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Streptonigrin inhibits β-Catenin/Tcf signaling and shows cytotoxicity in β-catenin-activated cells

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ABSTRACT

Background: Activation of β-catenin/T-cell factor (Tcf) signaling plays a role in human carcinogenesis. This suggests a possibility that the β-catenin/Tcf signaling activated by the accumulation of β-catenin in the nucleus is related to some type of human carcinogenesis. Therefore, if β-catenin's transcriptional activity can be markedly down-regulated, tumor growth will be suppressed in β-catenin activated types of cancer.
Methods: To investigate the activation or suppression of β-catenin/Tcf transcription, we established a transiently transfected cell line with a constitutively active β-catenin mutant gene whose product is not degraded. This cell line was also co-transfected with luciferase reporter gene constructs containing either an optimized (TOPflash) or mutant (FOPflash) Tcf-binding element.
Results: We identified the inhibitory effect of streptonigrin against β-catenin/Tcf signaling in β-catenin activated cells. Streptonigrin inhibited the transcriptional activity of β-catenin/Tcf in SW480 cells and HEK293 cells transiently transfected with a constitutively active mutant β-catenin gene. The growth inhibitory effect of streptonigrin was more evident in β-catenin-activated cancer cells than in non-activated cancer cells. The electrophoresis mobility shift assay showed that the binding of Tcf complexes with their specific DNA-binding sites was suppressed by streptonigrin. Conclusion: Streptonigrin is a negative regulator of β-catenin/Tcf signaling, and their inhibitory mechanism is related to the proliferation inhibitory effect on β-catenin-activated cancer cells.
General significance: This report reveals a molecular mechanism underlying the anti-tumor effect of streptonigrin from the perspective β-catenin/Tcf signaling. Given its function in inhibiting β-catenin/Tcf signaling, streptonigrin may be of interest as a leading compound for chemotherapeutic agent against β-catenin-activated tumorigenesis.

1. Introduction

Streptonigrin (also known as rufochromomycin and bruneomycin, Fig. 1, CAS registration number 3930-19-6) is a metal-dependent quinone-containing antibiotic produced by *Streptomyces flocculus* [1]. This antibiotic has been shown to exhibit inhibitory activity toward several tumors (e.g., lymphoma, melanoma, and breast and cervix cancers) as well as viruses in some early in vitro and clinical observations [2–4]. Although high toxicity and serious side effects of this drug limit its use only as an experimental antitumor agent, because of its antitumor potency and unique structure, streptonigrin has served as a lead compound for chemical modification and synthesis in order to correlate specific structure features with the biological activity of the molecule [2–6]. Recently, we have screened natural products containing quinone and flavanone moiety, to identify transcriptional factor specific inhibitors. Streptonigrin inhibited the β-catenin/Tcf transcriptional activity in β-catenin-activated cells.

β-Catenin plays a role in cell–cell adhesion in cooperation with the cytoplasmic domain of E-cadherin [7]. Cytosolic β-catenin is phosphorylated by the axin–adenomatous polyposis coli (APC)–glycogen synthase kinase (GSK)-3β complex and recognized by an F-box component of the E3 ubiquitin ligase complex that promotes ubiquitination of β-catenin and increases its susceptibility to degradation by an ubiquitin–proteasome system [8]. In normal cells, β-catenin in the cytoplasm is rapidly degraded by the proteasome [9]. However, the mutational inactivation of the APC gene or β-catenin mutations at regulatory amino-terminal serine residues, which stabilize cytosolic β-catenin protein by blocking degradation, result in an abnormal amount of β-catenin accumulation [9]. Also, if Wnt signaling is activated, β-catenin degradation is blocked due to the decreased ability of GSK-3β to phosphorylate β-catenin [8]. This failure of degradation in cells results

Abbreviations: APC, Axin-adenomatous polyposis coli; BCA, bicinchoninic acid; CBP, CREB binding protein; CRC, colorectal cancer; DMSO, dimethyl sulfoxide; Dsh, disheveled; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK, glycogen sythase kinase; IC_{50}, half maximal inhibitory concentration; Lef, lymphoid enhancer factor; NF-κB, nuclear factor-κB; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TBE, Tris base boric acid EDTA; TBS, Tris buffered saline; TBST, Tris buffered saline and Tween-20; Tcf, T-cell factor

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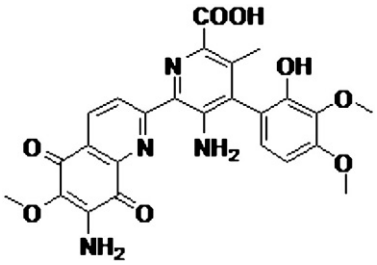

Fig. 1. Structure of streptonigrin.

in accumulation of β-catenin in the cytoplasm and translocation into the nucleus [8]. β-Catenin has transcriptional activity in cooperation with T-cell factor (Tcf)/lymphoid enhancer factor (Lef) transcription factor in the nucleus. Some of the genes activated by β-catenin/Tcf signaling are c-jun, c-myc, fibronectin, cyclin D1, and fra-1 [10–12]. Since the APC gene or the GSK-3β phosphorylation site within the β-catenin gene are mutated in many cancer cells including colorectal cancer, melanoma, hepatocellular carcinoma, and gastric carcinoma, the transcriptional activity of β-catenin is up-regulated in these cancer cells [13–15]. Studies have shown that β-catenin nuclear localization occurs in one third of gastric cancers and that β-catenin mutations occur in both diffuse- and intestinal-type gastric cancers at a higher rate [16]. This suggests a possibility that the β-catenin/Tcf signaling activated by the accumulation of β-catenin in the nucleus is related to some type of human carcinogenesis. Therefore, if β-catenin's transcriptional activity can be markedly down-regulated, tumor growth will be suppressed in β-catenin activated types of cancer.

