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Original Article

Inhibition of CYP4A by a novel flavonoid FLA-16 prolongs survival and normalizes tumor vasculature in glioma

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ABSTRACT

Glioblastomas rapidly become refractory to anti-VEGF therapies. We previously showed that cytochrome P450 (CYP) 4A-derived 20-hydroxyeicosatetraenoic acid (20-HETE) promotes angiogenesis. Here, we tested whether a novel flavonoid (FLA-16) prolongs survival and normalizes tumor vasculature in glioma through CYP4A inhibition. FLA-16 improved survival, reduced tumor burden, and normalized vasculature, accompanied with the decreased secretion of 20-HETE, VEGF and TGF-β in tumor-associated macrophages (TAMs) and endothelial progenitor cells (EPCs) in C6 and U87 gliomas. FLA-16 attenuated vascular abnormalization induced by co-implantation of GL261 glioma cells with CYP4A10^{high} macrophages or EPCs. Mechanistically, the conditional medium from TAMs and EPCs treated with FLA-16 enhanced the migration of pericyte cells, and decreased the proliferation and migration of endothelial cells, which were reversed by CYP4A overexpression or exogenous addition of 20-HETE, VEGF and TGF-β. Furthermore, FLA-16 prevented crosstalk between TAMs and EPCs during angiogenesis. These results suggest that CYP4A inhibition by FLA-16 prolongs survival and normalizes vasculature in glioma through decreasing production of TAMs and EPCs-derived VEGF and TGF-β. This may represent a potential therapeutic strategy to overcome resistance to anti-VEGF treatment by effects on vessels and immune cells.

Introduction

Glioblastoma (GBM) is the most common primary malignant brain tumor. Even after maximal safe resection and chemo-radiation, most patients survive little more than 1 year [1]. Vascular endothelial growth factor (VEGF) monoclonal antibody (bevacizumab, Avastin) was approved by the Food and Drug Administration in 2009 for the treatment of recurrent GBM (rGBM) [1–3]. However, bevacizumab confers an increase in progression-free survival but does not improve overall survival in three randomized phase III trials [4–6]. Similarly, sunitinib, an oral pan-VEGF receptor tyrosine kinase inhibitor fails to improve survival in a phase II trial in patients with rGBM [7]. Thus, there is an urgent need to develop novel strategies to increase survival in patients with GBM.

Angiogenic inhibitors transiently "normalize" GBM vasculature and improve the outcome of mice bearing GBM [8–10]. Unfortunately, resistance to anti-VEGF therapy elicits tumor adaptation and progression to stages of greater malignancy, with heightened invasiveness [11]. Tumor-recruited endothelial progenitor cells (EPCs) contribute to resistance to anti-VEGF therapy by indirect paracrine secretion of pro-angiogenic growth factors, including VEGF, TGF-β and HIF-1α [12,13]. Given that anti-VEGF therapy causes hypoxia, tumor-associated macrophages (TAM) might be recruited as a compensatory mechanism to provide tumor with pro-angiogenic growth factors to secure angiogenesis [12]. TAMs are increased in patients with rGBM after anti-angiogenic therapy and correlated with poor survival [12]. Therefore, inhibiting tumor angiogenesis by targeting TAMs and EPCs in the tumor microenvironment could represent a promising strategy to increase survival in GBM.

Cytochrome P450 (CYP) 4 family enzymes catalyze the hydroxylation of various fatty acids, such as arachidonic acid [14].

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20-Hydroxyeicosatetraenoic acid (20-HETE), a principal ω-hydroxylation product of arachidonic acid by CYP4A, is an important mediator of VEGF-mediated angiogenesis [15–17]. Our previous study demonstrated that CYP4A-derived 20-HETE activates EPCs functions associated with angiogenesis through synergism with VEGF [15]. Conversely, CYP4A inhibition blocks angiogenesis induced by human glioma cell U251 [18,19]. These data suggest that CYP4A could be an attractive target for anti-angiogenic therapy in glioma.

