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PBA2 exhibits potent anti-tumor activity via suppression of VEGFR2 mediated-cell proliferation and angiogenesis

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ABSTRACT

VEGFR2 (vascular endothelial growth factor receptor 2) is the main trigger of VEGF-mediated angiogenic signal and targeting VEGFR2 pathway to inhibit tumor angiogenesis represents a promising strategy for cancer therapy. We elucidated that a novel compound, PBA2 exhibited potent anti-tumor effects both *in vitro* and *in vivo* with limited toxicity. ELISA assay revealed that PBA2 had a strong suppressive activity against VEGFR2 related to angiogenesis. Furthermore, PBA2 considerably disrupted tube formation of endothelial cells *in vitro* and systemic administration of PBA2 exerted decreased tumor angiogenesis *in vivo*. Functional tests demonstrated that PBA2 concentration-dependently impeded the migration and proliferation of endothelial cells. PBA2 had no effects on the expression level of VEGF both in the detected cancer cells and endothelial cells. VEGFR2 and its downstream Akt and Erk pathways were blocked by PBA2 in a concentration-dependent manner both *in vitro* and *in vivo*. Overall, we first demonstrated that PBA2, targeting VEGFR2 related to angiogenesis, presented remarkable anti-angiogenic and anti-tumor activities through attenuating VEGFR2 mediated pathway *in vitro* and *in vivo* with limited toxicity. These observations posed that PBA2 could be a potential drug candidate for developing anti-angiogenic tyrosine kinase inhibitor in cancer therapy.

1. Introduction

Angiogenesis, the formation of neo-vessels from pre-existing vasculature in tumor tissue, containing degradation of the basement membrane, endothelial cells (ECs) proliferation, migration and formation of tubular structures [1], plays a critical role in tumor progression and metastasis [2]. Targeting angiogenesis to destruct vasculature and block supply of nutrients and oxygen in cancer represents a promising anti-cancer therapeutic strategy to inhibit tumor growth and metastasis [3]. Multiple kinases were identified involved in the process of angiogenesis, especially receptor tyrosine kinases (RTKs) such as vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR) and platelet-derived growth factor receptor (PDGFR) [4–6]. Special attention has been paid on the blockades of these RTKs to impede the formation of neo-vessels in cancer [7]. Anti-angiogenic tyrosine kinases inhibitors (TKIs) developed as a systemic therapy for cancers manifested beneficial anti-angiogenic effects as well as anti-tumor activities [8]. VEGF is a critical pro-angiogenic factor secreted by cancer cells [9] that facilitates the migration and proliferation of ECs, leading to the growth of neo-vessels into the tumor [10]. Therefore, VEGF signaling through its receptor is the major inducer of angiogenesis and VEGFR pathway blockade has been considered as a potential anti-tumor therapeutic approach [11]. VEGFR2 is proved to be the crucial mediator that regulates the angiogenic activity of VEGF via different signaling pathways, promoting the migration, proliferation and tube formation of ECs [12]. In addition, VEGFRs may also be expressed on cancer cells, as has been presented by several studies listed by Ellis and Hicklin [13]. Accordingly, it can be hypothesized that VEGF is capable of stimulating VEGFRs-expressing cancer cells [14]. There are various studies demonstrating that inhibition of VEGFR2 signaling in cancer cells leads to apoptosis and senescence of cancer cells [15,16]. VEGFR inhibitors may not only impede tumor growth by

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anti-angiogenesis of tumors, but exhibit additional suppressive activities on cancer cells.

Given that RTKs have the capability to regulate angiogenesis, significant efforts have been invested to discover and develop anti-angiogenic TKIs. At present, the three notable anti-angiogenic TKIs, sorafenib, sunitinib and pazopanib, targeting distinct angiogenic kinases, have been approved by FDA for several years for the treatment of patients with advanced cancers. However, there are two major problems appeared during the clinical applications of these angiogenesis inhibitors [17]. For one thing, intrinsic or acquired resistance to angiogenesis inhibitors occurred in some patients with continual tumor progression following drug interventions [18]. For another, in contrast to initial expectations, obvious clinical toxicities were observed during anti-angiogenic treatment including severe bleeding, gastro-intestinal perforation and hypertension [19]. There is still a long way to go in the development and optimization of angiogenesis inhibitors with strong activity and low toxicity. In our study, by means of combinatorial lead-optimization strategy, we synthesized 9-(2-chloro-phenyl)-6-ephyl-1-methyl-2,4-dihydro-2,3,4,7,10-pentaaza-benzo[*f*]azulene, designated as PBA2, which was identified as a novel, orally bioactive small molecular inhibitor. We sought to elucidate the anti-tumor effect of this novel compound and further investigate its potential anti-tumor mechanisms.

2. Materials and methods

2.1. Test materials

PBA2 (9-(2-chloro-phenyl)-6-ephyl-1-methyl-2,4-dihydro-2,3,4,7,10-pentaaza-benzo[*f*]azulene) was synthesized by Nanjing CSPC Pharmaceutical Group Limited (Nanjing, China) and purified to achieve a purity of more than 99%. Its molecular weight is 337.806. It was prepared as a 50 mM stock solution in dimethyl sulfoxide (DMSO) for research *in vitro* and was dissolved to appropriate concentration in purified water containing 0.5% hydroxypropyl methyl cellulose and 0.1% Tween 80 and the solvent was used as control for research *in vivo*. DMEM and RPMI 1640 medium were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). M199 medium and MCDB-131 medium were from GIBCO (USA). Antibodies against VEGFR2 and p-VEGFR2 (Tyr1175) were purchased from Cell Signaling Technologies (Cambridge, MA, USA). Antibodies against Akt (sc-8312), p-Akt (sc-7985-R), Erk (sc-514302) and p-Erk (sc-7383) were from Santa Cruz Biotechnology Inc. (Paso Robles, CA, USA). Antibodies against CD31 (ab28364) and VEGFA (ab46154) were obtained from Abcam Company (Cambridge, MA, USA). GAPDH antibody was from Kangchen Co. (Shanghai, China). VEGF (lot 113913012) was purchased from R&D Systems (Minneapolis, MN, USA). 1-(4, 5-dimethylthiazol-2-yl)-3, 5-diphenylformazan (MTT), DMSO, cyclophosphamide (CTX), taxol, su11248, hydroxypropyl methyl cellulose, Tween 80 and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell lines and cell culture