In this report, we investigated the inhibitory effect of streptonigrin using HEK293 cells transiently transfected with a constitutively active mutant β-catenin gene and reporter gene constructs containing either an optimized (TOPflash) or mutant (FOPflash) Tcf-binding element. We demonstrate here that streptonigrin acts as an inhibitor of β-catenin/Tcf signaling. The mechanism of reduced β-catenin/Tcf transcriptional activity for the streptonigrin is due to the decreased binding of β-catenin/Tcf complexes with consensus DNA. Moreover, streptonigrin suppressed the proliferation of β-catenin-activated cancer cells including SW480, AGS and HEK293 cells transiently transfected with a constitutively active mutant β-catenin gene. The growth inhibitory effect of streptonigrin was more evident in β-catenin-activated cancer cells than in non-activated cancer cells.

2. Materials and methods

2.1. Cell culture and treatments

The HEK293 (human embryonic kidney cell line), SW480 (human colon cancer cell line), AGS (human gastric cancer cell line) and SH-SY5y (human neuroblastoma cell line) cell lines were purchased from Korean Cell Line Bank (Seoul, Korea). TOPflash and FOPflash were provided by Dr. Hans Clevers (Hubrecht Institute, Netherlands), and the β-catenin mutant S33Y gene was provided by Dr. Eric R. Fearon (University of Michigan Medical School, Ann Arbor). Streptonigrin and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Transfection and luciferase assay

Transient transfection was performed using Lipofectamine Plus reagent (Invitrogen. Carlsbad, CA). Briefly, 1×10^4 HEK293 cells distributed in the medium were dispensed into wells of a 96-well plate. After 24 h, cells were transfected with 0.10 μg of the TOPflash or FOPflash luciferase reporter constructs, 0.04 μg of pcDNA β-catenin S33Y gene, and 0.08 μg of pGL4.74 (*Renilla* luciferase) gene for normalization. SW480 cells were transfected with 0.10 μg of the TOPflash or FOPflash luciferase reporter constructs and 0.08 μg of pGL4.74. After 24 h of transfection, inhibitors were added to the medium. Cells were incubated for another 24 h, lysed, and collected for assays of luciferase activity using Dual-Glio™ luciferase assay system (Promega, Madison, WI) with a luminescence multi reader (Zenyth 1100, Anthos, Austria). TOPflash luciferase activity was normalized to *Renilla* luciferase activity and represented as the relative value compared to control.

2.3. MTT assay

The cell proliferation was investigated using the MTT assay. The HEK293, SH-SY5y, and SW480 cells were seeded into 96-well plates at 5×10^3 cells/well. HEK293 cells were transfected with the pcDNA β-catenin S33Y gene. The next day, the media was replaced with fresh serum free media containing streptonigrin or DMSO for vehicle control. The serum free media in the blank well was used for background absorbance. After 24 h, cell cytotoxicity was determined using the CellTiter 96® AQueous One Solution Assay (Promega). The reagent was applied to the cultures and incubated for 3 h. The absorbance was recorded at 490 nm with a multi reader (Zenyth 1100).

2.4. Isolation of nuclear extracts

Nuclear extracts were prepared using a nuclear extraction kit (Panomics, Fremont, CA) according to the manufacturer's instructions. HEK293 cells transfected with β-catenin mutant gene were treated with lysis buffer (Panomics) for 10 min at room temperature with vigorous mixing followed by scraping. After centrifugation, the pellet was resuspended in extraction buffer (Panomics) and incubated on ice for 1 h. Nuclear extracts were used as described in the following EMSA.

2.5. Electrophoresis mobility shift assay

EMSA was performed using a gel shift kit according to the manufacturer's protocol (Panomics). Nuclear extract (4 μg) was incubated for 5 min at room temperature with poly dI-dC (1 μg), 5× binding buffer, and, if needed, inhibitors. Then, biotin-labeled β-catenin/Tcf binding probe (5′-CTTTGATCTTACC-3′) was added and incubated for 30 min at 15 °C in a thermal cycler. The samples were analyzed by nondenaturing 6% polyacrylamide gel electrophoresis (PAGE) in 0.5× TBE buffer at 120 V for 55 min. Separated proteins were transferred to Biodyne B nylon membrane (Pall Life Science, Port Washington, NY) for 1 h at 300 mA. Target proteins were visualized by autoradiography using streptavidin-horse radish peroxidase and substrate solution.

2.6. Total cell lysate extraction and Western blot analyses

Cells (1×10^6) were harvested and suspended with 1.0 ml of ice-cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 ng/ml PMSF, 0.03% aprotinin, and 1 μM sodium orthovanadate) and incubated on ice for 30 min. The protein content of the final extracts was estimated using the BCA kit according to the manufacturer's protocol (Bio-Rad). Total cell lysates were used for measuring phosphorylation of GSK-3β. Nuclear extracts were used to measure β-catenin level. The extracts were subjected to 12% sodium dodecyl sulfate (SDS)-PAGE. The separated proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). After transfer, the membrane was saturated by incubation, at 4 °C 1 h with 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS)–0.1% Tween-20 (TBST) and then incubated with antibody overnight at 4 °C. After washing with TBST, the membrane was incubated with an anti-rabbit immunoglobulin coupled with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). After 60 min of incubation at room temperature, the membrane was washed three times with TBST and the blots were developed using enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK).