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Taiwanin A targets non-steroidal anti-inflammatory drug-activated gene-1 in human lung carcinoma

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ABSTRACT

Taiwanin A (α,β-bis(piperonylidene)-γ-butyrolactone) is extracted from *Taiwania cryptomerioides*. Taiwanin A is extracted from tree bark and exhibits antitumor activity in breast, liver, and lung cancer cell lines. The objective of this study was to demonstrate the cytotoxicity of Taiwanin A against tumor cells by increasing the expression of non-steroidal anti-inflammatory drug-activated gene-1 (NAG-1). *NAG-1* has been reported to exhibit antitumor and proapoptotic activities, suggesting potential use in cancer therapy. Inhibiting *NAG-1* mRNA expression in A549 reduced the cytotoxicity caused by Taiwanin A. Furthermore, the c-Jun-N-terminal kinase/Ste20-related protein proline/alanine-rich kinase (JNK/SPAK) pathway played a key role in the influence of NAG-1 on cell viability, whereas the addition of the JNK pathway inhibitor SP600125 resulted in an inhibitory effect on NAG-1 and recovery of Taiwanin-A-treated cells. A xenograft tumor model demonstrated that Taiwanin A dose-dependently significantly decreases tumor-mediated growth in nude mice by increasing the NAG-1 expression accompanying tumor apoptosis. These data supported the hypothesis that Taiwanin A inhibits lung carcinoma growth by increasing NAG-1 expression through the JNK pathway both in vivo and in vitro. This result can contribute to a compound design for increasing cytotoxicity activity in the future.

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1. Introduction

The history of *Taiwania cryptomerioides* Hayata can be traced to the Pliocene, and is commonly bred for economic use in furniture and buildings, particularly for antimicrobial and anti-wood-decay fungi capabilities [1]. Taiwanin A (Fig. 1A) is one of the colored substances extracted from Taiwania heartwood. Researchers have observed that when exposed to light, the deep-orange-colored Taiwanin A changes to the pale-yellow-colored compounds Taiwanin C and Taiwanin E [2]. The potential therapeutic materials extracted from the bark of *T. cryptomerioides* include Taiwanin A, 6β-acetoxy-7α-hydroxyroyleanone, and 7-oxodehydropodocarpane, which possess respective antitumor, anti-inflammatory, and anti-oxidant properties [3].

One previous study demonstrated the in vitro anti-carcinogenic effects of Taiwanin A on breast, lung, and other

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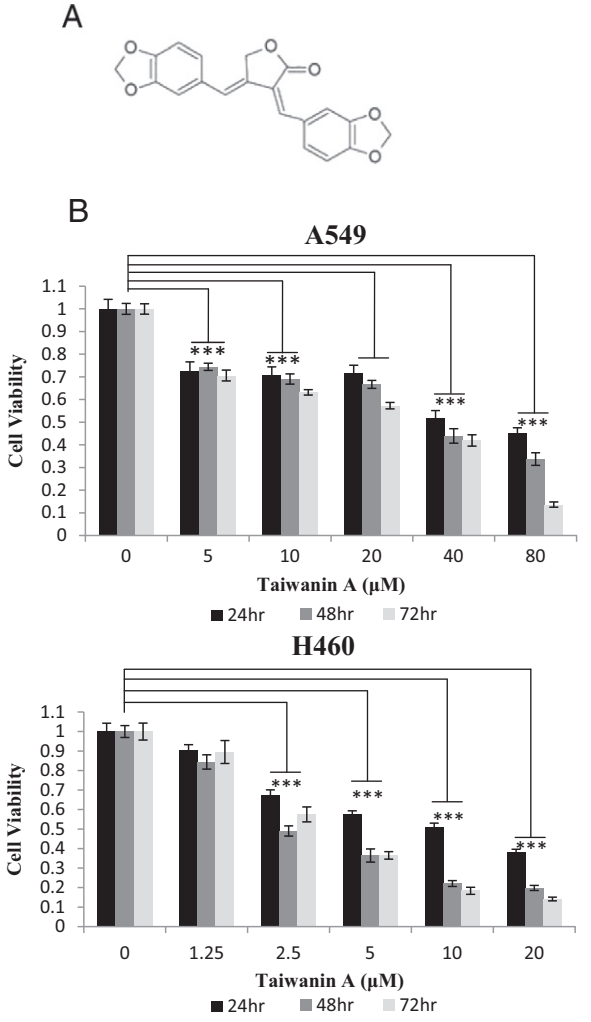

