Phytomedicine 23 (2016) 468–476

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Boehmenan, a lignan from the Chinese medicinal plant *Clematis armandii*, induces apoptosis in lung cancer cells through modulation of EGF-dependent pathways

Li-Long Pan[a,b], Xi-Ling Wang[a,b], Qiu-Yang Zhang[a,b], Xiao-Ling Luo[a,b], Peng Xu[a,b], Si-Yu Liu[a,b], Jin-Feng Hu[c,*], Xin-Hua Liu[a,b,**]

[a] Shanghai Key Laboratory of Bioactive Small Molecules, School of Pharmacy, Fudan University, Shanghai 201203, China
[b] Department of Pharmacology, School of Pharmacy, Fudan University, Shanghai 201203, China
[c] Department of Natural Products Chemistry, School of Pharmacy, Fudan University, 826, Zhangheng Road, Pudong New District, Shanghai 201203, China

ARTICLE INFO

Article history:
Received 10 October 2015
Revised 29 January 2016
Accepted 7 February 2016

Keywords:
Boehmenan
Non-small cell lung cancer cells
Apoptosis
Migration
Epidermal growth factor receptor

ABSTRACT

Background: Epidermal growth factor receptor (EGFR) is an effective molecular target for cancer treatment. Boehmenan, a lignan from the dried stems of *Clematis armandii*, exhibited the potent cytotoxic effects against many cancer cell lines in previous studies. However, the effects and underlying mechanism of boehmenan on non-small cell lung cancer (NSCLC) remains unclear.

Purpose: The present study was designed to determine the *in vitro* anti-cancer properties and underlying molecular mechanisms of boehmenan on A549 NSCLC cells.

Study design/methods: Cellular viability and chemoattractive properties of macrophages were investigated by using MTT and transwell migration assay, respectively. Mitochondrial membrane potential ($\Delta\Psi_m$), apoptotic ratio, and cell cycle were measured by flow cytometry. Protein expression was visualized by Western blot using specific antibodies.

Results: Boehmenan concentration-dependently suppressed proliferation and induced G_1 phase arrest in A549 NSCLC cells, which were accompanied by reduction of migration, colony formation and increase of apoptosis in A549 cells. In addition, boehmenan treatment markedly modulated apoptosis-related protein (p53, p21, cleaved caspase 3, and cleaved PARP) and cyclin D1 expression and induced $\Delta\Psi_m$ collapse in a concentration dependent manner. Furthermore, boehmenan concentration-dependently inhibited EGF-induced activation of EGFR and its downstream signaling molecules, including MEK, Akt, ERK1/2, and STAT3.

Conclusion: Taken together, our results suggested that boehmenan-mediated anti-tumor property was mediated by modulation of mitochondria and EGFR signaling pathway in A549 NSCLC cells.

Introduction

Cancer is one of the most serious clinical problems worldwide and lung cancer is the leading cause of cancer mortality worldwide (Vallieres et al. 2012). Among the patients of lung cancer, non-small cell lung cancer (NSCLC) constitutes around 80% of lung malignancies, and the 5-year survival of this highly aggressive disease is only 15% (Govender et al. 2013). Due to inherent and acquired resistance of NSCLC cells, intervention strategies (such as surgery, radiotherapy, and/or chemo-therapy) are not satisfactory (Schuurbiers et al. 2009; Seal et al. 2012). Therefore, novel and effective chemotherapeutic agents are highly desired, particularly those derived from natural products due to their intrinsic advantages (Lee 2010; Ma and Wang 2009).

Apoptosis induction of cancer cells is one of therapeutic strategies to manage the problem of NSCLC (Cotter 2009; Pan et al. 2014). Two major pathways have been described in the apoptosis induction mechanism: the extrinsic or the death-receptor pathway and the intrinsic or the mitochondrial pathway (Liu et al. 2010).

