http://dx.doi.org/10.5012/bkcs.2012.33.4.1405

Identification of Antimycin A as a Small Molecule Inhibitor of the Wnt/β-catenin Pathway

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Received January 13, 2012, Accepted January 30, 2012

Key Words : Chemical screening, Wnt/β-catenin pathway, Antimycin A, Rotenone

Wnts are secreted lipid-modified glycoproteins that regulate cell proliferation, cell polarity, and cell fate determination through a range of pathways.[1-3] The most characterized pathway is the Wnt/β-catenin pathway, which functions by regulating the phosphorylation and degradation of intracellular β-catenin.[4] The Wnt/β-catenin pathway is triggered by Wnts (Wnt1, Wnt3a and Wnt8) interacting with Frizzled (Fz) receptors and low density lipoprotein receptor-related protein5/6 (LRP5/6) co-receptor,[5] which then promotes the recruitment of Dishevelled (Dvl) to Fz. This leads to LRP5/6 phosphorylation, which induces an interaction of the Axin complex with phosphorylated LRP5/6, resulting in inhibition of the Axin complex-mediated β-catenin phosphorylation/degradation.[6] Accumulated β-catenin is translocated into the nucleus, where it forms a complex with the T cell factor/lymphocyte enhancer factor family of transcription factors to activate its target genes, such as *c*-myc, cyclin D1, and metalloproteinase-7.[7-10] In the absence of Wnt, casein kinase 1 (CK1) and glycogen synthase kinase-3β (GSK-3β) phosphorylate sequentially the amino-terminal region of β-catenin in a complex with the scaffolding protein Axin and the tumor suppressor protein adenomatous polyposis coli (APC).[11,12] Phosphorylated β-catenin is recognized by the F-box β-transducin repeat-

