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# Noscapine induces mitochondria-mediated apoptosis in human colon cancer cells in vivo and in vitro

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ABSTRACT

Noscapine, a phthalide isoquinoline alkaloid derived from opium, has been widely used as a cough suppressant for decades. Noscapine has recently been shown to potentiate the anti-cancer effects of several therapies by inducing apoptosis in various malignant cells without any detectable toxicity in cells or tissues. However, the mechanism by which noscapine induces apoptosis in colon cancer cells remains unclear. The signaling pathways by which noscapine induces apoptosis were investigated in colon cancer cell lines treated with various noscapine concentrations for 72 h, and a dose-dependent inhibition of cell viability was observed. Noscapine effectively inhibited the proliferation of LoVo cells in vitro ($IC_{50}$ = 75 μM). This cytotoxicity was reflected by cell cycle arrest at $G_2$/M and subsequent apoptosis, as indicated by increased chromatin condensation and fragmentation, the upregulation of Bax and cytochrome c (Cyt-c), the downregulation of survivin and Bcl-2, and the activation of caspase-3 and caspase-9. Moreover, in a xenograft tumor model in mice, noscapine injection clearly inhibited tumor growth via the induction of apoptosis, which was demonstrated using a TUNEL assay. These results suggest that noscapine induces apoptosis in colon cancer cells via mitochondrial pathways. Noscapine may be a safe and effective chemotherapeutic agent for the treatment of human colon cancer.



## 1. Introduction

Colorectal cancer is diagnosed in approximately 146,940 patients per year and is the second leading cause of cancer-related death in the United States [1–3]. Among patients with colorectal cancer (CRC) in the United States, 37.2% are diagnosed with stage III and 27.9% are diagnosed with stage II disease [3]. Adequate surgical resection is the only curative treatment, with overall survival rates of just under 50% at 5 years [4]. The survival depends on the pathologic stage and varies from 30%–60% for stage III to 60%–80% for stage II [3]. However, as many as 40%–50% of patients will relapse and require additional treatment, but the absolute benefit for survival obtained with adjuvant therapy compared with controls was approximately 6% [3]. Therefore, it is of great importance to discover and develop novel agents that have high efficacy and low toxicity, can overcome drug resistance and have improved pharmacologic profiles.

Noscapine, a phthalide isoquinoline alkaloid derived from opium, has been used as an oral antitussive agent and has shown very few toxic effects in animals and humans [5,6]. Noscapine showed little or no toxicity in the kidney, liver, heart, bone marrow, spleen, and small intestine at tumor-suppressive doses [7]. Noscapine binds stoichiometrically to tubulin, alters its conformation, affects microtubule assembly, and arrests mammalian cells in mitosis [6]. Furthermore, noscapine induces apoptosis in many cell types and has potent antitumor activity against a variety of solid tumors, including murine lymphoid tumors and human breast and bladder tumors implanted in nude mice [6]. Recently, the anti-cancer activity of noscapine was found to involve the induction of apoptosis via mitochondrial pathways in various cancers [7–9].

To date, there is no information available about the anti-cancer effects of noscapine on human colon cancer cells except that p21 plays a proapoptotic role and that p53 activity is necessary but not sufficient for noscapine-mediated apoptosis [10]. In this study, we addressed the hypothesis that noscapine plays an important role in mitochondria-mediated apoptosis in colon cancer cells. To examine this hypothesis, we investigated the mechanism of action by which noscapine induces apoptosis in colon cancer cells.

## 2. Materials and methods

### 2.1. Chemicals and cell culture

Noscapine (97% purity) was purchased from Sigma (Sigma–Aldrich, St. Louis, MO, USA). The noscapine stock solution was prepared at 100 mM in dimethyl sulfoxide (DMSO) and stored at

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−20 °C. Three human colon cancer cell lines (HT-29, LoVo and SW480) were obtained from the China Center for Type Culture Collection (Wuhan, China). The cells were cultured in DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator at 37 °C with a 5% $CO_2$/95% air atmosphere.

### 2.2. Cell proliferation assay

Cell proliferation was determined using the WST-8 tetrazolium salt assay (Cell Counting Kit-8, Beyotime Inst. of Biotech, China), which quantifies the amount of formazan dye formed when a tetrazolium salt is cleaved by cellular mitochondrial dehydrogenase present in viable cells. Cells were seeded in 96-well plates at a density of $5 \times 10^3$/well in 0.1 mL of culture medium. They were allowed to adhere for 12 h and were then incubated with 0.01, 0.1, 1, 10, 100, or 1000 μM noscapine for 0, 12, 24, 36, 48, or 72 h. At two hours before the end of the specified incubation periods, 10 μL of WST-8 reagent were added to the cells. At the end of the incubation, cell density was estimated by measuring the absorbance of the colored formazan reaction product at 450 nm using an iMark Microplate Absorbance Reader (Bio-Rad, USA). The percentage of cell survival as a function of drug concentration was then plotted to determine the $IC_{50}$ value.

