Berberine Can Target the VEGFR2/ERK Pathway to Inhibit Angiogenesis in Glioblastoma Xenografts

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Abstract: The purpose of this article is to investigate the concrete effect of berberine on angiogenesis of glioblastoma cells. The glioblastoma is the most malignant cancer with high mortality and it is also difficult to treat with modern medical methods. The fractionated radiotherapy, interstitial radiotherapy or stereotactic radiosurgery all have unstable effects on tumor. Previous study has reported that berberine can inhibit the angiogenesis in glioblastoma xenografts through targeting the VEGFR2/ERK pathway. And this study investigates the concrete effect of berberine on angiogenesis of glioblastoma cells. In the hypothetical experiment, we will treat glioblastoma xenograft mice with increasing amounts of injected Berberine (0, 0.1, 0.3, 1 mg/ml, 10 mg/ml) for 0 min, 1 day, 7 days, 2 weeks and then measure tumor weight and size, and perform confocal microscopy/ immunohistochemistry (IHC) for VEGFR2, or phospho-ERK and unphosphorylate ERK. The experiment will also measure VEGFR2, phosphorylate ERK, unphosphorylate ERK by western blot. Positive control is Temozolomide treatment.There are three most possible results: (1) Berberine inhibit the angiogenesis of glioblastoma cells in both *in vitro* and *in vivo* cell lines; (2) berberine can only inhibit the angiogenesis of glioblastoma cells in *in vitro* cell cultures; (3) berberine only inhibit the angiogenesis of glioblastoma cells in determined human and murine glioblastoma. In conclusion, the results of this study will provide important information for future clinical trials of berberine treatment. Future studies should focus on improving in vivo delivery methods, finding more inhibitors of the angiogenic pathway in glioblastoma, and exploring in detail the specific mechanisms of berberine.

1 INTRODUCTION

Malignant tumor is a potential killer of human life and seriously endangers people's healthy life. According to the report of World Health Organization, the incidence and mortality rate of malignant tumors are gradually increasing all over the world. 10 million new malignant tumors occur each year, and 6-7 million people die from this disease, accounting for 12% of the total, which becomes the second cause of human death. The main types of malignant tumors are lung cancer, stomach cancer, liver cancer, colorectal cancer and breast cancer, and their causative factors include environmental and life factors such as air pollution, smoking, poor living habits, food additives and drug abuse. In recent years, with the development of science and technology, chemotherapy for tumors has made some progress, and the life expectancy of tumor patients has been significantly extended, such as in leukemia, malignant lymphoma, etc., but there is still no effective approach for most solid tumors.

Scientific researchers have gradually realized that in order to make a breakthrough, we must start from the mechanism of tumor development in order to solve the problem at root. Anti-tumor drugs are gradually moving from traditional cytotoxic drugs to multi-targeted drugs and exploring various new drugs. The development of new natural drugs.

Glioblastoma is one of the most malignant astrocytomas. The tumor is located in the subcortical region and most grows over the cerebral hemispheres. It usually invades several lobes and deep structures of the brain. It can also spread through the corpus callosum to the contralateral cerebral hemisphere. Meanwhile, glioblastoma is the most common primary brain tumor in adults, with an annual incidence of 52.6 per 1 million people and approximately 17,000 new cases diagnosed each year. The etiology of most cases of glioblastoma and its prevention have not been clarified. Rare risk factors include genetic disorders (e.g.,

neurofibromatosis and Li-Fermini syndrome) and chemotherapy.

