European Journal of Medicinal Chemistry 59 (2013) 243–252

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Anti-angiogenesis and immunomodulatory activities of an anti-tumor sesquiterpene bigelovin isolated from *Inula helianthus-aquatica*

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ARTICLE INFO

Article history:
Received 4 July 2012
Received in revised form
5 November 2012
Accepted 19 November 2012
Available online 24 November 2012

Keywords:
Bigelovin
Inula helianthus-aquatica
Angiogenesis
Zebrafish
HMEC-1
Cell adhesion molecules

ABSTRACT

Bigelovin is a sesquiterpene lactone isolated from the plant *Inula helianthus-aquatica* which was traditionally used in cancer treatment in Yunnan, China. The potent apoptotic activities of bigelovin in human leukemia U937 cells were shown in our previous study. The present study investigated the anti-angiogenic and immunomodulatory effects of bigelovin using transgenic zebrafish *Tg(fli1a:EGFP)y1* with fluorescent blood vessels and human peripheral blood mononuclear cells (PBMCs), respectively. Furthermore, the inhibitory activities of bigelovin on the human endothelial cell adhesion molecules (CAMs) were also examined. Our results showed that the growth of subintestinal vessels of the bigelovin-treated zebrafish embryos was significantly inhibited and the gene expressions in angiogenesis signaling pathways (e.g. Ang2 and Tie2) of the zebrafish were down-regulated after bigelovin treatment. Besides, the proliferation and Th1 cytokines productions (e.g. IFN-γ, IL-2 and IL-12) were suppressed in bigelovin-treated PBMCs. On the other hand, bigelovin was shown to significantly inhibit the human monocyte adhesion to human endothelial cells and the gene expressions of inflammation-related CAMs (e.g. ICAM-1, VCAM-1 and E-selectin) were significantly down-regulated in bigelovin-treated human endothelial cells. In summary, our data provide the first evidence that bigelovin possesses anti-angiogenic and immunomodulatory activities, suggesting bigelovin may exert multi-target functions against cancer in animal models.

1. Introduction

Angiogenesis is tightly linked to chronic inflammation and cancer [1]. Chronic inflammation conditions have been found to mediate a wide variety of diseases including cancer [2]. The connection between inflammation and carcinogenesis was shown to be bridged partly by angiogenesis [3]. It is believed that blocking angiogenesis could be a strategy to arrest tumor growth and metastasis [4]. In the last decade, plenty studies showed that nonsteroidal anti-inflammatory drugs (NSAIDs) were effective to treat cancer [5,6]. On the other hand, cancer is always regarded as an inflammation site, the activated endothelium nearby will produce cytokines and cell adhesion molecules (CAMs) that recruit inflammatory cells, such as leukocytes [3]. The leukocytes then attach and extravasate to the target site [7]. Cell adhesion molecules such as intercellular cell adhesion molecule-1 (ICAM-1), and vascular endothelial cell adhesion molecule-1 (VCAM-1) mediate such process. Anti-angiogenesis, which are aimed at suppressing new blood vessel growth, as well as anti-migration of inflammatory cells and/or cancer cells, have the potential to become new targets focus or adjuvants for cancer treatment.

Botanical products, including traditional Chinese herbs, are rich source of angiogenesis-modulating [8] and CAMs-modulating [9] agents. Some compounds isolated from natural products, such as Taxol® (from *Taxus brevifolia* [10]), combretastatin (from *Combretum caffrum* [11]), triptolide (from *Tripterygium wilfordii* [12]) and farnesiferol C (from *Ferula assafoetida* [13]) have been shown to

Abbreviations: CAMs, cell adhesion molecules; PBMCs, peripheral blood mononuclear cells; SIV, subintestinal vessels.

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http://dx.doi.org/10.1016/j.ejmech.2012.11.029

have anti-angiogenic activities *in vitro* and *in vivo*. Several plant and fungal species, such as *Boswellia serrata*, *Canscora decussata*, *Ganoderma lucidum*, *Ginkgo biloba*, *Hypericum perforatum*, *Juglans regia*, *Panax notoginseng*, *Rheum undulatum* and *Salvia miltiorrhiza* have demonstrated modulation of multiple CAMs [9].

Bigelovin, a sesquiterpene lactone isolated from the plant *Inula helianthus-aquatica* C.Y. Wu ex Ling (family Asteraceae) which was used in folk remedies for cancer treatment and milk duct obstruction in Yunnan province of China. Bigelovin was isolated from *Inula hupehensis* and *I. helianthus-aquatica* and was shown to have cytotoxic activities in 1996 [14]. The crude aqueous extract of *I. helianthus-aquatica* has been shown to have potent antitumor activities in several human cancer cell lines and mouse Ehrlich ascites tumor model [15]. Besides, the *in vivo* anti-tumor activities of bigelovin were also previously reported [16]. In fact, the detailed mechanisms of actions of bigelovin in leukemia cells have been demonstrated in our previous study [17]. Bigelovin induced cell cycle arrest and apoptosis in leukemia U937 cells. On the other hand, bigelovin was shown to inhibit monocyte adhesion and adhesion molecule expression in mouse brain endothelial cells [18]. Their results also indicated that bigelovin blocked IκBα degradation and NF-κB activation. Nevertheless, other biological activities, such as anti-angiogenic and immunomodulatory effects of bigelovin have not been investigated.