All cancer cell lines used in this article were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The human oral epidermoid carcinoma cell KB, human lung cancer cell A549, GLC-82 and PC9, human gastric cancer cell SGC-7901, MGC-803, AGS and human colon cancer cell SW620 and SW480 were cultured in RPMI 1640 supplemented with 10% FBS and antibiotics. Human colon cancer cell S1 was cultured in DMEM supplemented with 10% FBS and antibiotics. Human umbilical vein endothelial cells (HUVECs) were isolated as previously described [20] and cultured in M199 medium with 10 ng ml^{-1} EGF, 20% heat-inactivated FBS and antibiotics. Human micro-vascular endothelial cells (HMECs) were obtained from the American Type Culture Collection (Rockville, MD) and cultured in MCDB-131 medium with 10% FBS and antibiotics. All cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.3. Cell cytotoxicity assay

The anti-proliferative activity of PBA2 was determined using MTT assay as described previously [21]. Cells growing in logarithmic phase were seeded in 96-well plates at the appropriate density. After culturing for 24 h, different concentrations of PBA2 were added for another 72 h at 37 °C. MTT (5 mg mL^{-1}) was added into each well and 4 h later, the medium was discarded followed by adding 150 μL of DMSO to dissolve the formazan product from the metabolism of MTT. Finally, the absorbance was measured at 540 nm, with background subtraction at 670 nm by a Model 550 Microplate Reader (Bio-Rad, Hercules, CA, USA). The Bliss method was used to calculate IC_{50} values of substances [22]. Data represent the mean ± SD of at least three independent experiments.

2.4. Animal ethics and procedures

Kunming mice (male equal female) for acute toxicity test, 4–6 week-old and athymic nude mice (BALB/c-nude, 4–6 week-old and weight at 16–18 g, half male and half female) were obtained from the Center of Experimental Animals, Sun Yat-sen University. The 8 groups of kunming mice were treated with different concentrations of PBA2. After observed for one week, the animals were fasted for 12 h before experiment but allowed free access to water. In addition, SGC-7901 and MGC-803 cells (1×10^7) were subcutaneously injected into the right flank of nude mice. When the tumors had reached a mean volume of about 110 mm^3, the animals were randomized into 5 groups and treated with different regimens: (a) vehicle control group (gavage, qd); (b) taxol ((20 mg kg^{-1}, intraperitoneal injection/i.p., q7d)) in SGC-7901 cell xenograft, CTX (100 mg kg^{-1}, intraperitoneal injection/i.p., q7d) in MGC-803 cell xenograft; (c) PBA2 (4 mg kg^{-1}, gavage, qd); (d) PBA2 (6 mg kg^{-1}, gavage, qd); (e) PBA2 (9 mg kg^{-1} gavage, qd). The body weights of mice and the two perpendicular tumor diameters (A and B) were measured every 3 or 4 days, and the tumor volume (V) was calculated according to the following formula: $V = (\pi/6)\ [(A + B)/2]^3$ [23]. The tumor volume and mice body weight were plotted against the time of treatment. When the mean of tumor weight was over 1 g in the control group, the mice were euthanized and the xenografts were excised, weighed and photographed. Efforts were made to minimize animal suffering as much as possible during experiments. The operator and data analysis were blinded. All experiments were performed in accordance with the guidelines for Use of Laboratory Animals of Sun Yat-sen University Cancer Center after the approval of Animal Experimental Ethics Review Committee of Sun Yat-sen University Cancer Center. The ratio of tumor growth inhibition (IR) was estimated according to the following formula [23]:

$$IR(\%) = \frac{1 - \text{Mean tumor weight of experiment group}}{\text{Mean tumor weight of control group}} \times 100.$$

2.5. ELISA kinase assay

The ability of PBA2 to inhibit the activity of a panel of kinases was tested using an ELISA *in vitro*. The kinase domain of VEGFR2, c-Kit, EGFR (epidermal growth factor receptor), ErbB4 (human epidermal growth factor receptor-4, HER4), $EGFR^{T790M/L858R}$, IGF1R (insulin-like growth factor 1R) and c-Met were expressed using the Bac-to-Bac™ baculovirus expression system (Invitrogen, Carlsbad, CA, USA) and purified on Ni-NTA columns (QIAGEN Inc., Valencia, CA, USA) as previously described [24]. Recombinant FGFR1, PDGFRα and PDGFRβ were obtained from Upstate Biotechnology. The 96-well ELISA plates were coated with the substrate, which contains 20 μg mL^{-1} Poly (Glu, Tyr)$_{4:1}$ (Sigma, St Louis, MO, USA). The experiment was performed according to standard ELISA procedures, and the plate was read at an absorbance of 490 nm. The inhibitory rate (%) was calculated using the following formula: $[1 - (A_{490}\text{treated}/A_{490}\text{control})] \times 100\%$. IC_{50}