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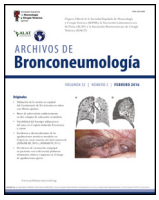

Original article

# Esculetin Attenuates the Growth of Lung Cancer by Downregulating Wnt Targeted Genes and Suppressing NF-κB

Xiangyun Zhu, Jiaping Gu, Hongxian Qian*

*Department of Respiratory Medicine, The Second People's Hospital of Wuxi, No. 68 Zhongshan Road, Wuxi 214002, China*

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ABSTRACT

*Introduction:* Esculetin was identified to inhibit cell proliferation and induce apoptosis or cell cycle arrest in several cancer cell lines. However, the effect of esculetin on lung cancer remains elusive.
*Methods:* The anti-proliferative role of esculetin in murine Lewis lung carcinoma (LLC) cells was evaluated by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and colony formation assays. BALB/c mice were subcutaneously injected with LLC cells to investigate the inhibitory effect of esculetin on the growth of lung cancer xenograft. Invasive ability was detected in esculetin treated and untreated LLC cells by transwell assay. The association between esculetin and Wnt/β-catenin signaling, as well as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), was confirmed by testing the expression of c-myc, Cyclin D1 and NF-κB using Western blot.
*Results:* Esculetin treatment in LLC cells led to significant decrease of cell proliferation in a time- and dose-dependent manner. After injection of LLC cells into mice, reduced size and weight of tumors were observed in esculetin treated mice compared to untreated mice. However, no difference in cell invasion was observed between the treated and untreated LLC cells. Notably decreased expression of c-myc, Cyclin D1 and NF-κB were observed in LLC cells with esculetin treatment compared to untreated cells.
*Conclusion:* Esculetin plays an inhibitory role in the growth of lung cancer by down-regulating c-myc, Cyclin D1 and NF-κB.

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## La esculetina atenúa el crecimiento del cáncer de pulmón por downregulation de los genes diana de Wnt y supresión de NF-κB

*Palabras clave:*
Esculetina
Cáncer de pulmón
Factor nuclear potenciador de las cadenas ligeras kappa de células B activadas (NF-κB)
C-myc
Ciclina D1

RESUMEN

*Introducción:* Se ha determinado que la esculetina inhibe la proliferación celular e induce la apoptosis o la detención del ciclo celular en varias líneas celulares de cáncer. Sin embargo, su efecto sobre el cáncer de pulmón es todavía desconocido.
*Métodos:* Se estudió el papel antiproliferativo de la esculetina en células murinas de carcinoma pulmonar de Lewis (LLC) mediante ensayos de bromuro de 3-(4,5-dimetil-2-tiazolil)-2,5-difenil-2H-tetrazolio (MTT) y de formación de colonias. Se inyectaron subcutáneamente células LLC a ratones BALB/c para investigar el efecto inhibidor de la esculetina sobre el crecimiento del xenoinjerto de cáncer de pulmón. La capacidad invasiva en células LLC tratadas o no tratadas con esculetina se evaluó mediante el ensayo transwell. La asociación entre la señalización de la esculetina y la de Wnt/β-catenina, y con el factor nuclear potenciador de las cadenas ligeras kappa de células B activadas (NF-κB) se confirmó midiendo la expresión de c-myc, de ciclina D1 y NF- κB usando Western blot.
*Resultados:* El tratamiento con esculetina de las células LLC disminuyó significativamente la proliferación celular de una manera dependiente del tiempo y de la dosis. Tras la inyección de células LLC en ratones, se observó que los tumores de los ratones tratados con esculetina eran de menor tamaño y peso que los de

* Corresponding author.
*E-mail address:* qianhongxianwuxi@126.com (H. Qian).

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los ratones no tratados. Sin embargo, no se observó diferencia en la invasividad celular entre las células LLC tratadas y las no tratadas. Se destacó la disminución de la expresión de c-myc, ciclina D1 y NF-κB en células LLC tratadas con esculetina en comparación con células no tratadas.
*Conclusión:* La esculetina desempeña un papel inhibitorio en el crecimiento del cáncer de pulmón a través de la regulación de c-myc, ciclina D1 y NF-κ B.
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## Introduction

Lung cancer is the third most prevalent cancer in the United States. Although less tobacco use contributes a reduced mortality rate,[1] lack of efficient treatment is still an obstruction to the survival of lung cancer patients. Surgery resection, chemotherapy and radiation therapy have been used as mainstay treatments, while recent advances in early detection and targeted therapy have improved the outcome and prognosis of lung cancer. However, several limits such as therapy resistance contribute to treatment failures and cancer recurrence.[2–4]

