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Protective role of gambogic acid in experimental pulmonary fibrosis *in vitro* and *in vivo*

Yubei Qu[1], Guanghua Zhang[1], Yunxia Ji, Haibo Zhua, Changjun Lv, Wanglin Jiang*

The Key Laboratory of Traditional Chinese Medicine Prescription Effect and Clinical Evaluation of State Administration of Traditional Chinese Medicine, School of Pharmacy, Binzhou Medical University, Yantai, P.R. China

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ABSTRACT

Background: Idiopathic pulmonary fibrosis (IPF) is a progressive disorder with poor prognosis. The treatment options for IPF are very limited. Gambogic acid (GA) has anticancer effect and anti-proliferative activity which is extracted from a dried yellow resin of the *Garcinia hanburyi* Hook.f. [Clusiaceae (Guttiferae)] in Southeast Asia. However, the anti-fibrotic activities of GA have not been previously investigated.

Methods: In this study, the effects of GA on TGF-β1-mediated epithelial-mesenchymal transition (EMT) in A549 cells and endothelial–mesenchymal transition (EndoMT) in human pulmonary microvascular endothelial cells (HPMECs), on the proliferation of human lung fibroblasts (HLF-1) were investigated *in vitro*, and on bleomycin (BLM)-induced pulmonary fibrosis was investigated *in vivo*.

Results: In TGF-β1 stimulated A549 cells, treatment with GA resulted in a reduction of EMT with a decrease in vimentin and p-Smad3 and an increase in E-cadherin instead. In TGF-β1 stimulated HPMECs, treatment with GA resulted in a reduction of EndoMT with a decrease in vimentin, and an increase in VE-cadherin instead. In the hypoxic HPMECs, treatment with GA reduced Vasohibin-2 (VASH-2), whereas increased VASH-1. In TGF-β1 stimulated HLF-1, treatment with GA reduced HLF-1 proliferation with a decrease in platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF-2) expressions. *In vivo*, treatment with GA for 2 weeks resulted in an amelioration of the BLM-induced pulmonary fibrosis in rats with a lower VASH-2. Instead, it was observed a higher VASH-1 expression at early stage of fibrosis at 1 mg/kg, with reductions of the pathological score, collagen deposition, α-SMA, PDGF and FGF-2 expressions at fibrotic stage at 0.5 mg/kg and 1 mg/kg.

Conclusion: In summary, GA reversed EMT and EndoMT, as well as HLF-1 proliferation *in vitro* and prevented pulmonary fibrosis *in vivo* by modulating VASH-2/VASH-1 and suppressing the TGF-β1/Smad3 pathway.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, irreversible and usually lethal lung disease, with unpredictable acute exacerbations that are often fatal. However, the cause is unknown (Ley et al. 2011; Raghu et al. 2011). The latest evidences indicated that the fibrotic response is driven by abnormally activated alveolar epithelial cells resulting in epithelial to mesenchymal transition (EMT) and formation of fibroblast and myofibroblast foci. The fibroblast and myofibroblast foci secrete amounts of extracellular matrix (ECM) resulting in scarring and destruction of the lung architecture (King et al. 2011). Several tyrosine kinase receptors were activated and involved the progression of IPF, and nintedanib (BIBF 1120) was approved for treatment of IPF (Myllärniemi et al. 2015).

Pulmonary arterial hypertension (PAH) is an increase of blood pressure in the pulmonary artery, pulmonary vein, or pulmonary capillaries, together known as the lung vasculature, leading to shortness of breath, dizziness, fainting, leg swelling and other symptoms. PAH is common in IPF patients, and the early development of PAH is associated with increased fibrotic cell mediators,

Abbreviations: α-SMA, alpha smooth muscle actin; AP, ponatinib; AT I, Human type I alveolar epithelial cell line; BLM, bleomycin; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; EndoMT, endothelial–mesenchymal transition; FGF-2, fibroblast growth factor; GA, gambogic acid; HE, hematoxylin and eosin; HPMEC, human pulmonary microvascular endothelial cells; Hyp, hydroxyproline; IPF, idiopathic pulmonary fibrosis; PAH, pulmonary arterial hypertension; PDGF, platelet-derived growth factor; VASH-1, vasohibin-1; VASH-2, vasohibin-2; vWF, von Willebrand factor.

