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Anti-angiogenic effect of furanodiene on HUVECs *in vitro* and on zebrafish *in vivo*

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ABSTRACT

Ethnopharmacological relevance: Furanodiene is an active ingredient of the traditional Chinese medicine, *Rhizoma Curcumae*, commonly used for the treatment of cancer in China.
Aim of the study: To investigate the anti-cancer property of *Rhizoma Curcumae*, this study describes the anti-angiogenic activities of furanodiene in human umbilical vein endothelial cells (HUVECs) *in vitro* and in zebrafish *in vivo*.
Materials and methods: HUVECs were treated with different doses of furanodiene in the presence or absence of vascular endothelial growth factor (VEGF). The anti-proliferative effect of furanodiene was measured using the XTT assay. The anti-migration and anti-invasion activities of this compound were investigated with a wound-healing migration model and a three-dimensional cell invasion model, respectively. The effects of furanodiene on HUVEC differentiation were assessed by *in vitro* tube formation in Matrigel™. The expression of related proteins was detected by Western blot. Morphological observations of zebrafish were evaluated in transgenic *Tg* (*fli1: EGFP*) zebrafish embryos.
Results: Our results showed that furanodiene exposure could significantly inhibit the proliferation of HUVECs in a dose-dependent manner and inhibit VEGF-induced proliferation at a low dose. Relative to the VEGF-induced control, the number of invading and migrating cells was significantly reduced in the furanodiene-treated groups. Furanodiene also dramatically suppressed tube formation and p-Akt (Ser473), p-Erk 1/2 (Thr202/Tyr204), ICAM-1, p-p85 (Ser428) as well as p85 protein expression. Furthermore, exposure to furanodiene inhibited angiogenesis in the zebrafish model.
Conclusions: This study demonstrated that furanodiene exposure exhibits a potential anti-angiogenic effect through suppression of endothelial cell growth, invasion, migration and tube formation via regulation of the PI3K pathway. This potential anti-angiogenic effect of furanodiene may play an important role in the anti-tumor activity of the traditional Chinese medicine, *Rhizoma Curcumae*.

1. Introduction

Furanodiene (Fig. 1A) is a sesquiterpene isolated from the essential oil of *Curcuma wenyujin* (Yang et al., 2005). Furanodiene is an active ingredient of the traditional Chinese medicine, *Rhizoma Curcumae*, commonly used for the treatment of cancer in China. Previous studies have reported that this compound exhibits hepatoprotective, anti-inflammatory and anti-tumor activities (Matsuda et al., 1998; Makabe et al., 2006; Xiao et al., 2007; Ma et al., 2008). Furanodiene induces apoptosis in HepG2 cancer cells through activation of mitochondrial and caspase-3-pathway which involved activation of p38 and inactivation of Erk 1/2 MAPK signaling cascades (Xiao et al., 2007). In additional to its antiproliferative effect in HL60 cancer cells, furanodiene-induced apoptosis is mediated by upregulation of TNF receptor 1 as well as induction of TNF-α production to activate TNFR1 signaling pathway (Ma et al., 2008).

Angiogenesis is essential for normal vascular development and malignant tumor growth. It is generally accepted that the modulation of angiogenesis (e.g., blocking the blood supply to a specific region) is an attractive therapeutic strategy for the treatment of a wide variety of human diseases, including cancer, inflammation and ischemic heart diseases (D'Amore and Ng, 2002). Angiogenesis is a complex process consisting of proliferation, migration and differentiation of endothelial cells (ECs) and is tightly regulated by growth factors, specific receptors, and intracellular signaling pathways (Bussolino et al., 1997). VEGF is a major angiogenic factor associated with cancer (Weis and Cheresh, 2005). These

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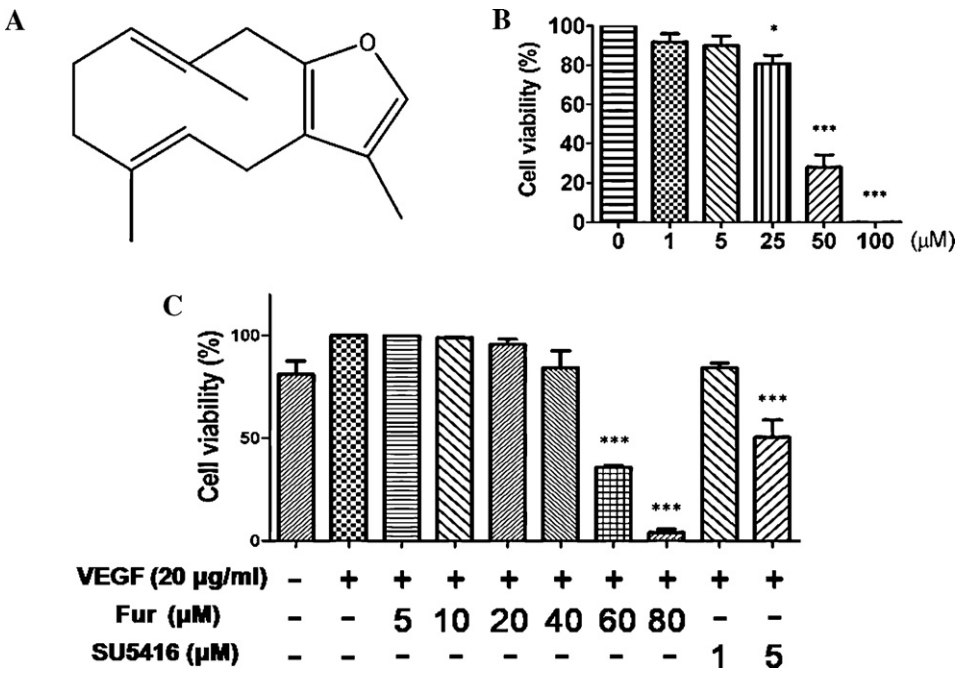

