Toxicology and Applied Pharmacology 272 (2013) 616–624

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

OSU-A9 inhibits angiogenesis in human umbilical vein endothelial cells via disrupting Akt–NF-κB and MAPK signaling pathways

Hany A. Omar [a,b], El-Shaimaa A. Arafa [b], Samir A. Salama [c], Hany H. Arab [d], Chieh-Hsi Wu [e,*], Jing-Ru Weng [f,**]

[a] Division of Medicinal Chemistry, College of Pharmacy, The Ohio State University, Columbus, OH 43210, USA
[b] Department of Pharmacology, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62514, Egypt
[c] Department of Biochemistry, Faculty of Pharmacy, Al-Azhar University, Cairo 11511, Egypt
[d] Department of Biochemistry, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt
[e] School of Pharmacy, China Medical University, Taichung 40402, Taiwan
[f] Department of Biological Science and Technology, China Medical University, Taichung 40402, Taiwan

ARTICLE INFO

Article history:
Received 2 May 2013
Revised 11 July 2013
Accepted 24 July 2013
Available online 3 August 2013

Keywords:
OSU-A9
Angiogenesis
Akt–NF-κB
MAPKs
VEGF
MMP-2

ABSTRACT

Since the introduction of angiogenesis as a useful target for cancer therapy, few agents have been approved for clinical use due to the rapid development of resistance. This problem can be minimized by simultaneous targeting of multiple angiogenesis signaling pathways, a potential strategy in cancer management known as polypharmacology. The current study aimed at exploring the anti-angiogenic activity of OSU-A9, an indole-3-carbinol-derived pleotropic agent that targets mainly Akt–nuclear factor-kappa B (NF-κB) signaling which regulates many key players of angiogenesis such as vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs). Human umbilical vein endothelial cells (HUVECs) were used to study the *in vitro* anti-angiogenic effect of OSU-A9 on several key steps of angiogenesis. Results showed that OSU-A9 effectively inhibited cell proliferation and induced apoptosis and cell cycle arrest in HUVECs. Besides, OSU-A9 inhibited angiogenesis as evidenced by abrogation of migration/invasion and Matrigel tube formation in HUVECs and attenuation of the *in vivo* neovascularization in the chicken chorioallantoic membrane assay. Mechanistically, Western blot, RT-PCR and ELISA analyses showed the ability of OSU-A9 to inhibit MMP-2 production and VEGF expression induced by hypoxia or phorbol-12-myristyl-13-acetate. Furthermore, dual inhibition of Akt–NF-κB and mitogen-activated protein kinase (MAPK) signaling, the key regulators of angiogenesis, was observed. Together, the current study highlights evidences for the promising anti-angiogenic activity of OSU-A9, at least in part through the inhibition of Akt–NF-κB and MAPK signaling and their consequent inhibition of VEGF and MMP-2. These findings support OSU-A9's clinical promise as a component of anticancer therapy.

Introduction

Pathological angiogenesis plays a major role in a number of diseases including cancer, rheumatoid arthritis, atherosclerosis, and other cardiovascular diseases (Folkman, 1995; Khurana et al., 2005). Since the approval of bevacizumab, an antibody specific for vascular endothelial growth factor (VEGF), for the treatment of metastatic colorectal carcinoma, the inhibition of angiogenesis has become a major goal of anticancer drug development (Hurwitz et al., 2004). Angiogenesis involves several key steps which include endothelial cell proliferation, migration, invasion and tube formation (Nacev and Liu, 2011). Effective antiangiogenic agents should target one or more of these processes (Omar et al., 2012).

VEGF, the key angiogenic cytokine, plays a key role in angiogenesis and neovascularization and stimulates the proliferation, migration as well as tube formation of plated endothelial cells (Byrne et al., 2005). VEGF production is up-regulated in cancer cells by oncogene expression, growth factors and hypoxia (Carmeliet, 2005). The production of VEGF by cancer cells is essential for the 'angiogenic switch', where neovascularization is formed within and around the tumor promoting exponential growth (Wang et al., 2012). However, the newly formed blood vessels under the influence of VEGF are irregularly shaped, convoluted, unorganized and leaky that cause hypoxia and further VEGF production. Thus, the central role of VEGF in tumor angiogenesis makes it an optimal target for anticancer therapy.