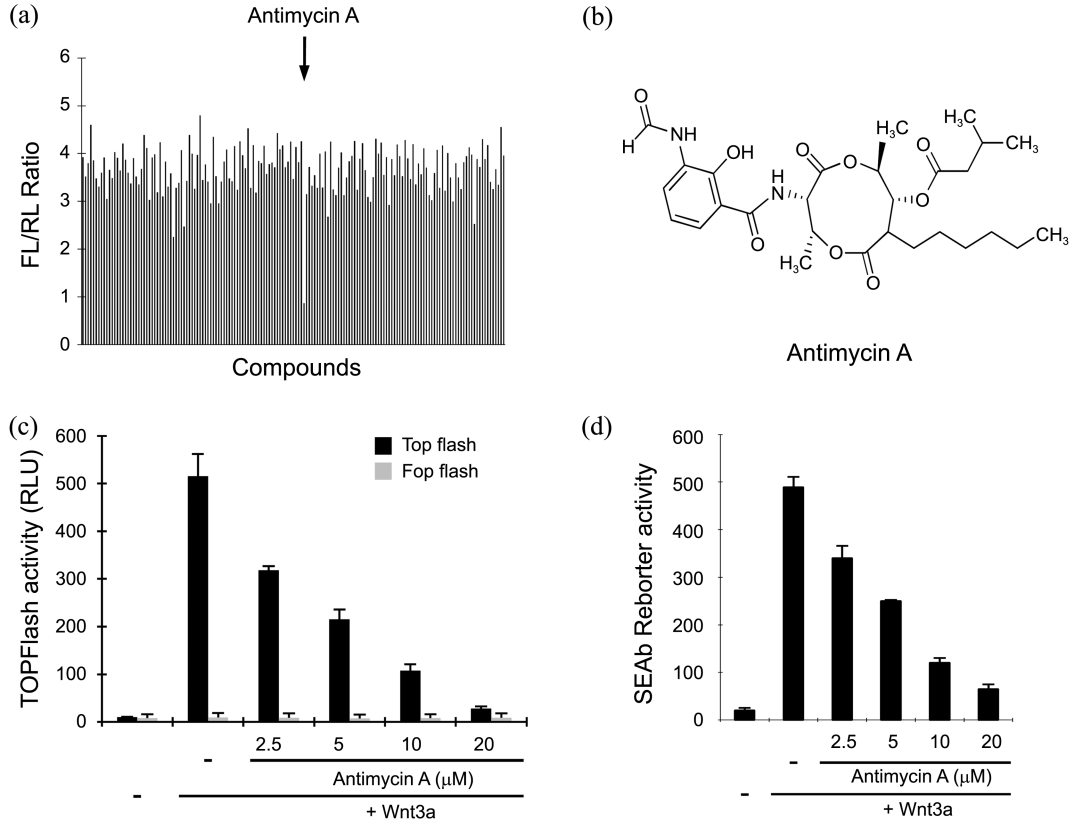

Figure 1. Identification of antimycin A as a small-molecule inhibitor of the Wnt/β-catenin pathway. (a) Screening of small molecules that inhibit the Wnt/β-catenin pathway. Compounds modulating the TOPFlash reporter activity were screened using the double readout system described as in Experimental Section. (b) Structure of antimycin A (c, d) HEK293-(TOPFlash and TOPSEAP) and control cells were incubated with indicated concentrations of antimycin A in the presence of Wnt3a CM. After 15 h, luciferase activity (c) or SEAP activity (d) was determined. In (c) and (d), the results are the average of three experiments, and the bars indicate standard deviations.

containing protein (β-TrCP), a subunit of the E3 ubiquitin ligase complex, leading to ubquitin-dependent proteolysis.[13,14]

In the present study, cell-based chemical screening with two reporter cell lines (HEK293-TOPFlash and HEK293-RL) was used to identify antimycin A, an inhibitor of complex III in the mitochondrial respiratory chain, as an antagonist of the Wnt/β-catenin pathway. Antimycin A suppressed β-catenin response transcription (CRT), which had been stimulated by Wnt3a-conditioned medium (Wnt3a-CM), and down-regulated the level of intracellular β-catenin. In contrast, 2-methoxyantimycin A3, an inactive analogue of antimycin A that does not inhibit mitochondrial electron transport chain, did not inhibit Wnt3a-CM-induced CRT or decrease the amount of intracellular β-catenin. Finally, the level of intracellular β-catenin was also decreased by blocking the mitochondrial electron transport chain with rotenone. These results suggest that a mitochondrial dysfunction due to metabolic inhibition negatively regulates the Wnt/β-catenin pathway.

To screen cell-permeable small molecule inhibitors of the Wnt/b-catenin pathway, I used two reporter cell lines: HEK293-TOPFlash cells, which were stably transfected with human Frizzled-1 (hFz-1) expression and a synthetic β-catenin/Tcf-dependent luciferase reporter plasmids, to assay β-catenin response transcription (CRT); and HEK293-RL cells, which stably harbored hFz-1 expression and CMV-*Renilla* luciferase plasmids, as a control. After adding the Wnt3a-CM and each compound to these reporter cells, the firefly luciferase (FL) and *Renilla* luciferase (RL) activities were measured using a microplate reader. The FL activity was normalized to the RL activity in each well to evaluate the specific effects of the screened compounds. As shown in Figure 1(a) and (b), antimycin A was found to inhibit the Wnt/β-catenin pathway. Treatment of HEK293-TOPFlash cells with increasing amounts of amtimycin A caused a concentration-dependent decrease in CRT that had been stimulated by Wnt3a-CM without affecting the activity of FOPFlash, a negative control reporter with mutated β-catenin/Tcf binding elements; a concentration of 20 μM antimycin A induced the near-complete inhibition of CRT relative to the control treatment (Fig. 1(c)). In addition, antimycin A consistently suppressed the Wnt3a-induced secreted alkaline phosphatase (SEAP) activity in the HEK293-TOPSEAP cells, which stably contained a β-catenin/Tcf-dependent SEAP reporter and hFz-1 plasmid (Fig. 1(d)). Overall, these results suggest that antimycin A is a specific antagonist of the Wnt/β-catenin pathway.

The Wnt/β-catenin pathway is controlled mainly by the intracellular β-catenin level.[4] Therefore, I examined whether antimycin A affects the cytosolic β-catenin level by Western blot analysis with the anti-β-catenin antibody. Incubation of HEK293-TOPFlash cells with antimycin A resulted in a decrease in the cytosolic β-catenin level that had been accumulated by Wnt3a-CM, which is consistent with its effect on CRT (Fig. 2(a)). In contrast, the mRNA level of β-catenin was unchanged by antimycin A regardless of the concentration (Fig. 2(b)), indicating that antimycin A suppresses the Wnt/β-catenin pathway by decreasing the β-catenin protein level.