Previous evidence showed that Wnt/β-catenin signaling was frequently activated in lung cancer, leading to the promotion of cell proliferation, survival and metastasis.[5,6] Overexpression of *Wnt* genes, such as *Wnt5a*, is predictive of aggressive non-small-cell lung cancer (NSCLC).[7] In fact, Wnt/β-catenin signaling is not only highly activated in lung cancer but also in other cancers such as colon cancer. It is an important oncogenic factor in tumorigenesis, progression and metastasis. Accumulation of nuclear β-catenin caused by oncogenic Wnt signaling leads to its nuclear translocation and the formation of a complex with transcription factors, such as T-cell factor 4 (TCF-4), to initiation the transcription of its downstream targets such as *c-Myc* and *cyclin D1*.[8]

Esculetin (6,7-dihydroxycoumarin) is a derivative of coumarin found in *Artemisia capillaries, Citrus limonia* and a number of other natural plants. It has been evidenced to inhibit cell proliferation and induce apoptosis or cell cycle arrest in several cancer cell lines including human malignant melanoma and colon cancer cells, and cancers including hepatocellular carcinoma.[9–13] The interaction between esculetin and β-catenin disrupts the formation of β-catenin-Tcf complex in colon cancer.[14] Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation has been documented to play an important role in the progression of epidermal growth factor receptor (EGFR) mediated lung tumor.[15] Previous studies have evidenced the inhibitory effect of esculetin on NF-κB in pancreatic cancer cells and vascular smooth muscle cells.[16,17] Additionally, it was reported that esculetin may protect mice from lipopolysaccharide-induced acute lung injury via suppressing NF-κB.[18] Thus, in this study, we investigated the effects of esculetin on lung cancer and the involvement of Wnt and NF-κB signaling pathways. We found that esculetin treatment exhibited inhibitory effects on the growth of lung cancer cells. Esculetin downregulated Wnt targeted genes and suppressed NF-κB.

## Methods

### *Compound*

Esculetin (6,7-dihydroxycoumarin, 98% purity) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO, 0.1%).

### *Cell Culture*

Murine Lewis lung carcinoma (LLC) cells were cultured in Dulbecco's modified Eagles medium (DMEM, Life Technology, Pleasanton, CA, USA) supplemented with and penicillin (60 IU/ml)/streptomycin (50 μg/ml) and 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37 °C in a 5% $CO_2$ humidified incubator.

### *Measurement of Cell Proliferation*

Cell proliferation was measured using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. LLC cells ($6\times10^3$/well) were plated in 96-well plates. After 24 h, phosphate buffered saline (PBS) or esculetin at various concentrations (20, 40 or 80 μmol/l) was added into each well. MTT assay was performed every 24 h of incubation for 5 days. Briefly, 20 μl MTT (Amresco, USA) was added to each well and incubated for 4 h. The formazan precipitation was dissolved in 150 μl DMSO and the results were observed using a microplate reader (BioTek Instruments, Inc., Vermont, USA) at 490 nm. Each test was repeated for three times.

### *Colony Formation Assay*

The colony formation assay was performed in 6-well plates covered with 1% low melting agarose and 0.25 ml 2× RPMI with 20% FBS. Cells (500 cells/well)) were mixed 0.7% low melting agarose and 0.25 ml 2× RPMI with 20% FBS and seeded onto the bottom layer of soft agar. The plates were incubated for 21–28 days at 37 °C in a 5% $CO_2$ humidified incubator. Colony numbers were counted after staining with crystal violet and photographed using a phase-contrast microscope (100×).

### *Mice Model*

The experimental protocols were approved by the Ethics Committee. BALB/c mice (6–8-week-old female) were purchased from SLAC (Shanghai, China). LLC cells ($1\times10^6$ cells/100 μl PBS) were subcutaneously injected in the right inguinal region of mice. Mice were divided into two groups, untreated vehicle group and esculetin (100 mg/kg) treated group for 20 days. At the end of the treatment, body weight was measured and mice were sacrificed for obtaining tumor size and weight.

### *Transwell Assay*

Invasion assay was performed to test the effect of esculetin on cell invasive ability using transwell invasion chambers coated or non-coated with Matrigel (Becton Dickinson Labware, USA) according to the manufacturer's instructions. Cells (5000 cells/well) were plated onto the chambers in 100 μl medium containing 0.1% DMSO or esculetin (20, 40 or 80 μmol/l). After 48 h incubation, the cells on the bottom surface of the membrane were fixed in 90% alcohol and stained with crystal violet staining for counting using a microscope.

### *Western Blotting*

Cells were treated with or without esculetin for 24 h. Cell were lysed by Lysis Buffer containing PMSF on ice. Cell lysate was separated by 10% SDS-PAGE gels and transferred onto a NC membrane (Amersham Pharmacia Biotech, USA). The membranes

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