Abbreviations: 5-Fu, 5-Fluorouracil; $\Delta\Psi_m$, mitochondrial membrane potential; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinase 1/2; MAPK, mitogen-activated protein kinase; MTT, 3-(4, 5-dimetrylthiazol)-2, 5-diphenyltetrazolium bromide; NSCLC, non-small cell lung cancer; PARP, poly (ADP-ribosyl) polymerase; PI3K, phosphatidylinositol 3-kinase; STAT3, signal transducer and activator of transcription 3; STS, staurosporine.

* Corresponding author. Tel./fax: +86 21 51980172.
** Corresponding author at: Department of Pharmacology, School of Pharmacy, Fudan University, Shanghai 201203, China. Tel.: +86 21 51980159.
E-mail addresses: jfhu@fudan.edu.cn (J.-F. Hu), liuxinhua@fudan.edu.cn, liuxinhuahua@gmail.com (X.-H. Liu).

http://dx.doi.org/10.1016/j.phymed.2016.02.006
0944-7113/

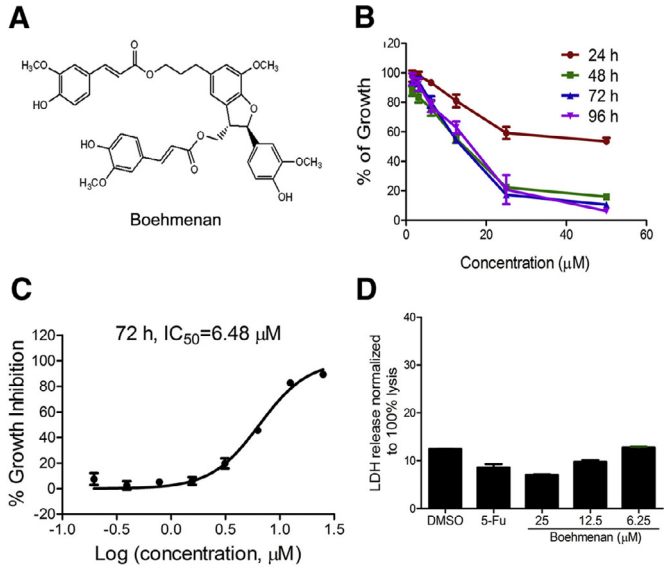

Fig. 1. Boehmenan inhibited proliferation of A549 cells. (A) The chemical structure of boehmenan. (B) A549 cells were treated with indicated concentrations of boehmenan for different periods and the proliferation of A549 cells was measured by MTT assay. (C) A549 cells were treated with indicated concentrations of boehmenan for 72 h and IC_{50} was calculated. (D) A549 cells were incubated with indicated concentrations of boehmenan (6.25, 12.5, 25 μM) or 5-Fu (25 μM) for 72 h. The levels of LDH in supernatant were measured by an LDH cytotoxicity assay kit. Data were from at least three independent experiments, each performed in duplicate; 5-Fu as a positive control.

The mitochondrial pathway is sensitive to various stress signals, including cytotoxic drugs (Yan et al. 2014). Mitochondrion is crucial for the execution of the apoptotic pathway because it activated caspase-9, which in turn activated caspase-3 (Hsiao et al. 2014). The dissipation of mitochondrial membrane potential ($\Delta\psi_m$) is one of the most important mechanisms of mitochondrial pathway. As $\Delta\psi_m$ collapse, mitochondrial permeability transition pores (PTP) are opened, leading to cytochrome *c* and other proapoptotic molecules released, from intermembrane space to cytosol. Finally, caspase-3 and poly (ADP-ribosyl) polymerase (PARP), DNA repair enzymes are activated, resulting in apoptosis (Pan et al. 2013a).The epidermal growth factor receptor (EGFR), a transmembrane receptor tyrosine kinase, activates the Ras/Raf/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and Janus kinase/signal transducer and activator of transcription (STAT) (Freudlsperger et al. 2011; Qu et al. 2014; Reungwetwattana et al. 2012), thereby stimulating tumor growth, angiogenesis, invasion, and survival (Freudlsperger et al. 2011; Qu et al. 2014). Increased EGFR activation and overexpression in various types of cancers (including NSCLC) is closely associated with tumorigenesis and cancer progression (Sharma et al. 2007). Thus, EGFR is an important target for anticancer treatment, and particularly of lung cancer (Burtness 2005; Sharma et al. 2007).