Angiogenesis is a process in which physiologically new microvessels develop into a blood supply system. Whereas Vasculogenesis usually refers to spontaneous blood vessel formation, Intussusception refers to the more general process of rapid formation of new blood vessels. This process is common in human growth and development, and in wound healing. In addition, angiogenesis is an important step in tumor progression, and in tumor growth, it can be the key to the transformation of a tumor from dormant to malignant, rapidly growing, and potentially invasive to other tissues. Neovascularization is an important way for tumors to obtain nutrients to ensure their own development, and tumor angiogenesis is the result of the combination of multiple factors induced by tumors. Tumor angiogenesis is the result of the combined action of multiple factors in the tumor-induced body, mainly promoters and inhibitors, which are in a balanced state under normal conditions. Among the various promoting factors, vascular endothelial VEGF is an important regulator of angiogenesis, and its receptors (VEGFR) are mainly VEGFR-1, VEGFR-2 and VEGFR-33. It has been suggested that inhibition of angiogenesis is a powerful strategy for cancer treatment, and when one of these factors is affected, angiogenesis will not occur properly. induced proliferation, migration, invasion and duct formation at sub-toxic doses. In addition, HDT significantly inhibited the in vivo production of villi allantoic membranes without showing cytotoxicity. Furthermore, HDT reduced not only VEGFR2 signaling in HUVECs but also hypoxia-inducible factor (HIF)-1 expression in hepatocellular carcinoma. The currently investigated antitumor peptide drugs in inhibiting mechanism of angiogenesis is a hot topic of research for most scholars. NT4 was shown to have important effects on endothelial cell proliferation, migration, and tube formation, especially when induced by FGF2 and coagulation. when induced by FGF2 and thrombin. In addition, NT4 has an important role in the migration and invasion of aggressive tumor cells. Therefore, the anti-angiogenic mechanism of anti-tumor peptides provides clues for their development as tumor-targeting drugs. The anti-angiogenic mechanism of antitumor peptides thus provides clues for their development as tumor-targeting agents.

EGFR is a transmembrane receptor that, when bound to a ligand, phosphorylates and binds some intracellular adapter molecules or forms homo- or heterodimers with other receptors, thereby activating a series of downstream signaling pathways that lead to cell proliferation, apoptosis, invasion, and metastasis. Several solid tumors are known to occur in association with aberrant activation of EGFR in tumor tissue. Gefitinib competitively binds to the Mg-ATP site in the EGFR-TK catalytic region on the cell surface, blocking intracellular signaling, thereby inhibiting cell proliferation and metastasis and producing an anti-tumor effect. This inhibits cell proliferation and metastasis, resulting in anti-tumor effects. The drug was launched in February 2005 for the treatment of locally advanced or metastatic non-small cell lung cancer in patients who have received prior chemotherapy or are unsuitable for chemotherapy. It has also been shown to inhibit microangiogenesis, modulate the cell cycle and increase chemotherapy sensitivity, and in some areas to potentiate the antitumor effects of cisplatin, carboplatin, platinum oxalate, adriamycin, topotecan, ralitrexed, paclitaxel, paclitaxel ester, glucosamine, and interferon.

Alkaloid is a naturally occurring basic compound that contains a nitrogen atom. Some compounds that are chemically synthesized but structurally similar to alkaloids are sometimes referred to as alkaloids. In addition to C, H, and N, alkaloids can also contain O, S, or other elements such as chlorine, bromine, and phosphorus. Alkaloids are mostly derivatives of amino acids and taste bitter and astringent. They are often found as secondary metabolites in plants, animals, and mushrooms. Most of the alkaloids can be obtained from their plant extracts by acid-base extraction. Among the plant derivatives with biological properties, berberine, an isoquinoline quaternary alkaloid isolated mainly from Huanglian, has a wide range of therapeutic effects on a variety of diseases. In recent years, berberine has been reported to inhibit cell proliferation and be cytotoxic to cancer cells. Therefore, many derivatives have been synthesized to improve the efficiency and selectivity of berberine.(Ortiz, Lombardi, Tillhon, Scovassi 2014). In this study, we tested the inhibitory activity of berberine on angiogenesis in cell-based experiments and in a mouse xenograft model of human glioblastoma, and clarified the involvement of the VEGFR2/ERK pathway (Jin, Xie, Huang & Zhao 2018).

