasome-dependent mechanism.[18] However, several lines of evidence from the present study suggest that CGK-101 promotes β-catenin degradation through a mechanism independent of CK1/GSK-3β-mediated β-catenin phosphorylation. CGK-101 promoted the degradation of β-catenin in the HCT116 colon cancer cells, which contain mutant β-catenin protein at the CK1 phosphorylation site (Ser45), suggesting that CK1 is not involved in CGK-101-mediated β-catenin degradation. In addition, in the presence of LiCl, which is a pharmacological inhibitor of GSK-3β, cytosolic β-catenin was still degraded by treatment with CGK-101, which indicates that GSK-3β activity is not required for β-catenin down-regulation by CGK-101. We plan to further investigate the mechanism underlying CGK-101-induced β-catenin degradation.

Abnormal up-regulation of the Wnt/β-catenin pathway is major pathological events in intestinal epithelial cells during the development of human colon cancer.[3] Small molecules that attenuate the pathogenic activation of the Wnt/β-catenin pathway have been identified. We previously reported that curcumin, which is a component of turmeric, inhibits the Wnt/β-catenin pathway through downregulation of the transcriptional coactivator p300, thereby suppressing the proliferation of colon cancer cells.[19] On the other hand, quercetin, which is the main representative of flavonol, has been found to inhibit the Wnt/β-catenin pathway by decreasing nuclear β-catenin and Tcf-4 protein levels in SW480 colon cancer cells.[20] In this study, MG132, which is a proteasome inhibitor, abolished CGK-101-mediated β-catenin down-regulation. This finding suggests that CGK-101 inhibits the Wnt/β-catenin pathway through proteasome-dependent β-catenin degradation.

In summary, we identified CGK-101 as an antiproliferative agent that acts against HCT116 colon cancer cells. CGK-101 induced the degradation of intracellular β-catenin, thereby suppressing the Wnt/β-catenin pathway. Therefore, CGK-101 can be developed as a preventive or therapeutic agent against various cancers that involve abnormal β-

catenin accumulation.

Experimental Section

Cell Cultures, Reporter Assays, and Chemicals. HEK293, HCT116, 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-FL reporter (TOPFlash), control (FOPFlash) cells were established as previously described.[11,19] Wnt3a-conditioned medium (Wnt3a-CM) was prepared as previously described.[21] Luciferase assay was performed using the Dual Luciferase Assay Kit (Promega). LiCl and MG-132 were purchased from Sigma-Aldrich.

Plasmid Constructs and Transfection. The pTOPFlash reporter plasmid was obtained from Upstate Biotechnology. pCMV-RL and pSV-FL plasmids were purchased from Promega. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Screening for a 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 compounds were added to the wells. After 15 hours, the plates were assayed for firefly luciferase activity and cell viability.

Western Blot and Antibodies. The cytosolic fraction was prepared as previously described.[22] 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 *anti*-body against β-catenin was purchased from BD Transduction Laboratories. β-Actin was purchased from Cell Signaling Technology.

Cell Viability Assay. HCT116 colon cancer cells were inoculated into 96-well plates and treated with CGK-101 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.

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