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TXA9, a cardiac glycoside from *Streptocaulon juventas*, exerts a potent anti-tumor activity against human non-small cell lung cancer cells *in vitro* and *in vivo*

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ABSTRACT

Non-small cell lung cancer is the most common type of lung cancer and the most common cause of cancer-related death in humans. TXA9, which is a natural product separated from an anti-tumor-active fraction of the roots of *Streptocaulon juventas*, may possess potent anti-proliferative activity according to the present study. In this study, the anti-tumor effects and toxicity of TXA9 were tested against human non-small cell lung cancer cell lines (A549, NCI-H1299, Ltep-α2, PC-9, and Lu99) and a normal human lung embryonic fibroblast cell (HE-lung) *in vitro*, and then toward A549 cells *in vivo* in a murine xenograft model. The results show that TXA9 exhibits potent cytotoxic activities against non-small lung cancer cells and has no toxic effect on the normal human lung embryonic fibroblast cells. The mechanistic studies demonstrate that TXA9 can induce the apoptosis of A549 cells through the extrinsic pathway. The *in vivo* study results reveal that the intravenous administration of TXA9 at high-dose (15 mg kg^{-1}) induces significant tumor growth inhibition of non-small cell lung cancer xenografts with tumor inhibition rate up to 64.2%, compared with mice in the control group. The inhibitory effect was similar to that of taxol (62.5%). In particular, no significantly adverse effects were exerted by TXA9, which suggests that it is well tolerated. This promising natural product may be useful as a potential novel anti-tumor candidate.

1. Introduction

Non-small cell lung cancer (NSCLC), accounts for approximately 85% of all lung cancers, and almost 65–70% of NSCLC patients present with advanced disease at the time of diagnosis [1]. The prognosis for NSCLC patients is very poor with a five-year survival rate of only 15%, and this malignancy remains difficult to treat [2]. Due to the limited therapeutic effect of standard chemotherapy, radiotherapy and surgery, no effective treatment has been developed to date. For many years, natural products have played a very important role in anticancer drug discovery and development because they offer a large structural diversity [3]. As a result, both terrestrial and marine organisms are presently considered important sources of novel lead compounds.

Well known for their positive inotropic effect, cardiac glycosides (CGs) have been used clinically for many years for the treatment of heart failure and atrial arrhythmias. Since the middle of the 1960s, initial observations from epidemiological studies have shown that cancer patients receiving cardiac glycosides exhibit significantly lower mortality rates than patients without

Abbreviations: NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; CGs, cardiac glycosides; SJ, *Streptocaulon juventas*; MTT, 3-(4,5-dimethylibiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; AO, acridine orange; PI, propidium iodide; *i.v.*, intravenous; LTXA9, TXA9 at a dose of 5 mg kg^{-1}; MTXA9, TXA9 at a dose of 10 mg kg^{-1}; HTXA9, TXA9 at a dose of 15 mg kg^{-1}; RBC, red blood cell; WBC, white blood cell; PLT, platelets; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactic dehydrogenase; BUN, blood urea nitrogen; TBIL, total bilirubin; Crea, creatinine; TP, total protein; FADD, Fas-associated death domain.

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treatment with such drugs. Consequently, it is generally accepted that CGs have a putative anti-cancer effect, and the anti-proliferative activity of CGs, such as digitoxin on human cancer cells has been widely reported [4–8]. However, it is well known that the most significant adverse effect of digitoxin, which is frequently encountered in clinical treatment, is cardiotoxicity [9–10]. The narrow therapeutic window dramatically limits the application of digitoxin, because its toxicity is a major concern whenever it is considered for therapy [11]. Therefore, it is necessary to discover new candidate drugs that exhibit a significant anti-tumor effect but low toxicity.

Streptocaulon juventas (SJ) (Asclepiadaceae), which is mainly distributed in Southeast Asia, is used in folk medicine to stimulate the spleen, tonify the body, and strengthen the kidney [12]. We previously reported that the 75% ethanol extract of SJ exhibits potent inhibitory activity *in vivo* on the growth of the human lung A549 adenocarcinoma cell line with only mild adverse effects [13]. A subsequent phytochemical study of the active extract confirmed that cardiac glycosides contribute to the observed anti-tumor activity [14–15]. Among the numerous cardiac glycosides obtained, TXA9 was chosen as the lead compound for further research due to its potent *in vitro* anti-proliferative activity and relatively simple structure.

In the present study, the potential anti-proliferative activities of TXA9 against NSCLC *in vitro* and *in vivo* were evaluated, and any possible treatment-related toxic effects were assessed through a series of hematology and serum biochemistry tests, investigations of the visceral organs, and monitoring of body weight. Additionally, the apoptosis mechanism of TXA9 on A549 cells was investigated.

