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Toosendanin induces caspase-dependent apoptosis through the p38 MAPK pathway in human gastric cancer cells

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ABSTRACT

Although many advances have been made in the treatment of gastric cancer (GC), numerous difficulties, such as the emergence of chemo-drug resistance, continue to lead to disappointing GC prognoses. Thus, novel alternative strategies are urgently needed. The use of natural products could be a viable option to treat GC. Toosendanin (TSN) is a triterpenoid derived from the bark of *Melia toosendanin* Sieb. et Zucc that has been shown to be highly cytotoxic to multiple cancer cells. As the underlying impact of TSN on GC and its molecular mechanism remain poorly understood, in this study, we performed a series of experiments involving the use of TSN to treat GC cells. In the present study, we showed that TSN suppressed cell viability, inhibited cell proliferation by causing G_1/S arrest and induced caspase-dependent apoptosis in AGS and HGC-27 cells. The possible mechanism of TSN-induced apoptosis may be associated with the activation of the p38 MAPK pathway. These results demonstrated the potential of TSN as a promising therapeutic compound to treat gastric cancer.

1. Introduction

Gastric cancer (GC) is the fifth most common cancer and the second leading cause of cancer-related deaths worldwide [1]. GC has a high rate of incidence in Eastern Asia, peaking primarily in China [1,2]. Early stage gastric tumors are generally asymptomatic, and nearly 80% patients diagnosed with GC are in advanced stage when it is detected, with the overall 5-year survival rate of GC reported to only be 5–27.4% [2–4]. To date, surgery is still the primary curative therapy of GC, and radiotherapy and chemotherapy can help improve the disease outcome [3]. However, the drug resistance and side effects of chemotherapeutic drugs severely limit their efficacy [5]. Thus, it is crucial that novel chemotherapeutic agents with superior efficacy and selectivity be developed.

An increasing number of traditional Chinese medicines derived from natural products have been shown to have strong anticancer effects and few side effects [6,7]. Toosendanin (TSN), a triterpenoid isolated from the bark of *Melia toosendanin* Sieb. et Zucc, has been used as a vermifuge against ascaris for decades in China. Recently, TSN was shown to possess many bioactive activities, including prominent antibotulismic and antioxidant activities [8]. Importantly, TSN was reported to induce a strong cytotoxic effect on a variety of cancer cells. Accumulating evidence indicates that the molecular mechanism for the anticancer effects of TSN involves the regulation of intracellular Ca^{2+} homeostasis and suppression of mitogen-activated protein kinases (MAPKs) pathways [9]. In this study, we demonstrated that TSN inhibits proliferation and induces caspase-dependent apoptosis in the human GC cell lines AGS and HGC-27 via the activation of the p38 MAPK signaling pathway. The excellent efficacy of TSN at inducing cancer cell apoptosis demonstrates the potential of developing this compound as a chemotherapeutic agent against gastric cancer.

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2. Materials and methods

2.1. Cell culture

The human gastric cancer cell lines AGS and HGC-27 were acquired from the Culture Collection of Chinese Academy of Science (Shanghai, China). Cells were maintained in RPMI 1640 (Gibco Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Gibco Life Technologies), 100 units/ml penicillin and 10 μg/ml streptomycin (Gibco Life Technologies). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Reagents

SB203580 (Selleck chemicals, Houston, TX, USA) was dissolved in DMSO to make a 50 mM solution, which was stored at −20 °C. z-VAD-fmk (Selleck chemicals) was dissolved in DMSO to make a 100 mM solution, which was stored at −20 °C. Toosendanin (ChemFaces, Wuhan, China) was dissolved in DMSO to make a 10 mM stock solution, which was stored at −20 °C. Antibodies against poly ADP-ribose polymerase (PARP), caspase-3, cleaved-caspase-3, caspase-8, Mcl-1, Bcl-xl, Bcl-2, Bax, Xiap, survivin, cyclin D1, p21, p38 and phospho-p38 MAPK (Thr180/Tyr182) were obtained from Cell Signaling Technology (Beverly, MA, USA), cleaved-caspase-9 was obtained from Abcam (Cambridge, MA, USA). Caspase-9, β-actin, anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies were obtained from Proteintech Group (Chicago, IL, USA).