Clematis armandii Franch. (Ranunculaceae) (*Caulis clematidis armandii,* also named "Chuan-Mu-Tong" in China), a flowering climbing plant, is often found in southwestern China, especially in Si-Chuan (Szechwan) Province. In China, *Clematis armandii* has been long used in the treatment of inflammation conditions such as rheumatism and urinary tract infection, as well as for its diuretic and lactation promoting (Chawla et al. 2012). As a part of our ongoing effort to search for new anti-neuroinflammatory and anti-aging agents (Tang et al. 2013), the chemical components of the stems have been recently reinvestigated and 17 ligans including boehmenan (Fig. 1A) are isolated from *Clematis armandii* (Xiong et al. 2014). In previous studies, boehmenan has been reported to exert the potent cytotoxic effects on many cancer cells (Chin et al. 2006; Shono et al. 2015), it is unclear whether it has anti-cancer activity on NSCLC. Therefore, this study was then conducted to investigate the role and underlying mechanism of boehmenan on A549 NSCLC cells.

Materials and methods

Reagents and antibodies

RPMI-1640 medium and fetal bovine serum (FBS) were from GIBCO-BRL (USA). Antibodies against total- and phosphor (p)-STAT3 (Tyr705), total- and p-Akt (Ser473), total- and p-MEK (Ser217/221), total- and p-extracellular signal-regulated kinase 1/2 (ERK,Thr202/Tyr204), total- and p-EGFR (Tyr1068), cyclin D1, p53, and p21 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against β-actin, caspase-9, and cleaved caspase-3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against cleaved PARP-1 was from Epitomics (Burlingame, CA). 5-Fluorouracil (5-Fu), staurosporine (STS), 3-(4,5-dimetrylthiazol)-2,5-diphenyltetrazolium bromide (MTT), and all other chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO). Boehmenan was provided by Prof. Jin-Feng Hu (Department of Natural Products Chemistry, School of Pharmacy, Fudan University) and the purity was over 99% determined by high performance liquid chromatography. Boehmenan was dissolved in dimethyl sulfoxide (DMSO, 50 mM) and the final concentration of DMSO was less than 0.1%.

Cell culture

Human lung cancer cell line A549 cells, obtained from American Type Culture Collection (Manassas, VA, USA), were cultured in RPMI-1640 containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO) in 5% CO_2 at 37 °C.

Cell viability

Cell viability was evaluated by MTT assay as described previously (Liu et al. 2009). In brief, A549 cells in 96-well plates were incubated with different concentration of boehmenan for 24, 48, 72, or 96 h. The medium was changed before the assay. MTT dissolved in phosphate buffered saline, was added to the culture media to reach a final concentration of 0.5 mg/ml. After incubation at 37 °C for 4 h, the culture media containing MTT were removed. DMSO was then added into each well, and the absorbance at 570 nm was measured using a microplate reader (M1000, TECAN, Austria GmbH, Austria).

Colony formation assay

A549 cells were seeded at low density in complete media (1000 cells per well in six well plates) and treated with vehicle, 5-Fu (25 μM), or boehmenan (3.125–25 μM) for six days. After the treatment period, cells were washed with PBS and fixed with 4% formaldehyde for 20 min, washed again, stained with 0.1% crystal violet and individual clones were manually counted under the microscope. The colony formation efficiency was calculated as (number of colonies/number of cells inoculated) × 100%. All assays were independently performed in triplicate.

Cytotoxicity assay

The lactate dehydrogenase (LDH) based cytotoxicity detection kit (Jiancheng Biotechnology, Nanjing, China) was utilized.