Steroids 115 (2016) 160–168

Contents lists available at ScienceDirect

Steroids

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

Synthetic stigmasterol derivatives inhibit capillary tube formation, herpetic corneal neovascularization and tumor induced angiogenesis Antiangiogenic stigmasterol derivatives

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ARTICLE INFO

Article history:
Received 5 April 2016
Received in revised form 21 July 2016
Accepted 2 September 2016
Available online 10 September 2016

Keywords:
Stigmasterol derivative
Neovascularization
HUVEC
Antiangiogenic activity
VEGF
Tumor
Herpetic keratitis

ABSTRACT

Angiogenesis plays a critical role in initiating and promoting several diseases, such as cancer and herpetic stromal keratitis (HSK). Herein, we studied the inhibitory effect of two synthetic stigmasterol derivatives on capillary tube-like structures and on cell migration in human umbilical vein endothelial cells (HUVEC): (22*S*,23*S*)-22,23-dihydroxystigmast-4-en-3-one (compound **1**) and (22*S*,23*S*)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one (compound **2**). We also studied their effect on VEGF expression in IL-6 stimulated macrophages and in LMM3 breast cancer cells. Furthermore, we investigated the antiangiogenic activity of the compounds on corneal neovascularization in the murine model of HSK and in an experimental model of tumor-induced angiogenesis in mice.

Both compounds inhibited capillary tube-like formation, but only compound **1** restrained cell migration. Compound **1**, unlike compound **2**, was able to reduce VEGF expression. Only compound **1** not only reduced the incidence and severity of corneal neovascularization, when administered at the onset of HSK, but it also restrained the development of neovascular response induced by tumor cells in mice skin.

Our results show that compound **1** inhibits angiogenesis *in vitro* and *in vivo*. Therefore, compound **1** would be a promising drug in the treatment of those diseases where angiogenesis represents one of the main pathogenic events.

1. Introduction

Angiogenesis is an essential process in both embryonic development and adulthood that if not tightly regulated, it frequently becomes imbalanced and it is then associated with several pathological situations [1,2]. In this sense, angiogenesis leads to tumor neovascularization by promoting tumor growth and metastatic spread [3]. Particularly, vascular endothelial growth factor-A (VEGF-A) is primarily responsible for the "angiogenic switch" that allows tumor to grow and invade surrounding tissue [4–6]. Interleukin-6 (IL-6) is another mediator of angiogenesis in inflammatory and malignant conditions that stimulates the production of VEGF-A [7].

Another example of pathological angiogenesis can be found in Herpetic stromal keratitis (HSK), the first cause of infectious blindness in developed countries, which is caused by the infection of Herpes simplex virus type 1 (HSV-1) in the cornea [8]. The emergence of this HSV-1-induced ocular disease correlates with the development of corneal vascularization, normally avascular [9]. Polymorphonuclear cells (PMN) invade the cornea through new vessels and clear the virus but, at the same time, they enable the entry to various inflammatory cells and factors that provoke corneal scarring [8].

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http://dx.doi.org/10.1016/j.steroids.2016.09.001
0039-128X/

We have reported that some 28-homobrassinosteroid analogues have *in vitro* antiviral activity against several viruses [10–15]. Two of them, (22*S*,23*S*)-22,23-dihydroxystigmast-4-en-3-one (compound **1**) and (22*S*,23*S*)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one (compound **2**) (Fig. 1) prevent HSV-1 multiplication and reduce TNF-α and IL-6 secretion [16–20]. Both compounds strongly decrease the incidence and severity of HSK in the murine model of HSV-1 corneal infection [16,19,20]. Many natural brassinosteroids (BRs), such as brassinolide and *S,S*-homobrassinolide, that significantly inhibit the formation of capillary tubes in endothelial cells have been reported [21].

Since IL-6 is involved in neovascularization, which is crucial for the development of HSK and, besides, compounds **1** and **2** restrain IL-6 secretion *in vitro* and also reduce the signs of HSK, we decided to investigate their potential antiangiogenic activity on capillary tube-like structure formation in HUVEC and on VEGF expression [16–20]. Likewise, we also studied their antiangiogenic effect on tumor-induced angiogenesis and neovascularization in HSK.

2. Materials and methods

2.1. Cells, viruses, compounds and animals

Murine macrophage cell line J774A.1 (ATCC® Number TIB-67TM) was kindly provided by Dr. Osvaldo Zabal, from Instituto Nacional de Tecnología Agropecuaria (I.N.T.A.), Buenos Aires, Argentina, and it was grown in RPMI 1640 medium supplemented with 10% inactivated fetal bovine serum (FBS) (RPMI 10%) and maintained in RPMI supplemented with 2% inactivated FBS (RPMI 2%).

Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from InVitrogen Argentina S.A. (catalog number C-003-5C), Buenos Aires, Argentina, and they were propagated and maintained in Medium 200 supplemented with Low Serum Growth Supplement (LSGS).

