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5-Acetyl goniothalamin suppresses proliferation of breast cancer cells via Wnt/β-catenin signaling

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3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (PubChem CID:64965)
Ellipticine (PubChem CID:3213)
5-bromo-2′-deoxyuridine (BrdU) (PubChem CID: 6035)
Propidium iodide (PI) (PubChem CID: 104981)
Doxorubicin (PubChem CID: 31703)

ABSTRACT

Styryl lactones are plant-derived compounds from genus *Goniothalamus* with promising anti-proliferation and anticancer properties. However, the exact mechanism and the target for their activities remained unclear. In the present study, we investigated the effect of 5-acetyl goniothalamin (5GTN) from *Goniothalamus marcanii* on Wnt/β-catenin signaling pathway which is a key regulator in controlling cell proliferation in breast cancer cells (MCF-7 and MDA-MB-231). 5GTN, a naturally occurring derivative of goniothalamin (GTN) mediated the toxicity to MCF-7 and MDA-MB-231 cells in a dose- and time- related manner, and was more potent than that of GTN. 5GTN strongly inhibited cell proliferation and markedly suppressed transcriptional activity induced by β-catenin in luciferase reporter gene assay. In consistent with this view, the expression of Wnt/β-catenin signaling target genes including c-Myc, cyclin D1 and Axin2 in MCF-7 and MDA-MB-231 cells were suppressed after treatment with 5GTN. It was concomitant with cell cycle arrest at G_1 phase and cell apoptosis in MCF-7 cells. In addition, 5GTN enhanced glycogen synthase kinase (GSK-3β) activity and therefore reduced the expression of active form of β-catenin protein in MCF-7 and MDA-MB-231 cells. Taken together, 5GTN exhibited a promising anticancer effect against breast cancer cells through an inhibition of Wnt/β-catenin signaling. This pathway may be served as a potential chemotherapeutic target for breast cancer by 5GTN.

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1. Introduction

Wnt/β-catenin signaling pathway plays a central role in regulating cell proliferation in normal and various cancer cells (Amado et al., 2011). Mutation of Wnt/β-catenin pathway causing deregulation of the expression of Wnt/β-catenin target genes was demonstrated in mammary carcinoma and colorectal cancer (Holland et al., 2013). Approximately 40% of breast cancer cases showed increases in β-catenin (Incassati et al., 2010). The dysregulation of Wnt/β-catenin signaling in cancer cells has been reported on various steps along the complex regulating pathway including increased β-catenin expression, inactivated negative regulators, and altered levels of Wnt ligands or other components of Wnt signaling (Incassati et al., 2010). Thus, the defect of Wnt signaling is one of the promising targets for chemoprevention and chemotherapy for cancers including breast cancer (Dakeng et al., 2012). In the absence of Wnt ligand, β-catenin, which is a key component of Wnt/β-catenin signaling pathway, is degraded via the proteasome as a result of the phosphorylation by the destruction complex proteins including the APC (adenomatous polyposis coli), axin, glycogen synthase kinase (GSK) 3-β, and casein kinase 1 (Amado et al., 2011). In the presence of Wnt ligand, on the other hand, binding of Wnt to a Frizzled receptor in the

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presence of the co-receptor, LRP5/6 (low density lipoprotein-related protein 5/6), subsequently reduces the activity of destruction complex to degrade β-catenin (Incassati et al., 2010). The stabilized cytoplasmic β-catenin is then translocated to the nucleus to bind to lymphoid enhancer-binding factor 1/T-cell factor (TCF/LEF) transcription factor, and stimulates the expression of TCF responsive target genes (Bao et al., 2012). The downstream transcriptional targets of β-catenin/TCF including cyclin D1 and c-Myc are important regulators of cell cycle process and proliferation particularly at the transition of G_1/S and G_2/M phases (Barcelos et al., 2014, Pooja et al., 2014).