2. Experimental

2.1. Ethics statement

All of the animal care and experimental procedures were approved by the Animal Ethical Committee of Shenyang Pharmaceutical University. All tumor implantations, blood collections, and sacrifices were performed under anesthesia and all efforts were made to minimize suffering.

2.2. Plant material

Roots of SJ were collected in Simao, Yunnan Province in 2009. No specific permissions were required for the collections because the crude drug is wild and is not an endangered or protected species. The roots were identified by Prof. Jun Yin (Division of Pharmacognosy, Shenyang Pharmaceutical University), and voucher samples (SPU 2162-2165) were deposited at the herbarium of Shenyang Pharmaceutical University, Shenyang, China.

2.3. Preparation of TXA9

TXA9 was isolated from the roots of SJ collected in Yunnan Province of China in 2009. The air-dried roots were extracted with EtOH–H_2O (75:25, v/v) for 1.5 h, and this process was repeated three times. The combined solution was concentrated under reduced pressure until there was no trace of alcohol and then extracted with *n*-butanol four times. The concentrated extract was then subjected to silica-gel column chromatography with CH_2Cl_2–MeOH (25:1, 12:1, 1:1, v/v) to yield three fractions (A, B and C). Fraction B was applied to a silica-gel column, eluted with EtOAc–Me_2CO–H_2O (10:1:0.1, v/v/v), subjected to ODS silica-gel column chromatography with MeOH–H_2O (50:50, v/v), and recrystallized using MeOH to obtain TXA9 (purity: 99%).

2.4. Cell culture

The human lung A549 adenocarcinoma cell line, human non-small cell lung carcinoma NCI-H1299 cell line, human lung PC-9 adenocarcinoma cell line, and lung giant cell Lu99 carcinoma cell line were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The human lung Ltep-α2 adenocarcinoma cell line was ordered from Typical Culture Preservation Commission Cell Bank (Chinese Academy of Sciences, Shanghai, China). The HE-lung embryonic fibroblast cell line was provided by Prof. Ikuo Saiki (Department of Bioscience, Institute of Natural Medicine, University of Toyama, Sugitani, Toyama, Japan) as a gift. All of the cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in an atmosphere of 5% CO_2 and 95% air.

2.5. Cell proliferation

The effect of TXA9 on cell proliferation was assessed using the 3-(4,5-dimethylibiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, 96-well microtitre plates were seeded with 5×10^3 cells per well and incubated for 24 h. Culture media containing different concentrations of the samples were then added. After incubation for 12 h, 24 h, and 48 h, 100 μL of MTT from a stock solution (0.5 mg/mL) was added to each well, and the plate was incubated for 4 h. The absorbance of the resulting formazan product was measured at 492 nm using a microplate reader (Tecan, Austria).

2.6. Observation of cell morphological changes and acridine orange staining analysis

The morphological changes were observed as described previously [16]. Briefly, A549 cells were cultured at a density of 8×10^5 cells/flask in 25-mm^2 cell culture flasks. After 24 h of incubation, the cells were treated with 600 nM TXA9 and incubated for an additional 24 h. At the end of the incubation period, the morphological changes in the cells were observed through photomicrography using a phase-contrast microscope.

Acridine orange (AO) staining was used as an indicator of cell apoptosis. A549 cells were plated at 3×10^5 cells/well in six-well plates. After 24 h of incubation, the cells were treated with TXA9 at a concentration of 600 nM and incubated for 24 h. The prepared cells were washed once with PBS. After 10 mg/L AO was added to the cells, the cells were incubated for 30 min at 37 °C and then observed under a fluorescence microscope at an excitation wavelength of 488 nm and an emission wavelength of 515 nm.

2.7. Flow cytometry analysis by annexin V and propidium iodide staining

Annexin V-FITC/PI double staining was employed for the quantitative determination of the percentage of apoptotic cells. A549 cell at a density of 3×10^6 cells in each dish (ID: 6 cm) were incubated overnight, and then treated with sample or vehicle. After incubation for 6, 12, or 24 h with TXA9, the cells were digested with trypsin and suspended with PBS. After centrifugation, the PBS was removed, and the cells were stained with FITC-conjugated annexin V and propidium iodide (PI) for 15 min at 20 °C in a Ca^{2+}-enriched binding buffer (apoptosis detection kit, R&D Systems, Abingdon, UK; 20). The cells were immediately analyzed with a flow cytometer in their staining solution (Becton Dickinson FACScan, Franklin Lakes, NJ, USA).