2 METHOD AND MATERIALS

I predict that Berberine inhibits angiogenesis in glioblastoma xenografts by targeting the VEGFR2/ERK pathway. I will treat glioblastoma

xenograft mice will increasing amounts of injected Berberine (0, 0.1, 0.3, 1 mg/ml, 10 mg/ml) for 0 min, 1 day, 7 days, 2 weeks and then measure tumor weight and size, and perform confocal microscopy/ immunohistochemistry (IHC) for VEGFR2, or phospho-ERK and unphosphorylate ERK. Also measure VEGFR2, phosphorylate ERK, unphosphorylate ERK by western blot. Positive control is Temozolomide treatment.

① Reagents

Berberine: Berberine powder was dissolved in phosphate buffered saline (PBS), then sterilized using a 0.22 μm pore filter and stored at 4 °C until use. Temozolomide: Compounds were resuspended in DMSO and stored at room temperature (O6BG) or -20°C (all others).

② Immunocytochemistry

Immunocytochemistry of VEGFR2, or phospho-ERK and unphospho-ERK were performed as described previously.

3 POSSIBLE RESULT

Possible Result 1: Berberine was applied to inhibit the VGEFR2/ERK pathway in defined human and murine glioblastoma cell lines, cell lines from clinical samples and cell lines from in vivo animal models. (Luan, 2020)

Berberine inhibits glioblastoma in all of the *in vitro* and *in vivo* cell samples, decreasing VEGF-C or VEGF-D binding to VEGFR2, and decreasing activity of ERK outside the cells, as shown in table 1. The proliferations of cell samples are inhibited significantly. The animal experiments display that berberine has therapeutic effect on angiogenesis of glioblastoma cells, as shown in table 2. In the simulation experiment, to investigate the possible molecular mechanism of berberine-induced inhibition of angiogenesis, this experiment will analyze the protein expression of VEGFR2 and MAPK pathways by Western blots. The total expression of VEGFR2 will not change after berberine treatment, while the phosphorylation of VEGFR2 will be significantly reduced ($p < 0.001$, Figure 1). Likewise, phosphorylation of ERK and p38 will also reduce after berberine treatment ($p < 0.001$ and $p < 0.01$, respectively, Figure 1). (Jin, Xie, Huang & Zhao 2018)
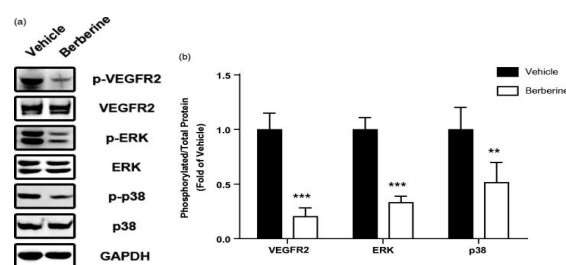

Figure 1: Molecular mechanisms involved in antiangiogenic effect of berberine. Tumor tissue from ectopic xenograft model was isolated and homogenized for Western blot analysis. ***$p < 0.001$ vs. vehicle group, **$p < 0.01$ vs. vehicle group. $N = 6$ for each group (Jin, Xie, Huang & Zhao 2018) .

Possible Result 2: Berberine was applied to inhibit the VGEFR2/ERK pathway in defined human and murine glioblastoma cell lines, cell lines from clinical samples, but not in cell lines from in vivo animal models. (Wang, Zhou, Xu, Song, Qian, Lv, Luan 2019)

Berberine inhibits glioblastoma in all of the *in vitro* cell samples, decreasing VEGF-C or VEGF-D binding to VEGFR2, and decreasing activity of ERK outside the cells, as shown in table 1. The proliferations of *in vitro* cell samples are inhibited significantly. However, the berberine does not successfully decrease *in vivo* VEGF-C or VEGF-D binding to VEGFR2 or decreasing activity of ERK outside the cells, or the animal experiments do not display a significant therapeutic effect of berberine inhibits VEGFR2/ERK pathway of angiogenesis, as shown in table 2.

Possible Result 3: Berberine was applied to inhibit the VGEFR2/ERK pathway in identified human and murine glioblastoma cell lines, but not cell lines derived from clinical samples.