2.3. Cell viability

The effect of TSN on cell viability was tested through a Cell Counting Kit-8 (CCK-8) assay (Nanjing KeyGen Biotech Co., Ltd., Nanjing, Jiangsu, China). Briefly, cells were seeded into 96-well plates (3×10^3 cells per well) and were treated with TSN (0.5–2 μM). After incubating for 12, 24, 48, or 72 h, 20 μl of CCK-8 solution was added into each well. The absorbance was measured using a 96-well plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 450 nm after a 4 h incubation.

2.4. Colony formation

AGS and HGC-27 cells were seeded into a 6-well plate at a density of 500 cells per well. Cells were treated with various concentrations of TSN and incubated under a 5% CO_2 atmosphere at 37 °C for 7 days. To terminate the assay, cells were washed, fixed in ice-cold methanol for 5 min, and stained with crystal violet for 30 min. Images of the cell colonies were captured using an Epson scanner (Epson America, Inc., Long Beach, CA, USA).

2.5. Real-time cell impedance

An xCELLigence system (Roche Applied Science, Mannheim, Germany) was used to measure cell proliferation rates dynamically in a real-time cell analysis (RTCA). Experiments were carried out according to the standard protocol provided by Roche Applied Science. Each well of an E-Plate was seeded with 2×10^3 of cells in 100 μl of media, and cell proliferation was followed at set intervals by detecting the electrical impedance across microelectrodes on the bottom of the E-Plate. The cell impedance was evaluated using the RTCA software supplied by the manufacturer, and impendence values were converted to cell index (CI), an arbitrary unit.

2.6. Cell cycle analysis

AGS and HGC-27 cells were incubated with TSN for the indicated times, after which the cells were collected, washed and fixed with 66% cold ethanol at 4 °C overnight. After the 66% ethanol was removed, cells were washed with phosphate-buffered saline (PBS) and stained with propidium iodide (BD Biosciences, Franklin Lakes, NJ, USA). The cell cycle distribution analysis was performed using a BD FACSCanto II flow cytometer (Franklin Lakes, NJ, USA).

2.7. Cell apoptosis analysis

An Annexin V-FITC/PI dual staining kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was used to detect cell apoptosis. Cells were treated with or without TSN for the indicated times, after which the cells were collected, washed and suspended in 500 μl of binding buffer and mixed with 5 μl of Annexin V-FITC and 5 μl of propidium iodide. The mixtures were incubated in the darkness at room temperature for 15 min prior to analysis. Cell apoptosis rates were measured using a BD FACSCanto II flow cytometer. Annexin V-FITC-positive and Annexin V-FITC-plus-PI positive cells were reported as undergoing apoptosis.

2.8. Western blot

Cells were lysed in lysis buffer supplemented with protease and phosphatase inhibitors (Nanjing KeyGen Biotech Co., Ltd.). A Bio-Rad assay kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to determine protein concentrations. Protein electrophoresis was performed on 8, 10 and 15% SDS polyacrylamide gels and proteins were transferred to PVDF membranes, blocked with 5% nonfat dried milk at room temperature for 2 h, then were probed with the appropriate primary antibody overnight at 4 °C. After membranes were washed with 0.1% TBS-T and probed with the corresponding secondary antibody at room temperature for 2 h, the protein bands were observed via ECL detection.

2.9. Statistical analysis

All reported results are representative of three independent experiments, with the data expressed as the means ± SD. For statistical analyses, one-way analysis of variance followed by Tukey's test was performed using GraphPad Prism (San Diego, CA, USA). Differences were considered significant at $p < 0.05$.

3. Results

3.1. TSN inhibits proliferation and induces G_1/S cell cycle arrest in human GC cells

To investigate the anti-GC activity of TSN, we first used a CCK-8 assay to assess the ability of TSN to decrease the viability of AGS, HGC-27, MGC-803, MKN-45 and SGC-7901 cells. As shown in Fig. 1, TSN decreased the viability of GC cells in an apparent dose- and time-dependent manner. After being exposed to TSN for 72 h, the observed IC_{50} values of cells were 0.04631–0.8255 μM. To further evaluate the dose-response effect of TSN, we used the AGS and HGC-27 cell lines to perform colony formation assays. As shown in Fig. 2A, cells were treated with various concentrations of TSN (10–25 nM for AGS and 1–2.5 nM for HGC-27) for 7 days. Compared with the control groups, cells treated with TSN showed fewer and smaller colonies. Moreover, according to the RTCA results, as the concentration of TSN increased, the proliferation of AGS and HGC-27 cells was progressively inhibited (Fig. 2B). From these

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