Fig. 1. NAG-1 expression activated by Taiwanin A (TiA) in lung carcinoma cell lines A549 and H460. (A) Structure of Taiwanin A. (B) MTT assay for 24-, 48-, and 72-h treatment of Taiwanin A, and the IC_{50} values of Taiwanin A in A549 cells were 60 μM at 24 h, and 30 μM at 48 h; in H460 cells, at 24 h and 48 h, IC_{50} values were 10 μM and 2.5 μM, respectively (***, $P < 0.001$, compared to the control groups).

cancers [4,5]. In MCF-7 breast carcinoma cells, Taiwanin A upregulates p53 signaling, causes G2/M arrest, and induces apoptosis [4]. HepG2 hepatocellular carcinoma cells exhibited similar results; pifithrin-alpha blocked p53 and attenuated Taiwanin A cytotoxicity [5]. The mechanism of apoptosis induced by Taiwanin A was further examined in both intrinsic and extrinsic pathways [1], resulting in p53 [6] and Bcl-2 phosphorylation [7]. However, researchers have not yet determined the specific target gene.

NAG-1 belongs to the transforming growth factor-beta (TGF-β) superfamily [8], which induces apoptosis in cancer cells [9]. NAG-1 is identified and cloned from NSAID-treated HCT-116 cells [10], and the cells expressing NAG-1 exhibit increased apoptosis, increased response to NSAIDs, and reduced numbers of colonies in soft agar. Furthermore, NAG-1 transgenic mice colons exhibited smaller numbers of preneoplastic lesions after azoxymethane (AOM) carcinogen induction [11]. Recently, researchers have focused on NAG-1 for use against diverse cancers and a variety of natural agents [12]. NAG-1 has been reported to play a pivotal role in regulating NAG-1 through multiple transcriptional mechanisms [13].

In this study, we demonstrated the potential target gene of cytotoxicity induced by Taiwanin A in A549 adenocarcinoma and H460 large-cell carcinoma lung cell lines. By using microarray analysis (data not shown), we determined that NAG-1 can be induced by Taiwanin A in tumor cells. We evaluated the induction of cell death based on JNK signaling and NAG-1 expression. We also observed that the inhibitor and siRNA of JNK phosphorylation and NAG-1 can recover the viability of Taiwanin-A-treated cells. Moreover, we determined that treatment with Taiwanin A reduced tumor size in xenograft animal models, and the expression of NAG-1 and phosphor-JNK was elevated in tumor tissue. The results of this study might be useful for application in a Taiwanin A compound design for targeted therapy.

2. Materials and methods

2.1. Chemicals and antibodies

Taiwanin A was isolated from *T. cryptomerioides* Hayata heartwood by National Taiwan University and is soluble in DMSO. The AKT, JNK, p38, and ERK inhibitor wortmannin, SP600125, SB203580, and PD98059 were purchased from Merck KGaA (Darmstadt, Germany). Antibodies for phospho-SAPK/JNK, SAPK/JNK, phospho-ERK, ERK, phospho-PKC, PKC, phospho-p38 MAP kinase, p38 MAP kinase, phospho-Akt, Akt, and anti-rabbit IgG horseradish peroxidase-conjugated antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Monoclonal β-actin antibodies were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). NAG-1/PTGF-β antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA).

2.2. Cell lines and culture

A549 human lung non-small cell lung carcinoma and H460 human lung large-cell carcinoma cells were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). A549 and H460 cells were maintained in RPMI1640 medium containing 10% fetal bovine serum, 1% HEPES, 1% sodium pyruvate, 1% sodium bicarbonate, and 100 ng/mL of penicillin/streptomycin. Cell culture medium and supplements were purchased from Thermo Scientific HyClone (Logan, UT, USA). Cells were cultured at 37 °C in 5% CO_2, and the medium was changed every 2 days.

2.3. Inhibitor treatment and siRNA transfection

Pathway inhibitors were added 1 h before adding the respective IC_{50} concentrations of Taiwanin A. NAG-1 siRNA was synthesized by Thermo Scientific Dharmacon (Lafayette, CO, USA). TurboFect™ siRNA Transfection Reagent was purchased from Fermentas Inc. (Glen Burnie, MD, USA). To determine transfection efficacy, the same cell that carried the EGFP was

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