The present study aimed to investigate the anti-angiogenic effects of bigelovin in zebrafish *in vivo* model as well as human microvascular endothelial cell HMEC-1. The immunomodulatory activities of bigelovin were examined in human peripheral blood mononuclear cells (PBMCs). In addition, the inhibitory effects of bigelovin on human inflammatory monocyte adhesion to endothelial cells and the mRNA expression of CAMs were also evaluated in this study.

2. Materials and methods

2.1. Materials

The transgenic zebrafish line *Tg(fli1a:EGFP)y1* was purchased from the Zebrafish International Resource Center in University of Oregon, USA. The use of zebrafish for experiment was approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong. Human peripheral blood mononuclear cells (PBMCs) were isolated from human buffy coat preparations, obtained from the Hong Kong Red Cross Blood Transfusion Service. The use of human buffy coat for experiment was approved by The Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee. The human microvascular endothelial cells (HMEC-1) and human acute monocytic leukemia cells (THP-1) were purchased from American Type Culture Collection (MD, USA). Cell culture medium RPMI-1640, fetal bovine serum (FBS), penicillin, streptomycin, trypsin-EDTA, phosphate-buffered saline (PBS), Trizol, SuperScript III Reverse Transcriptase, dNTP, CellTracker Red CMTPX were purchased from Life Technologies (NY, USA). MCDB131 medium, epidermal growth factor, hydrocortisone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), phytohemagglutinin (PHA), were obtained from Sigma (MO, USA). The ELISA kits for IL-2, IL-12, IFN-γ were purchased from BD Pharmingen (CA, USA). Real-time PCR reagent iTaq Fast SYBR Green Supermix was from Bio-Rad (Hong Kong). [Methyl-^{3}H]-thymidine and unifilters were from PerkinElmer (MA, USA). Human recombinant TNF-α was obtained from PeproTech (USA).

2.2. Plant material

Leaves and flowers of *I. helianthus-aquatica* were collected in 2004 in Yunnan, China, and identified by Prof. Xi-Wen Li of Kunming Institute of Botany. The voucher specimen (KUN0495655) was deposited in the Herbarium (KUN) of Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China.

2.3. Extraction and isolation of bigelovin

Leaves and flowers of *I. helianthus-aquatica* (500 g) were successively extracted with petroleum ether, and then concentrated *in vacuo*. The petroleum ether extracts were treated with 60% ethanol under reflux for two times, then combined and filtered to get the ethanol-soluble portion [17]. Bigelovin was obtained together with ergolide as a crystal mixture from the ethanol-soluble portion after repeated crystallization in 100% ethanol. Then the mixture was further separated by preparative HPLC [Agilent 1100 HPLC system, Zorbax ODS, 21.2 × 250 mm, 5 μm, U.S.A., MeOH-H_2O (45:55), wavelength: 210, 230 nm] to provide 1.5 g pure bigelovin (Fig. 1). The structure was elucidated by spectroscopic analysis including 2D-NMR spectroscopy and HR-EI-MS [17].

2.4. Zebrafish (Danio rerio) model

2.4.1. Zebrafish husbandry

The transgenic zebrafish line *Tg(fli1:EGFP)y1*, in which the endothelial cells express enhanced Green Fluorescent Proteins (eGFP), was ordered from Zebrafish International Resource Center, University of Oregon (USA) and maintained as described in the Zebrafish Book (http://zfin.org/zf_info/zfbook/zfbk.html). Briefly, the fish were maintained in a controlled environment at a temperature of 28 °C on a 10 h: 14 h light/dark cycle (lights on at 09:00am daily). Zebrafish were housed in 9 L tanks, continuously supplied with filtered reverse osmosis water with Instant Ocean Salt at 0.06 g/L. The fish were fed twice daily in the morning and the afternoon, with general tropical fish food, and also with brine shrimp three times per week.

2.4.2. Embryo collection

Embryos were collected by natural pair-wise mating, as described in the Zebrafish Book (http://zfin.org/zf_info/zfbook/zfbk.html) with minor modifications. Fourteen hours before mating, female and male fish were housed in two different compartments in a small tank partitioned with a spacer. At the time of mating, the lights were switched on and the spacer was removed. The fish were left undisturbed for 15–30 min. The embryos were then transferred to clean Petri dishes via a fine fishing net.

2.4.3. Bigelovin treatment on zebrafish embryos

Healthy, limpid, and regular embryos were picked out at the 1–4 cell stage and were transferred into a 24-well microplate, with 20

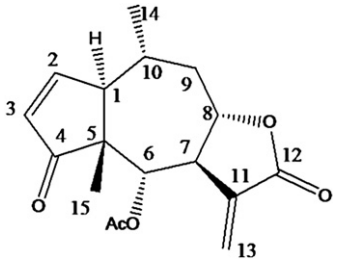

Fig. 1. Chemical structure of bigelovin.