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Inhibition of angiogenesis involves in anticancer activity of riccardin D, a macrocyclic bisbibenzyl, in human lung carcinoma

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ABSTRACT

Riccardin D is a novel macrocyclic bisbibenzyl compound extracted from Chinese liverwort plant *Dumortiera hirsuta*. Our previous studies showed that riccardin D is a DNA topo II inhibitor and has therapeutic potential for treatment of cancers. In this combined *in vitro* and *in vivo* study, we examined the inhibitory effects of riccardin D on tumor angiogenesis and the subsequent effect of anticancer activity was evaluated. Incubation with riccardin D weakly inhibited the proliferation of human umbilical vascular endothelial cells (HUVEC) as estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The scratch wound experiment showed that riccardin D effectively decreased the motility and migration of HUVEC cells. Riccardin D inhibited the formation of capillary tube as demonstrated by decrease of branch points formed by HUVEC cells on 3-D Matrigel. We examined the levels of angiogenic factors including vascular endothelial growth factor (VEGF), VEGF receptor 2, epidermal growth factor receptor (EGF receptor), and matrix metalloproteinase (MMPs) in HUVEC cells. The expressions of VEGF, phospho-VEGF receptor 2, EGF receptor and MMP-2 were significantly reduced by riccardin D as estimated by Western blot assay and real-time quantitative PCR analysis. The decrease of VEGF was also detected in riccardin D-treated human lung cancer H460 cells. The anticancer activity of riccardin D was then evaluated in a mouse model in which riccardin D delayed the growth of H460 xenografts without obvious toxicity to animals after three weeks injection. To evaluate the role of antiangiogenesis of riccardin D in mice, CD34 immunohistochemical staining was employed to analyze the mean vascular density in H460 xenograft tissues. The number of blood vessels was significantly decreased after riccardin D treatment. These results suggest that riccardin D display the inhibitory effect on growth of human lung carcinoma cells and that the inhibition of angiogenesis may involve in anticancer activity of riccardin D.

1. Introduction

Macrocyclic bisbibenzyls, which belong to the family of phenolic compounds, are a class of characteristic components from liverworts. They are attracting more and more attention for their wide range of biological activities such as cytotoxicity, antioxidation and antifungi (Bringmann et al., 2004; Li et al., 2009). In our group, we isolated a series of macrocyclic bisbibenzyl compounds and their biological activities were evaluated. Marchantin C, which was extracted from *Dumortiera angust*, was found to inhibit the proliferation of human nasopharyngeal carcinoma KB cells and human glioblastoma A172 cells (Shen et al., 2010; Xi et al., 2010). Marchantin C induced mitotic arrest of cancer cells by depolymerizing microtubules (Shi et al., 2008a, 2008b; Shi et al., 2009). Another homologue of macrocyclic bisbibenzyl plagiochin E, which was isolated from *Marchantia polymorpha*, was found to suppress the activity of P-glycoprotein (P-gp) and displayed the reversal effect on multidrug resistance in adriamycin-induced resistant K562/A02 cells (Shi et al., 2008a, 2008b).

Riccardin D, a novel macrocyclic bisbibenzyl, was isolated from Chinese liverwort plant *Dumortiera hirsuta* (Lu et al., 2006). Our previous study showed that riccardin D had great effects against the proliferation of some cancer cells, such as K562, K562/A02 and HL60. Riccardin D induced apoptosis of human leukemia cells by targeting DNA topoisomerase II (Xue et al., 2010). Thus, riccardin D has been considered as a potential candidate compound for treatment of cancers. However, the inhibitory effect of riccardin D on cancer growth has not been completely evaluated and the mechanism of action has not been investigated. In this study, we examined the inhibitory effect of riccardin D on the formation of capillary tube in human vascular endothelial cells (HUVEC) *in vitro* and the expressions of vascular endothelial cell

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growth-related angiogenic factors were also measured. We evaluated the therapeutic potential effect in human lung cancer H460 xenografts in mice and then examined the mean vascular density in the xenograft tissues. These experiments offer an objective assessment of riccardin D involvement of inhibition of angiogenesis in anticancer activity.

2. Materials and methods

2.1. Chemical

Riccardin D was isolated from the Chinese liverwort plant *Dumortiera hirsuta* and its structure was identified as reported previously (Fig. 1) (Lu et al., 2006). The purity of riccardin D was 98.6% as measured by high performance liquid chromatography (HPLC). Riccardin D was dissolved in dimethylsulfoxide (DMSO, Sigma, USA) for the *in vitro* assays. For animal experiment, riccardin D nanosuspension was prepared by the HPH method as described previously (Xiong et al., 2008). Riccardin D was dispersed in an aqueous surfactant solution, containing 0.2% (w/v) lecithin and 0.1% (w/v) poloxamer 188 under magnetic stirring. The suspensions were pre-milled at 13,000 rpm and were further processed via HPH by an EmulsiFlex-C3 equipped a heat exchanger applying 5 homogenization cycles at 500 bar, and then by 15 homogenization cycles at 1800 bar for nanosuspension (Zheng et al., 2011). Dried riccardin D nanosuspension was prepared by spray drying. Riccardin D nanosuspension was dissolved in the distilled water before use.