There is an increasing interest in exploring dietary flavonoids as anti-angiogenic agents [20–24]. Our previous study showed that isoliquiritigenin, a chalcone-type flavonoid isolated from the roots of *Glycyrrhiza glabra*, prevents human breast cancer growth through CYP4A inhibition [25,26]. 2, 3′, 4, 4′-tetrahydroxy-3, 5′-diprenylchalcone, termed FLA-16, is a novel chalcone-type flavonoid isolated from *G. glabra* roots [27]. However, its effect on tumor progression has not been reported. In this study, we investigated whether FLA-16 prevents glioma angiogenesis and induces vascular normalization through inhibition of CYP4A in TAMs and EPCs. This provides opportunities for treatment of patients' refractory to VEGF-targeting drugs.

Materials and methods

Plant material and the preparation of FLA-16

The roots of *G. glabra* used in this study were collected in Yili Prefecture, Xinjiang Province, China and authenticated by Dr. Mingxi Jiang, Wuhan Institute of Botany, Chinese Academy of Sciences. Isolation, purification and identification of flavonoid FLA-16 were performed as previously described [27]. The purity of FLA-16 was determined to be higher than 99% based on analysis using high-performance liquid chromatography.

Cell cultures

Human EPCs (hEPCs), human peripheral blood mononuclear cells (PBMCs) and T cells were isolated from the blood of healthy volunteers according to the Helsinki Declaration. All donors gave informed consent. Our study was approved by the ethics boards of the Medical School of Wuhan University. Isolation, culturing, and characterization of hEPCs and mouse EPCs (mEPCs) from peripheral blood were performed as previously described [15,28]. Briefly, $CD133^+$ cells were isolated from peripheral blood by immunomagnetic selection, and then incubated in Stemline II medium (Sigma Aldrich, St. Louis, MO) supplemented with 40 ng/ml stem cell factor, 40 ng/ml FLT3, and 10 ng/ml thrombopoietin (all from CellGenix, Antioch, IL) for 7 days. Cells were isolated and characterized by the expression of CD34, CD133 and VEGFR2.

Human PBMCs were isolated as previously described [29], and then incubated with macrophage-colony stimulating factor (50 ng/ml) for 7 days to be M0 macrophages, and treated with tumor supernatants containing IL-4/IL-13 (20 ng/ml) for 12 h to be M2 macrophages. Isolation, culturing, and characterization of mouse bone marrow-derived macrophages (BMDMs) were performed as previously described [30,31], and treated with tumor supernatants containing IL-4/IL-13 (20 ng/ml) for 12 h. TAMs were isolated from C6 or U87 glioma tissues using immunomagnetic selection as described previously [32].

Tumor models and treatment regimes

All animal studies were approved by the Animal Research Committee of Wuhan University, and maintained in accordance with the guidelines by the Association for Assessment and Accreditation of Laboratory Animal Care International.

In C6 or U87 subcutaneous glioma model, C6 glioma cells (5×10^6) or U87 glioma cells (2×10^6) were injected subcutaneously into the right flank of Wistar rats or BALB/c nude mice. When tumors reached a size of about 100 mm^3, FLA-16 was administered intraperitoneally at the dose of 10 and 20 mg/kg once daily. After sacrificing the rats or mice on day 8 of treatment, tumors were collected and analyzed.

In C6 or U87 intracranial glioma model, 10 μl of cell suspension containing 1×10^6 rat C6 glioma cells or 3 μl of cell suspension containing 5×10^5 human U87 glioma cells was implanted into the right caudatum of Wistar rats or BALB/c nude mice. FLA-16 was administered intraperitoneally at the dose of 10 and 20 mg/kg once daily after tumor implantation. The survival time for each animal after inoculation was measured.

In co-injection model, GL261 cells (9×10^5) mixed with parental or $CYP4A10^{high}$ (BMDMs or mEPCs) (3×10^5) were injected subcutaneously into the right flank of C57BL/6 mice in a ratio of 3:1. When palpable tumors formed approximately 0.5 cm in diameter, FLA-16 was administered intraperitoneally at the dose of 10 and 20 mg/kg once daily. After sacrificing the mice on day 8 of treatment, tumors were collected and analyzed.

Statistical analysis

All values were expressed as mean ± SEM, and statistical analyses were performed using a one-way ANOVA followed by the Student–Newman–Keul's test or two-tailed Student's t test. *P* values of 0.05 or less were considered significant.