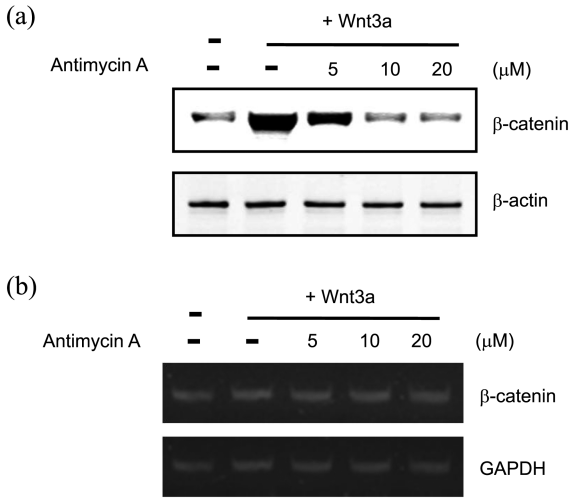

Figure 2. Antimycin A down-regulates the level of intracellular β-catenin. (a) Cytosolic proteins were prepared from HEK293-TOPFlash cells treated with either vehicle (DMSO) or antimycin A in the presence of Wnt3a-CM for 15 h and then subjected to Western blotting with β-catenin antibody. To confirm equal loading, the blots were re-probed with anti-actin antibody. (b) Semi-quantitative RT-PCR for β-catenin, and GAPDH was performed with total RNA prepared from HEK293-TOPFlash cells treated with either vehicle (DMSO) or antimycin A in the presence of Wnt3a-CM for 15 h.

Antimycin A causes a mitochondrial dysfunction by inhibiting the mitochondrial electron transport system through its binding to complex III.[15] 2-Methoxyantimycin A3, an inactive derivative of antimycin A that maintains Bcl-2/Bcl-x_L binding activity but does not inhibit electron transport,[16] was used to determine if an antimicin A-induced mitochondrial dysfunction inhibits the Wnt/β-catenin pathway directly. When HEK293-TOPFlash cells were incubated with various concentrations of 2-methoxyantimycin A3, 2-methoxyantimycin A3 did not affect CRT at any of the concentrations tested (Fig. 3(a)). In parallel with this experiment, the effect of 2-methoxyantimycin A3 on the levels of cytosolic β-catenin in HEK293-TOPFlash cells was examined by Western blot analysis. Consistent with the results from reporter assays, the addition of 2-methoxyantimycin A3 to the HEK293-TOPFlash cells did not down-regulate the intracellular β-catenin levels (Fig. 3(b)). These results suggest that the Wnt/β-catenin pathway is specifically suppressed by a mitochondrial dysfunction induced by the inhibition of the electron transport chain.

The effect of rotenone, an inhibitor of complex I in mitochondrial electron transport chain,[17] on the Wnt/β-catenin pathway was examined to confirm that the mitochondrial dysfunction induced by metabolic inhibition negatively regulates the Wnt/β-catenin pathway. As expected, treatment of HEK293-TOPFlash cells with various amounts of rotenone resulted in a significant decrease in CRT, which had been activated by Wnt3a-CM, in a concentration-dependent manner (Fig. 4(a)). Consistent with this result, rotenone de-