2.2. Cell lines and cell culture

The human umbilical vein vascular endothelial cell line (HUVEC) was purchased from American Type Culture Collection (Manassas, Virginia, USA). HUVEC cells were maintained in Medium 199 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, USA), penicillin-streptomycin, fungizone (Gibco, USA) and endothelial cell growth factor (BD Biosciences, USA) at 37 °C in a humid atmosphere (5% CO_2–95% air). The human lung cancer cell line H460 was purchased from Cell Bank, China Academy of Sciences (Shanghai, China). H460 cells were maintained in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, and 10 mM HEPES buffer at 37 °C in a humid atmosphere (5% CO_2–95% air) and were harvested by brief incubation in 0.02% (w/v) EDTA in PBS.

2.3. HUVEC cell proliferation assay

HUVEC cells (5×10^3 per well) seeded in 96-well plates were exposed to increasing concentrations of riccardin D (2.5–30 μM) for the indicated time. The medium was then removed and the wells were washed with PBS. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μl of MTT (5 mg/ml, Sigma, USA) for 4 h (Chen et al., 2008). Light absorbance of the solution was measured at 570 nm on THERMOmax microplate reader (Molecular Devices, USA). Triplicate experiments with triplicate samples were performed.

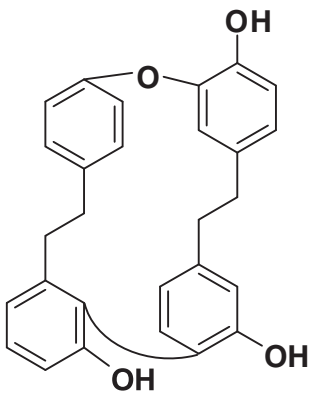

Fig. 1. Chemical structure of riccardin D.

2.4. Scratch assay

The migratory activity of HUVEC cells was examined in the presence of riccardin D using a wound scratch assay (Wang et al., 2009). HUVEC cells were seeded into 6-well culture dishes at 2.0×10^5 cells/well. After HUVEC cells were allowed to attach and reach 80% confluence, a scratch (1 mm) was made through culture dish with a sterile plastic 200 μl micropipette tip to generate one homogeneous wound. After wounding, the peeled off cells were removed with twice PBS washes. HUVEC cells were further incubated without or with riccardin D for 12 and 24 h and then the wound widths were measured under microscope using an ocular grid. The experiments were repeated in triplicate wells at least three times. Cell migration = 0 time wound width (1 mm) -12 or 24 h wound width. The migration thus observed is represented as percentage migration considering migration in untreated control as 100%. Images of wound were taken using a microscope at 100× magnification (Olympus IX51, Japan).

2.5. Capillary tube formation assay

The capillary tube formation assay was performed to evaluate the inhibitory effect of riccardin D on the formation of capillary tube by HUVEC cells on 3-D Matrigel (Xiao and Singh, 2007). Matrigel (100 μl, Sigma, USA) at 4 °C was used to coat each well of a 96-well plate and allowed to polymerize for 1 h at 37 °C. HUVEC cells were suspended in medium at a density of 2×10^5 cells/ml, and 0.1 ml of cell suspension was added to each well, together with or without riccardin D and incubated at 37 °C in a humidified chamber with 5% CO_2. The morphogenesis of capillary tube was visualized after 18 h under an inverted microscope using a 10× objective lens. Images from a total of five microscopic fields per well were analyzed by Motic Image Plus 2.0 software (Motic Instruments Inc., Richmond, Canada). The tube formation was defined by counting the branch points of the formed tubes and average numbers of branch points were calculated. The experiments were repeated three times.

2.6. Western blot analysis

HUVEC cells (3.0×10^5) seeded in 6-well plates were incubated with increasing concentrations of riccardin D for 48 h. The cells were harvested and cell lysates (30 μg of protein per lane) were fractionated by 10% SDS-PAGE. The proteins were electro-transferred onto nitrocellulose membranes and then protein levels were detected using dilutions of the primary antibodies (Gao et al., 2008; Qi et al., 2008). The primary antibodies included anti-EGF receptor (Boster, China), anti-VEGF (sc-152, Santa Cruz, USA), anti-phospho-VEGF receptor 2 (19A10, Cell Signaling Technology, USA) and anti-MMP-2 (sc-10736, Santa Cruz, USA). The primary antibodies were washed in 0.05% Tween-20/PBS and then incubated with horseradish peroxidase-conjugated secondary antibody. The bound antibodies were visualized using an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) and quantified by densitometry using an electrophoresis image analysis system. The data is expressed as the relative density of the protein normalized to β-actin. Triplicate experiments with triplicate samples were performed.