Berberine inhibits glioblastoma in CUTLL1, HPBALL, NS2, and W44, as shown in table 1. The proliferations of these *in vitro* cell samples are inhibited significantly. However, the berberine does not successfully decrease VEGF-C or VEGF-D binding to VEGFR2, or decrease activity of ERK outside the cells in the clinical samples. The animal experiment involving the NOD-CID model will fail as described in Possible Result 2 since this model has glioblastoma cells from clinical samples, as shown in table 2.

Possible Result 4: Application of berberine inhibited the VGEFR2/ERK pathway in defined human cell lines and clinical samples, but had no effect on murine cell lines.

Berberine inhibits glioblastoma in CUTLL1, HPBALL, and the majority of the clinical samples

and reduces VEGF-C or VEGF-D binding to VEGFR2 and decrease activity of ERK outside the cells, as shown in table 1. The proliferations of these *in vitro* cell samples are inhibited significantly. However, applying berberine to NS2, and W44 does not successfully decrease the reduces VEGF-C or VEGF-D binding to VEGFR2 and decrease activity of ERK outside the cells. Since the animal model is constructed using human cell lines, there are still possibility that berberine will decrease *in vivo* VEGF-C or VEGF-D binding to VEGFR2 and decrease activity of ERK outside the cells in animal experiments, as shown in table 2.

Possible Result 5: Application of berberine did not inhibit the VGEFR2/ERK pathway in any cell line.

The berberine does not significantly reduce VEGF-C or VEGF-D binding to VEGFR2 and decrease activity of ERK outside the cells in any cell lines, as shown in table 1. The animal experiment will not be successfully conducted in this scenario, as shown in table 2. Additional Possible Results on VEGFR2/ERK Pathway Different from Previous Researches

Possible Results 6: Berberine inhibits the VGEFR2/ERK pathway, but does not have effects on angiogenesis levels. (Luan 2020)

The level of angiogenesis of glioblastoma determined by VGEFR2/ERK pathway is low. The cell proliferation decreases. However, the level of angiogenesis of tumors does not change significantly, as shown in table 1 and table 2.

Table 1: Possible Results.

Cell Line	Result I	Result II	Result III	Result IV	Result V	Result VI
Tumor size decreases with Increasing berberine	+	-	-	-	No inhibition(+)	Negative inhibition(-)
VEGFR WB level	+	-	+	-	No reduction(-)	No reduction(-)
Phosphor-AKT decrease	+	+/-	+/-	-	Partly agree	-

Note. "+" represents a significant decrease in cell proliferations. "-" represent not significantly different from negative control.

Table 2: Possible Results on Cell Proliferation.

Cell Line	Result I	Result II	Result III	Result IV	Result V	Result VI
VEFG/HIF-1 expression	+	+	+	+	+	+
Angiogenesis of HUVECs	+	+	+	+	-	-
Tumor growth in vivo	+	+	-	-	-	-
PI3K pathway	Y	N	Y	N	Y	N

Note. "+" represents a significant decrease in cell proliferations. "-" represent not significantly different from negative control.

4 CONCLUSION

The Possible Results 1 are consistent with previous studies on the effect of berberine on glioblastoma angiogenesis. To gain insight into the structure and function of berberine, the specific gene regulatory mechanisms of berberine should be further investigated. The relationship between berberine and RNA interference with VGEFR should also be

studied to investigate more specific pathways that can be used to treat glioblastoma angiogenesis. Preclinical testing on more complex and representative animal models should also be conducted before transitioning to clinical testing of berberine treatment. Better delivery platforms, such as nanodrugs or mechanisms involving endocytosis, should also be applied in order to improve this therapeutic approach.

What makes the failure of the experiments in vivo in Possible Result 2 is most likely due to unsuccessful delivery of berberine: either the cells in the animal did not take up berberine or berberine was not maintained in vivo long enough to perform its function. The final result would indicate a high expression level of berberine and a low expression level of berberine. In order to improve this experiment, an efficient and reliable delivery method should be developed. The experiment can be repeated again with the traditional retroviral infection method as it has proven successful in previous experiments. The safety level of retroviral gene therapy should be improved prior to clinical trials. In addition, pharmacological angiogenic inhibitors of glioblastoma could be developed for berberine.