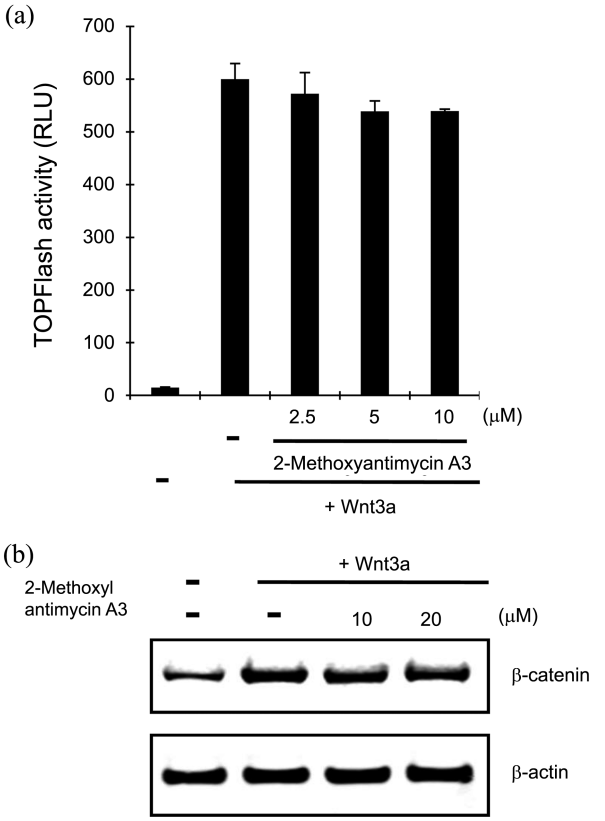

Figure 3. 2-Methoxyantimycin A3 does not affect the Wnt/β-catenin pathway. (a) HEK293-TOPFlash cells were incubated with indicated concentrations of 2-methoxyantimycin A in the presence of Wnt3a CM. After 15 h, luciferase activity was determined. The results are the average of three experiments, and the bars indicate standard deviations. (b) Cytosolic proteins were prepared from HEK293-TOPFlash cells treated with 2-methoxyantimycin A3 in the presence of Wnt3a-CM for 15 h and then subjected to Western blotting with β-catenin antibody. To confirm equal loading, the blots were re-probed with anti-actin antibody.

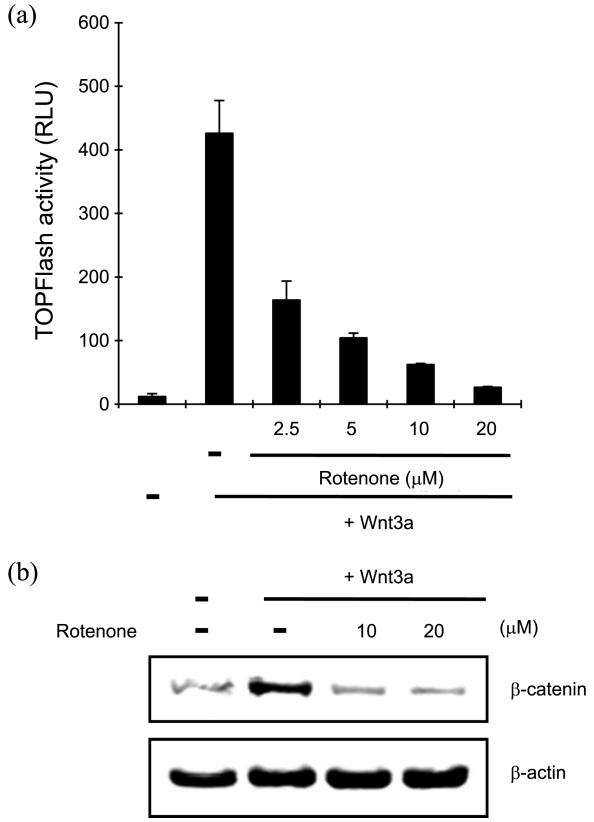

Figure 4. Rotenone inhibits the Wnt/β-catenin pathway. (a) HEK293-TOPFlash cells were incubated with indicated concentrations of rotenone in the presence of Wnt3a-CM. After 15 h, luciferase activity was determined. The results are the average of three experiments, and the bars indicate standard deviations. (b) Cytosolic proteins were prepared from HEK293-TOPFlash cells treated with either vehicle (DMSO) or rotenone in the presence of Wnt3a-CM for 15 h and then subjected to Western blotting with β-catenin antibody. To confirm equal loading, the blots were re-probed with anti-actin antibody.

creased the amount of cytosolic β-catenin in HEK293-TOPFlash cells (Fig. 4(b)), suggesting that rotenone also inhibits the Wnt/β-catenin pathway by down-regulating the intracellular β-catenin level.