* Corresponding authors. Tel.: +86 535 6912036; fax: +86 535 6912036.
E-mail addresses: Lucky_lcj@sina.com (C. Lv), jwl518@163.com (W. Jiang).

[1] These authors contributed equally to this work and should be considered co-first authors.

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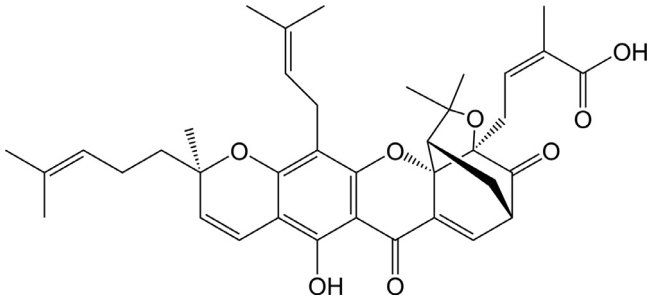

Fig. 1. *Structure of gambogic acid.*

abnormal vasculature or response to intermittent hypoxia. IPF patients benefit from treatment of PH (Corte et al. 2009). Vasohibin-1 (VASH-1), a unique endogenous angiogenesis inhibitor is induced in endothelial cells by proangiogenic factors and is a negative feedback regulator of angiogenesis (Nasu et al. 2009), knockdown of VASH-1 in cancer cells promoted cell growth, adhesion and migration *in vitro* and enhanced angiogenesis *in vivo* (Liu et al. 2015). Vasohibin-2 (VASH-2), an endogenous and vascular endothelial growth factor (VEGF)-independent angiogenic factor is highly expressed in tumor vessels which regulates tumor angiogenesis (Kitahara et al. 2014).

Gamboge is a dry resin secreted by *Garcinia hanburyi* Hook.f. [Clusiaceae (Guttiferae)] in Southeast Asia, and gambogic acid (GA, Fig. 1) is the main active compound of gamboge with the content from 22.5% to 34.58% in different areas of China (Yang et al. 1999). GA has various bioactivities including detoxification and anti-inflammatory (Zhao et al. 2010), anti-tumor and anti-proliferation (Wu et al. 2004; Cascão et al. 2014). Up to now, no pharmacological activity of GA has been reported in experimental pulmonary fibrosis. Therefore, we investigated the effects of GA on experimental pulmonary fibrosis *in vitro* and *in vivo* and proposed a mechanism of action.

Materials and methods

Chemicals

GA (CAS NO: 116064-77-8, molecular formula: $C_{18}H_{18}O_2$, purity >98.9%, provided by Paipai biological company, (Guangzhou, PR China); ponatinib (AP, purity >99.0%, CAS NO.: 1232836-25-7, provided by NCE biomedical company, Wuhan, PR China.); SIS3 (CAS NO: 1009104-85-1, molecular formula: $C_{28}H_{27}N_3O_3$, purity >98.9%, Santa Cruz Biotechnology, sc-222318); TGF-β1 (T7039, purity >98.0%, Sigma).

Cell culture

Human lung fibroblasts (HLF-1), human pulmonary microvascular endothelial cells (HPMECs) and human type II alveolar epithelial cells (A549 cell line) were purchased from the cell bank of the Chinese Academy of Sciences.