The LMM3 cell line was derived from the parental metastatic MM3 murine adenocarcinoma aroused in BALB/c mice in the Instituto de Oncología Angel H. Roffo, (Universidad de Buenos Aires, Argentina), and it was cultured in DMEM/F12 medium with 2 mM L-glutamine, 80 μg/ml gentamycin, supplemented with 10% FBS (Internegocios S.A., Buenos Aires, Argentina).

Five to seven-week-old male BALB/c mice purchased from I.N.T.A. (Buenos Aires, Argentina) were used in the HSK model. For the tumor-induced angiogenesis assay, we worked with three-month-old female BALB/c mice, obtained from the Facultad de Veterinaria, Universidad de Buenos Aires (CABA, Argentina). All mice were handled according to the Animal Care Guidelines from the National Institute of Health (USA) and the Association for Research in Vision and Ophthalmology (ARVO, USA). The protocol was approved by the Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL), Facultad de Ciencias Exactas y Naturales (FCEyN), Universidad de Buenos Aires, that follows EU Directive 2010/63/EU for animal experiments.

Compounds **1** and **2** were dissolved in dimethylsulfoxide (DMSO) and diluted with culture medium. The maximum concentration of DMSO tested was 1% and exhibited no cytotoxicity under experimental conditions.

Anti-VEGF antibody bevacizumab (Avastin) was obtained from Roche Laboratories, San Francisco, USA.

2.2. Cytotoxicity assay

Cell viability was determined using the cleavage of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, USA) by the mitochondrial enzyme succinate dehydrogenase to give a blue product (formazan), according to the manufacturer's instructions.

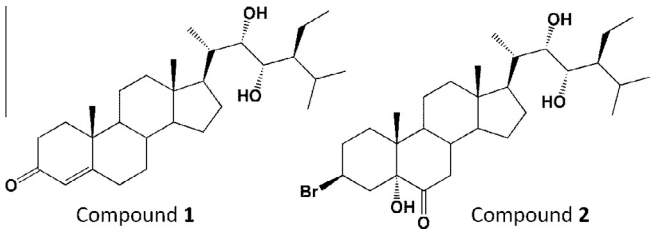

Fig. 1. Structures of compounds **1** and **2**.

LMM3 cells were seeded at a concentration of 10^4 cells/well in 96-well plates with DMEM/F12 medium supplemented with 5% FBS and they were left to adhere for 4 h. Then, the culture medium was replaced by fresh medium without serum and the cells were treated with different concentrations of compound **1** or **2** during 1, 2 or 6 h by triplicate. Supernatants were discarded, viable cells were detected by adding 10 μl MTT (5 mg/ml in distilled water) to each well and the production of formazan was evaluated by measuring the absorbance at 540 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad Laboratories Inc., Oakland, CA) after 4 h at 37 °C. Values are mean ± S.E. and results were expressed as percentage of cell viability relative to control untreated cells.

2.3. Capillary tube formation

The formation of capillary tube-like structures by HUVEC cells was analyzed on 24-well cell culture plates, coated with an extracellular membrane matrix (Matrigel; BD Biosciences). Matrigel was thawed at 4 °C. Precooled plates and tips were used and 100 μl/well of Matrigel was distributed and allowed to jellify at 37 °C for at least 30 min. Cells were seeded on the polymerized Matrigel ($5–8 \times 10^4$ cells/well). The plate was incubated at 37 °C and tube formation was observed under an optic inverted microscope. Digital pictures were taken at different times with a camera. Tubular structures were quantified by manual counting of low power fields (25×), in at least three fields, and the percentage of inhibition was calculated with respect to untreated cells.

2.4. Cell invasion assay

HUVECs' invasion was evaluated using 24-well transwell cell culture chambers with 8 μm pore polycarbonate filter inserts. Cultured HUVECs were trypsinized and they were suspended in Medium 200, supplemented with LSGS at a concentration of 8×10^5 cells/ml. A total of 4×10^4 cell suspension was applied to insert filters. 100 ng/ml of IL-6 was used to stimulate cell migration through the inserts. For that, 600 μl of medium alone or with the stimulus in the lower chamber were added and was then incubated for 24 h at 37 °C to allow cell migration. The inserts were removed and migrated cells on the wells were fixed and stained with crystal violet. The wells were examined under a microscope. Migration was quantified by measuring the total stained cells in every well. Percent inhibition of migrating cells was calculated with respect to untreated cells.

2.5. Breast tumor angiogenesis in vivo

2.5.1. Western blot analysis of VEGF-A production by tumor cells

LMM3 cells were seeded in 6-well plates (2×10^6/well) with 1 ml of DMEM/F12 and treated with compound **1** or **2** (25 ng/μl), during 1, 2 or 6 h. Supernatants were replaced by fresh medium