Abbreviations: NF-κB, nuclear factor-kappa B; VEGF, vascular endothelial growth factor; MMPs, matrix metalloproteinases; HUVECs, human umbilical vein endothelial cells; MAPKs, mitogen-activated protein kinases; EGM-2, Endothelial Cell Growth Medium-2; DMSO, dimethyl sulfoxide; PARP, poly (ADP-ribose) polymerase; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide]; RA, Retinoic acid; CAM, chorioallantoic membrane; DAPI, 4′,6-diamidino-2-phenylindole.

* Correspondence to: C.-H. Wu, School of Pharmacy, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan. Fax: +886 4 22073709.
** Correspondence to: J.-R. Weng, Department of Biological Science and Technology, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan. Fax: +886 4 22071507.
E-mail addresses: chhswu@mail.cmu.edu.tw (C.-H. Wu), columnster@gmail.com (J.-R. Weng).

0041-008X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2013.07.014

Equally important, the matrix metalloproteinase (MMP) family is another key player in the angiogenesis process (Artacho-Cordon et al., 2012). MMPs are frequently overexpressed in malignant tumors and are implicated in the processes of tumor growth, invasion, and metastasis (Coussens et al., 2002; Roy et al., 2009). Among the structurally related human MMPs, the expression of gelatinases such as MMP-2 and MMP-9 increases in malignant cancers compared to benign cancers (Klein et al., 2004; Roomi et al., 2010). The association of MMPs with aggressive malignant phenotype and poor prognosis in cancer highlights the need for the development of MMP inhibitors (Szinwong et al., 2013).

Although few anti-angiogenic agents are currently available in the clinic, their efficacy is limited by the up-regulation of alternative and compensatory signaling pathways during the course of treatment (Abdollahi and Folkman, 2010; Bergers and Hanahan, 2008). One of the most successful strategies to minimize resistance in cancer therapy is the employment of polypharmacology to simultaneously modulate more than one target involved in a network of signaling (Reddy and Zhang, 2013). A well-recognized example of polypharmacology is sorafenib, an oral multikinase inhibitor that has been approved for the treatment of patients with advanced renal cell carcinoma and unresectable hepatocellular carcinoma (Wilhelm et al., 2008).

The unique ability of OSU-A9 to selectively induce apoptosis in various types of cancer, including prostate, breast, liver, and oral cancer with high safety margins supports its clinical development into a cancer therapeutic agent (Omar et al., 2009; Weng et al., 2007, 2009, 2010). Given that OSU-A9 has been reported to target Akt–NF-κB signaling that mediates the action of VEGF and MMPs, the key players in angiogenesis process (Cheung et al., 2011; Morais et al., 2009; Shiojima and Walsh, 2002), we aimed to investigate its potential anti-angiogenic activity that may highlight the usefulness of OSU-A9 in cancer therapy. The current work, describes for the first time, the in vitro and in vivo anti-angiogenic efficacy of OSU-A9 along with its dual inhibition of Akt–NF-κB and MAPK signaling.

Material and methods

Material. OSU-A9 [1-(4-chloro-3-nitrobenzenesulfonyl)-1*H*-indol-3-yl]-methanol was synthesized as described before (Fig. 1A) (Weng et al., 2007). The identity and purity (≥99%) of OSU-A9 were verified by proton nuclear magnetic resonance, high-resolution mass spectrometry, and elemental analysis. OSU-A9 was dissolved in dimethyl sulfoxide (DMSO) and diluted in culture medium. Endothelial Cell Growth Medium-2 (EGM-2) was purchased from Lonza (Walkersville, MD) and fetal bovine serum (FBS) from Invitrogen (Carlsbad, CA). Matrigel and 24-well modified Boyden chambers (8 μm pore size) were obtained from BD Biosciences (Bedford, MA) and Corning Costar (Cambridge, MA), respectively. Antibodies against the following biomarkers were obtained from the indicated commercial sources: procaspase-8, procaspase-3, poly (ADP-ribose) polymerase (PARP), proliferating cell nuclear antigen (PCNA), p-^{473}Ser Akt, p-^{308}Thr Akt, Akt, MMP-2, MMP-9, IKKα and p-^{108}Ser IKKα and p-^{217}Ser/^{221}Ser mitogen-activated protein kinase (MEK) from Cell Signaling Technology (Beverly, MA); p-^{180}Thr/^{182}Tyr p38, p38, Bcl-2, MEK, NF-κB, mammalian target of rapamycin (mTOR), p-^{2448}Ser mTOR, ERK, jun N-terminal kinase (JNK), p-$^{183/185}$Thr/Tyr JNK, and p-$^{202/204}$Thr/Tyr ERK from Millipore (Bedford, MA); and β-actin was obtained from Sigma-Aldrich (St. Louis, MO). Other chemicals and reagents were obtained from Sigma-Aldrich unless otherwise mentioned.