Naturally occurring compounds from plant have long been an attractive source of anticancer agents (Harvey, 2008). Of these, a styryl lactone goniothalamin (GTN), which is a low molecular weight phenolic compound from the genus *Goniothalamus* (Annonaceae), has been reported to exhibit a promising antiproliferative activity against a broad array of cancer cells including leukemia, kidney, pancreas, colon and breast cancer cells (Alabsi et al., 2013; Chen et al., 2005; Tantithanaporn et al., 2011). Its anticancer activities have been reported to be associated with induction of apoptosis, ROS production, DNA damage, and interruption of mitochondrial transmembrane potential in a variety of cancer cells (Alabsi et al., 2012; Inayat-Hussain et al., 2003; Yen et al., 2012). Although many anticancer activities of GTN have been proposed, the more specific signaling target to improve the efficiency and specificity of treatment has not been investigated. In an effort to search for more specific therapeutic target, the present study aimed to investigate the involvement of antiproliferative activity of a naturally occurring derivative of goniothalamin, 5-acetyl goniothalamin (5GTN) on Wnt/β-catenin signaling pathway, a critical pathway for regulating proliferation in cancer cells.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM), antibiotic-antimycotic agents, trypsin, Lipofectamine 2000 and Trizol reagents were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS), RIPA, and SuperSignal West Pico Chemiluminescent Substrate were from Thermoscientific (Cramlington, UK). SDS, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and ellipticine were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dual-luciferase reporter assay was from Promega (Madison, WI, USA). SYBR kit was from Applied Biosystems (Carlsbad, CA, USA). Protease inhibitor cocktail and cell proliferation ELIZA, BrdU (colorimetric) were from Roche (Mannheim, Germany). Propidium iodide (PI)/RNase Staining Buffer and Annexin-V FITC apoptosis detection kit were obtained from BD Biosciences (San Jose, CA, USA). Doxorubicin was from Guanyu Bio-Tech Co. (Xijan, Republic of China). Phosphatase inhibitor cocktail and active-β-catenin (anti-ABC) clone 8E7 monoclonal antibody were from Millipore Corporation (Darmstadt, Germany). Beta-catenin (H-102) and β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-GSK-3β (Ser9) antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). GSK-3β antibody was from Abcam (Cambridge, MA, USA). The T-cell factor (TCF)-reporter plasmids (TOPFLASH and the negative control FOPFLASH), β-catenin-FLAG and S33Y plasmids were generated as previously described (Bhukhai et al., 2012).

Two styryl lactones; Goniothalamin (GTN) and 5-acetyl goniothalamin (5GTN) were isolated from *Goniotalamus tapis* and *Goniothalamus marcanii*, respectively as previously described (Ahmad et al., 1991). They were obtained as white crystals, m.p. 80–82 °C and 90–93 °C, respectively. The GTN and 5GTN were assigned to the formula $C_{13}H_{12}O_2$ and $C_{15}H_{14}O_4$ by EIMS. The molecular structures were elucidated by spectroscopy technique such as NMR, IR, and UV. Their spectral data were similar to those previously reported (Ahmad et al., 1991). The chemical structures are shown in Fig. 1A.

2.2. Cell culture

Human breast cancer (MCF-7), triple-negative breast cancer (MDA-MB-231) and human embryonic kidney (HEK-293) cells were obtained from American Type Culture Collection (ATCC) and cultured in DMEM and MEM, respectively. They were supplemented with 10% fetal bovine serum and 1% antibiotic (100 U/ml of penicillin and 100 μg/ml of streptomycin), and incubated at 37 °C in a 5% CO_2 incubator with humidified atmosphere (Invitrogen, Carlsbad, CA, USA).

2.3. Cell viability and cell proliferation assay

Cell viability was determined by using the colorimetric MTT assay. MCF-7 and MDA-MB-231 cells (3×10^3 cells/well) were seeded in 96-well plates and incubated for 24 h prior to treatment with GTN, 5GTN, ellipticine or doxorubicin at concentrations ranging from 0 to 50 μM for 24, 48, and 72 h. The compounds were dissolved in DMSO and the final concentration of DMSO in the medium was less than 0.05%. After treatment, the medium was removed and fresh medium containing 0.5 mg/ml MTT was added and incubated at 37 °C for 4 h. The dark blue formazan crystal product was dissolved with DMSO and measured at 540 nm using a Multiskan™ GO Microplate Spectrophotometer (Thermoscientific, Cramlington, UK). The result was calculated as % of cell viability.

Cell proliferation was determined by a Cell Proliferation ELISA, BrdU colorimetric assay according to the manufacturer's instructions (Roche, Mannheim, Germany). Briefly, MCF-7 and MDA-MB-231 cells were plated at 3×10^3 cells/well in 96-well plates and cultured for 24 h. After treatment with 5GTN or doxorubicin for 24 h, BrdU labeling solution was added and incubated for 2 h at 37 °C followed by fixation and DNA denaturation with FixDenat solution. BrdU-POD antibody was incubated for 90 min at room temperature. BrdU incorporation with newly synthesized DNA was detected by adding substrate solution and incubated at room temperature for developing optimal color. The optical density was measured by a Multiskan™ GO Microplate Spectrophotometer (Thermoscientific, Cramlington, UK).

2.4. Cell cycle analysis

The proportion of cells at different phases of the cell cycle after treating with 5GTN was determined by flow cytometry. Briefly, MCF-7 cells (6×10^5 cells/well) were seeded in 6-well plates for 24 h and treated with 5GTN or doxorubicin for 24 h. Cells were then harvested, washed with PBS, and fixed in cold 70% ethanol overnight at −20 °C. Fixed cells were then washed and re-suspended in PI/RNase Staining Buffer in the dark for 30 min at 37 °C. Cells were monitored using a BD FACSCanto™ flow cytometer (BD Bioscience, San Jose, CA, USA) and DNA histograms of each sample were further analyzed with BD FACSDiva software.

2.5. Analyses of apoptosis (Annexin V-propidium iodide staining)

Cell apoptosis was analyzed by annexin V-FITC and PI staining. MCF-7 cells were seeded in 6-well plates and incubated with 5GTN or doxorubicin for 24 h. Cells were washed twice with PBS and followed by gentle pipetting to detach cells. Cells were then co-

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