### 2.3. Cell cycle analysis

Cell cycle status was determined by measuring cellular DNA content after staining with propidium iodide (PI) by flow cytometry. Cells were seeded in culture dishes and grown to 70% confluence before the addition of the $IC_{50}$ dose of noscapine for 0, 12, 24, 36, 48, or 72 h. After drug incubation, cells were centrifuged, washed twice with ice-cold PBS, and fixed in 70% ethanol at 4 °C for 24 h. Cells were then centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. The pellets were then washed twice with 4 mL PBS and then stained with 0.5 mL RNase A (2 mg/mL) and 0.5 mL of PI (0.1% in 0.6% Triton-X in PBS) for 30 min in the dark. Samples were then analyzed with a FACSCalibur flow cytometer (Beckman Colter, Inc., Fullerton, CA).

### 2.4. Annexin V staining for apoptosis

Cells were grown in culture dishes, and 24 h after seeding, they were exposed to the $IC_{50}$ of noscapine for 0, 12, 24, 36, 48, or 72 h. After the incubation period, adherent cells were harvested by mild trypsinization, washed twice with cold PBS and pooled together with detached cells in 500 μL of binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). The cells were then incubated with 5 μL of Annexin V-FITC and 10 μL of PI at room temperature for 5 min in the dark. Flow cytometric analysis was performed with a FACSCalibur using the CellQuest software (BDIS). The density plots illustrate four cell populations (live, apoptotic, necrotic, and late apoptotic/dead), defined by their fluorescence characteristics. Live cells are Annexin V- and PI-negative, early apoptotic cells are Annexin V-positive and PI-negative and late apoptotic and dead cells are both Annexin V-positive and PI-positive.

### 2.5. Western Blot analysis

Proteins were resolved by SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA) using a wet transfer system (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 10% non-fat dry milk in TBST and incubated first with primary antibodies at 4 °C overnight and then with horseradish peroxidase-conjugated anti-mouse secondary antibody for 2 h at room temperature. The dilutions used were: survivin, Bax, Bcl-2, Cyt-c (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:1000; Caspase-9 (Abcam, USA) 1:1000; Caspase-3 (Abcam, USA), 1:250; and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:2000. Specific proteins were visualized using an enhanced chemiluminescence (ECL) system (Millipore, Bedford, MA, USA) and then exposed with Kodak X-ray film. Protein band intensities were determined densitometrically using the CMIASWIN video imaging system (Bio-Rad, Hercules, CA, USA).

### 2.6. Xenograft tumor model

All experiments were conducted according to the NIH Guide for the Care and Use of Laboratory Animals, and the study protocol was approved by the Ethics Committee for Animal Research of Wuhan University, China. Male BALB/c-nu/nu nude mice, 4–6 weeks old, were purchased from the Center for Animal Experiment of Wuhan University and used in the experiments. LoVo cells, suspended in 100 μL PBS, were subcutaneously inoculated into the lower right flank of the nude mice. When the tumors reached approximately 100 $mm^3$ in size, the nude mice were divided into four groups (6 in each group): a control group, a low-dose group (10 mg/kg), a mid-dose group, (20 mg/kg), and a high-dose group (40 mg/kg), and noscapine was administered via intratumoral injection every 3 days [8]. There was no significant difference among these groups at the beginning of treatment. Tumor growth was measured using calipers every 3 days. Tumor volume (TV) was calculated using the formula: TV ($mm^3$) = $d^2 \times D/2$, where d and D are the shortest and the longest diameters, respectively. At the end of the experiment, tumors were harvested for additional analyses as described below.

### 2.7. HE staining and TUNEL assay

For histologic analysis, tumor tissues were fixed in 4% formaldehyde, dehydrated with an ethanol gradient, and embedded in paraffin, and the paraffin tumor tissue sections were stained with hematoxylin and eosin (HE). Then, an in situ apoptosis detection kit (Roche Diagnostics, Branchburg, NJ, USA) was used to detect apoptotic cells in paraffin tumor tissue sections. The positive cells were identified, counted (six random fields per slides), and analyzed by light microscopy (Olympus, Japan).

### 2.8. Statistical analysis

All data were expressed as the mean ± standard error of the mean. The data were analyzed with the unpaired Student's t-test, and differences were considered significant at a $p$ value of less than 0.05.

## 3. Results

### 3.1. Noscapine inhibits colon cancer cell proliferation

Using the tetrazolium salt (WST-8) cell viability assay (see "Section 2"), we generated a dose-response curve by incubating cultures of HT-29, LoVo, and SW480 cells with various concentrations of noscapine for 72 h and observed a dose-dependent inhibition of cell viability (Fig. 1A). Of the three colon cancer cell lines, LoVo was the most sensitive to noscapine. Noscapine inhibited the viability of LoVo cells with an $IC_{50}$ of 75 μM at 72 h (Fig. 1A). Because exposure to 75 μM noscapine resulted in a significant inhibition of LoVo cell growth, we selected this dose to examine how this concentration affects cell viability at various timepoints