Animals

Adult male Sprague–Dawley rats (180–200 g, body weight) were housed individually under constant temperature (22 ± 2 °C) and humidity with a 12 h light/dark cycle and with free access to chow and water. All animal experimental procedures in this study were performed in accordance with the Institutional Animal Care and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Maryland, USA). The protocol was approved by the Committee on the Ethics of Animal Experiments of Binzhou Medical University (Permit Number: SCXK 20110003).

Epithelial-to-mesenchymal transition (EMT) of A549 cells in vitro

The A549 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) /F12 containing 10% (v/v) fetal bovine serum, 100 kU/l penicillin and 100 mg/l streptomycin at 37 °C in a humidified 5% CO_2. The cells were cultured at approximately 70% confluency and starved in the serum-free DMEM overnight. GA was incubated at concentrations of 0, 0.15 and 0.5 μM or AP at 0.3 μM or a Smad3 inhibitor, SIS3 with or without TGF-β1 5 ng/ml for 48 h. Pictures were taken in five random fields under inverted microscope, then the proteins were extracted to detect the expression of vimentin, E-cadherin, Smad3 and p-Smad3 by western blots. Data were normalized against those of the corresponding β-actin bands. Results were expressed as fold increase over the normal.

Endothelial–mesenchymal transition (EndoMT) of HPMECs in vitro

HPMECs were maintained in DMEM (high glucose) containing 10% (v/v) fetal bovine serum, 100 kU/l penicillin and 100 mg/l streptomycin at 37 °C in a humidified 5% CO_2. The cells were cultured at approximately 70% confluency and starved in serum-free DMEM overnight. GA was incubated at concentrations of 0, 0.15 and 0.5 μM or AP at 0.3 μM with or without TGF-β1 5 ng/ml in A549 cells for 48 h, pictured five random fields in the inverted microscope camera, then extracted the protein to detect the expression of vimentin (ab45939) and VE-cadherin (ab119785) by western blots. Data were normalized against those of the corresponding β-actin bands. Results were expressed as fold increase over the normal.

HPMECs were cultured in vitro during the hypoxic condition

The HPMECs were maintained in DMEM/F12 containing 10% (v/v) fetal bovine serum, 100 kU/l penicillin and 100 mg/l streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. The cells were cultured at approximately 80% confluency in DMEM, GA were incubated at concentrations of 0, 0.15 and 0.5 μM for 48 h in the hypoxic condition (5% CO_2, 93% N_2, 2% O_2), then collected the HPMECs, lysed and analyzed VASH-1 and VASH-2 expression. Data were normalized against those of the corresponding β-actin bands. Results were expressed as fold increase over the normal.

HLF-1 proliferation assay

For the proliferation assays *in vitro*, HLF-1 were seeded into 96-well (1×10^5 cells/well) flat bottom plates with medium alone (control) or medium containing the different concentrations of GA (0, 0.15, 0.5 and 1 μM), with or without TGF-β1 5 ng/ml. Cell proliferation was tested by MTT methods. Briefly, the serum-starved cells were treated with GA for 48 h. The absorbance was recorded at 490 nm (Spectramax/M5 multi-detection reader, molecular devices, USA), and calculated as a *ratio* against the untreated cells. The cells were treated in the same way. PDGF and FGF-2 expression were measured by Western blot.

HLF-1 cells were exposed to cobalt chloride ($CoCl_2$, 100 μM) in order to mimic hypoxia. The viability of HLF-1 cells was determined by MTT methods. These HLF-1 cells were seeded into 6-well flat bottom plates. One blank well in every plate was left which was filled with normal HLF-1 cells without $CoCl_2$ and with medium alone as the control. The others were with medium containing the different concentrations of GA (0, 0.15, 0.5 μM) or AP at 0.3 μM (Qu et al. 2015) for 48 h maintained in DMEM (high glucose) containing 10% (v/v) fetal bovine serum, 100 kU/l penicillin and 100 mg/l streptomycin at 37 °C in a humidified 5% CO_2. The expression of PDGF and FGF-2 was measured by Western blot. Data