Several studies suggested the possible interaction between Wnt signaling and the mitochondrial physiology. A recent study reported that Wnt signaling activates mitochondrial biogenesis and the generation of reactive oxygen species (ROS).[18] In addition, treatment with LiCl, an agonist of the Wnt/β-catenin pathway, increases mitochondrial biogenesis.[19] Moreover, stimulation of the Wnt/β-catenin pathway inhibits mitochondria-mediated apoptosis in colon cancer cells.[20] Nevertheless, the effects of a change in the mitochondrial function on the Wnt/β-catenin pathway have not been studied. In the present study, a screen of the chemical library, consisting of bioactive compounds, produced antimycin A, an inducer of mitochondrial dysfunction, which specifically suppressed CRT stimulated by Wnt3a-CM. In contrast, 2-methoxyantimycin A3, which is unable to inhibit the mitochondrial electron transport chain, did not affect the Wnt/β-catenin pathway. Moreover, rotenone, a complex I inhibitor, significantly down-regulated Wnt3a-CM-induced CRT, suggesting that metabolic inhibitor-induced mitochondrial dysfunction causes the inhibition of the Wnt/β-catenin pathway.

The level of intracellular β-catenin is regulated by GSK-3β-dependent and -independent pathways. Several small molecules that down-regulate the β-catenin level through GSK-3β-independent pathway have been identified by high-throughput screening. We previously reported that hexachlorophene induced β-catenin degradation by up-regulating the expression of Siah-1, which interacts with the carboxyl terminus of APC and promotes the ubiquitination of β-catenin, in HCT116 and LS174T colon cancer cells containing a β-catenin mutation at the CK1/GSK-3β phnosphorylation sites.[21] In addition, galangin, a naturally occurring bioflavonoid, reduces the intracellular β-catenin level by an APC/Axin/GSK-3β-independent mechanism in CRT-positive cancer cells.[22] In the present study, antimycin A was unable to down-regulate the intracellular β-catenin protein level when the GSK-3β activity was blocked by pharmacological inhibitors, LiCl and 6-bromoindirubin-3'-oxim (BIO), suggesting that a mitochondrial dysfunction causes a decrease in

the amount of β-catenin protein through a mechanism dependent of GSK-3β. Future studies will further investigate the mechanism of metabolic inhibitors-mediated β-catenin down-regulation.

In summary, the mitochondrial dysfunction-induced suppression of the Wnt/β-catenin pathway was identified by cell-based small molecule screening. Antimycin A and rotenone, which are inhibitors of the mitochondrial electron transport chain, inhibited the Wnt/β-catenin pathway by reducing the intracellular β-catenin protein level *via* a mechanism dependent on GSK-3β.

Experimental Section

Cell Cultures. HEK293 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. Wnt3a-conditioned medium (Wnt3a-CM) was prepared as described previously.[21] HEK293-TOPFlash, HEK293-FOPFlash, and HEK293-TOPSEAP cells were established as previously described.[21,23] The HEL293-RL cell line was established by selecting HEK293 cells co-transfected with the plasmid expressing hFz-1 and pRL-CMV vector (Promega) using media containing G418 (1 mg/mL).

Chemicals, Transfection and Reporter Assays. Antimycin A, rotenone, and BIO were purchased from Sigma-Aldrich. 2-methoxyantimycine A3 were obtained from Enzo Life Sciences. The transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Luciferase assays were performed using a Dual luciferase assay kit (Promega) and a secreted alkaline phosphatase assay was carried out using Phospha-Light™ Assay kit (Applied Biosystems).

Screening for Small-Molecule Inhibitors of the Wnt/β-Catenin Pathway. The HEK293-TOPFlash and HEK293-RL cells were mixed and plated into 96-well plates at 15,000 cells per well in duplicate. 24 h later, Wnt3a-CM and the compounds (Genesis Plus Collection, MicroSource Discoverty Inc.) were added to the wells at a final concentration of 20 μM. After 15 h, the plates were assayed for the firefly luciferase and *Renilla* luciferase activities.

Western Blot and Antibodies. The cytosolic fraction was prepared using a methodology reported elsewhere.[24] The 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 the 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 by ECL chemiluminescence (Santa Cruz Biotechnology). The antibody against β-catenin was purchased from BD Transduction Laboratories. β-Actin antibody was obtained from Cell Signaling Technology.

RNA Extraction and Semiquantitative RT-PCR. The 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.[21] The amplified DNA was separated on 1.5% agarose gels and stained with ethidium bromide.

Acknowledgments. This study was supported by a grant from the National R&D Program for Cancer Control, Minstry for Heath, Welfare and Family Affairs, Republic of Korea (0920090) and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0072221).

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