Cell culture and hypoxic conditions. Human umbilical vascular endothelial cells (HUVECs, ScienCell Research Laboratories, Carlsbad, CA) were grown in EGM-2 supplemented with 10% FBS. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2. For hypoxic conditions, cells were incubated at 5% CO_2 with 1% O_2 balanced with N_2 in an anaerobic chamber (Forma Scientific). The passage number of all the used cells was between 3 and 5.

Cell cycle analysis. Treated HUVECs were harvested and washed with cold 1 × PBS and then fixed with 70% ice-cold ethanol overnight. After centrifugation at 1200 ×*g* at 4 °C for 5 min, the ethanol was removed and the pellets were resuspended in 500 μL of DNA staining buffer (4 μg/mL of propidium iodide, 1% Triton X-100, and 0.1 mg/mL of RNase A) and incubated for 30 min at room temperature in the dark. Samples were subjected to flow cytometry using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA), and the cell cycle profile was analyzed using the Modfit LT Program (Verify Software House, Topsham, HE).

Cell viability analysis. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described before (Bai et al., 2011). In brief, HUVECs were seeded at 1×10^4 cells/well in 96-well flat-bottomed plates in EGM-2. After 24 h, cells were treated with different concentrations of OSU-A9 in the presence and absence of 50 μM Pan Caspase Inhibitor Z-VAD-FMK (R&D Minneapolis, MN) in a total volume of 100 μL of 10% FBS-containing culture medium. At the end of the treatment, the media were removed, replaced by 200 μL fresh EGM-2 containing 0.5 mg/mL of MTT and cells were incubated in the CO_2 incubator at 37 °C for 2 h. Supernatants were removed by aspiration, and the reduced MTT was dissolved in 200 μL/well DMSO. Absorbance at 570 nm was determined using a plate reader. The results were calculated by subtracting blank readings, in which cells were absent, from sample readings. The cell viability was expressed as a percentage of the vehicle-treated control group. For cell count analysis, HUVECs were seeded overnight in 6-well plates at 1.2×10^5 cells/well and were treated at the indicated concentrations of OSU-A9 or vehicle for 24 h. At the end of the treatment, cells were washed three times with PBS and trypsinized. Cell numbers were calculated using Coulter Z1 cell counter (Beckman Coulter).

Western blotting. Lysates of HUVECs treated with OSU-A9 at the indicated concentrations for 24 h were prepared for immunoblotting of procaspase-8, procaspase-3, PARP, p-^{473}Ser Akt, p-^{308}Thr Akt, Akt, MMP-2, MMP-9, IKKα and p-^{108}Ser IKKα and p-^{217}Ser/^{221}Ser MEK, p-^{180}Thr/^{182}Tyr p38, p38, Bcl-2, MEK, NF-κB, mTOR, p-^{2448}Ser mTOR, ERK, JNK, p-$^{183/185}$Thr/Tyr JNK, p-$^{202/204}$Thr/Tyr ERK, PCNA and β-actin. Western blot analysis was performed as previously reported (Kulp et al., 2006).

Preparation of nuclear extracts. Cells were treated with different concentrations of OSU-A9 for 24 h. Nuclear extracts were prepared using NEPER Nuclear and Cytoplasmic Extraction Reagents Kit (PIERCE®, Rockford, IL) according to the manufacturer's instructions. Nuclear extracts were then analyzed for NF-κB levels by western blotting as described above.

DAPI staining assay. HUVECs at the density of 4×10^4 cells/well in 24-well plates were incubated with the indicated concentrations of OSU-A9 or vehicle (DMSO) for 24 h in EGM-2 medium containing 10% FBS. Harvested HUVECs were stained by 4′,6-diamidino-2-phenylindole (DAPI) then examined and photographed by using a fluorescence microscope.

Matrigel tube formation assay. *In vitro* anti-angiogenic activity of OSU-A9 was conducted using Matrigel tube formation assay as described before (Chabut et al., 2003). In summary, Matrigel (10 mg/mL, 100 μL) was incubated for 30–45 min at 37 °C for polymerization to take place. HUVECs suspended in 300 μL EGM-2 supplemented with 10% FBS at a density of 4×10^4 cells/mL were added to Matrigel-coated plates. Different concentrations of OSU-A9 or vehicle were added