Fig. 1. Characterization of furanodiene (Fur) screening in HUVECs. (A) Chemical structure of furanodiene. (B) Furanodiene inhibits HUVEC proliferation (*$P < 0.05$, ***$P < 0.001$). (C) Furanodiene suppresses VEGF-induced proliferation of HUVECs (***$P < 0.001$).

pro-angiogenic factors induce pre-existing endothelial progenitors and ECs to proliferate and differentiate. Stimulated ECs then invade and migrate into the extracellular matrix (ECM) and form an interconnected network with the surrounding cells (Risau, 1997). The disruption of any of these processes may contribute to successful cancer therapy.

It is still unclear, however, whether furanodiene has any activities pertaining to angiogenesis, a process crucial for the progression of cancer and other diseases. In this study, the effect of furanodiene on the process of vasculature formation, including cell growth, cell migration, invasion and tube formation, was evaluated both *in vitro* and *in vivo* using vascular endothelial cells and transgenic *Tg* (*fli1: EGFP*) zebrafish embryos, respectively. The present study was the first to investigate the potential anti-angiogenic properties of furanodiene.

2. Materials and methods

2.1. Chemicals and reagents

Furanodiene (>96%) was isolated and identified by Prof. Shaoping Li (University of Macau, Macau, China) as previously described (Yang et al., 2005). HUVECs were purchased from American Type Cell Culture (Manassas, VA). Kaighn's modification of Ham's F-12 medium (F-12K), fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin–streptomycin (PS) and 0.25% (w/v) trypsin/1 mM EDTA were purchased from Invitrogen (Carlsbad, CA, USA). Endothelial cell growth supplement (ECGS), heparin and gelatin were supplied by Sigma (St. Louis, MO). Growth factor reduced (GFR) Matrigel™ basement membrane matrix, VEGF, Matrigel™ 24-well plates and the BioCoat™ Matrigel™ invasion chamber were obtained from BD Biosciences (Bedford, MA). SU5416 was purchased from Calbiochem (Darmstadt, Germany). Primary antibodies against p-Akt (Ser473), p-Erk 1/2 (Thr202/Tyr204), ICAM-1, p-P85 (Ser428), p85 and β-actin and secondary antibodies were obtained from Cell Signaling (Danvers, MA, USA).

2.2. Cell culture and drug treatment

HUVECs were cultured as previously reported (Lam et al., 2008; Hong et al., 2009). The stock solution of furanodiene (100 mM) was dissolved in DMSO and then diluted to various concentrations as needed. The stock solution of VEGF (10 μg/ml) was dissolved in sterilized Milli-Q water, and the stock solution of SU5416 (5 mM) was dissolved in DMSO.

2.3. Collection of zebrafish embryos

Embryos were generated by natural pair-wise mating when the fish were 3–12 months old. The filters were switched off, and breeding boxes were placed into the tanks, meanwhile switch on the lights. The fish were left undisturbed for 15–30 min. Breeding boxes were collected, and the embryos were transferred into clean petri dishes with a fine fishing net. The embryos were maintained in Milli-Q water. Healthy, transparent and regular embryos were selected at the 1–4 cell stage and were distributed into a 24-well microplate. Each well contained 8–10 embryos, depending on the assay.

2.4. Cytotoxicity assay in the absence of growth factors

The effect of furanodiene on HUVEC viability was assessed by the XTT assay. Cells were trypsinized and seeded at 10^4 cells/well in 96-well gelatin coated plates. After a 24-h incubation at 37 °C, the complete medium was removed, and the cells were deprived of angiogenic growth stimulation for 24 h in low serum (0.5% FBS) medium. Following these pre-incubations, the medium was replaced with fresh medium containing various concentrations (0–80 μM) of furanodiene. After 24 h of furanodiene treatment, cell proliferation was assessed with the Cell Proliferation Kit II (Roche, Mannheim, Germany) using a previously described method (Economou et al., 2008). The absorbance of the orange colored formazan product at 490 nm and the reference wavelength at 690 nm were measured using a Multilabel counter (Perkin Elmer,