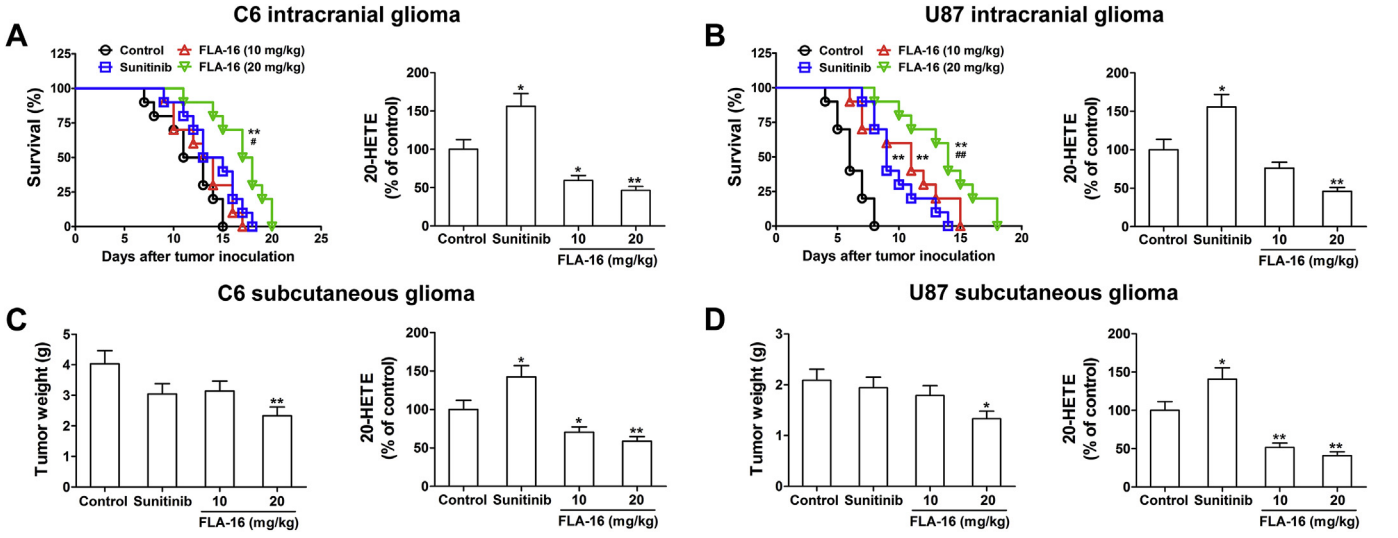

Fig. 1. FLA-16 prolongs survival and delays growth in C6 and U87 gliomas with the decreased intratumoral level of 20-HETE. (A) In the C6 glioma intracranial model, the survival time of the rats ($n = 10$) gavaged with FLA-16 (10 and 20 mg/kg), Sunitinib (80 mg/kg) or vehicle was measured, and 20-HETE in the tumor tissues from each group was determined at day 12 by LC-MS/MS. (B) In the U87 glioma intracranial model, the survival time of the mice ($n = 10$) gavaged with FLA-16 (10 and 20 mg/kg), Sunitinib (80 mg/kg) or vehicle was measured, and 20-HETE in the tumor tissues from each group was determined at day 6 by LC-MS/MS. (C) In the C6 glioma subcutaneous model, rat C6 glioma cells (5×10^6) were injected subcutaneously into the right flank of Wistar rats. When tumors reached a size of about 100 mm^3, the rats ($n = 8$) received FLA-16 (10 and 20 mg/kg), Sunitinib (80 mg/kg) or vehicle by intraperitoneal injection once daily for a week. Tumor weight was measured, and 20-HETE was determined by LC-MS/MS. (D) In the U87 glioma subcutaneous model, human U87 glioma cells (2×10^6) were injected subcutaneously into the right flank of BALB/c nude mice. When palpable tumors formed approximately 0.5 cm in diameter, the mice ($n = 8$) received FLA-16 (10 and 20 mg/kg), Sunitinib (80 mg/kg) or vehicle by intraperitoneal injection once daily for a week. Tumor weight was measured, and 20-HETE was determined by LC-MS/MS. The values are presented as the mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* sunitinib (80 mg/kg)-treated group.