Only if the berberine reduces the activity of angiogenesis in the tumor, the berberine treatment targeting the VGEFR2/BRK pathway will potentially have therapeutic effects and should be carried on to clinical trial. Possible Result 3 suggests that berberine does not qualify as a universal treatment for glioblastoma because a subset of clinical samples do not have abnormal gap expression or because they have different types of gaps and angiogenic mechanisms. This requires future studies to reassess the relationship between berberine, the VGEFR2/ERK pathway, and the general type of glioblastoma.

The unlike Possible Results 4 and 5 indicate potential systematic errors in the experimental designs. Possible Result 4 indicate the berberine used in this experiment cannot be applied on mice. The redesign of potential drug will be required for future studies on animal cell lines. Possible Results 5 is likely to be caused by the offtarget of the berberine used in this experiment.

The Possible Results 6 contradicts with the current understanding of berberine's effects on VGEFR/ERK pathway. Result 6 indicates that an alternative pathway that is crucial for the angiogenesis of glioblastoma maintenance. Future studies should use experiments like dual-luciferase reporter assay to verify the relationship between berberine and other potential glioblastoma oncogenes.

In conclusion, white tyrosine kinases occupy a very important position in the cell signaling pathway, regulating a series of physiological and biochemical processes such as cell growth, differentiation and death. More than 50% of proto-oncogenes and oncogenes are tyrosine kinases, and their abnormal expression usually leads to disruption of cell proliferation regulation, resulting in tumorigenesis. More than 20 different families of receptor and non-receptor tyrosine kinases have been used as targets for antitumor drug screening, including epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), insulin receptor (InsR), Src , Abl, etc. (representative drugs: Imatinib, Gefitinib, Erlotinib, Exatinib, Sorafenib, Sunitinib, Crizotinib).

Recently, another challenging concept has been proposed for molecularly targeted antitumor drug therapy: the strategy of mul-tipletargeted tyrosine kinase inhibition. Based on the complexity of tumor development, the vast majority of tumors do not rely on a single signaling pathway to maintain their growth and survival; there are crossovers and compensations between signaling pathways. Multi-targeted drugs can achieve the dual function of synergistic treatment and overcoming drug resistance by inhibiting multiple signaling pathways or multiple molecules upstream and downstream in one pathway (representative drugs: lapatinib, afatinib, daclatinib, axitinib, ceritinib, etc.).

Mechanism of tumor neovascularization inhibition: Targeting VEGFR, FGFR, EGFR and other receptor tyrosine kinase inhibitors with tumor neovascularization-promoting effects represents another important direction in antitumor targeted drug research - inhibition of tumor neovascularization. Blocking tumor neovascularization to varying degrees can slow down the growth of solid tumor tissue (representative drugs: bevacizumab, sorafenib, sunitinib).

Stimulated by cancer cell growth, VEGF binds to specific endothelial cell receptors, leading to angiogenesis and the production of new blood vessels to feed tumor tissue. VEGF inhibitors destroy tumor tissue by blocking this process. Everolimus and pazopanib are used clinically in the treatment of renal cell carcinoma, and bevacizumab, which has the structure of a human-derived antibody structural region and the complementary decision region of a murine-derived monoclonal antibody that binds VEGF, is used in the treatment of non-small cell lung

cancer. Due to the tolerability of this class of drugs, tumor recurrence and metastasis can occur after discontinuation of the drug, rendering the treatment ineffective. Adverse effects include increased blood pressure, delayed wound healing, bleeding, thrombosis, intestinal perforation, heart failure, and heart disease. Some of these drugs currently interfere with the regulation of the activity of other cellular pathways. The mechanism of action is not well understood.

In addition, HER-2 has a transmembrane tyrosine kinase receptor, and its positive expression is closely related to tumor cell development, progression and prognosis, of which only the intracellular ligand-binding region has tyrosine kinase activity. Her-2 oncogene amplification causes receptor overexpression, activates the intracellular region and phosphorylates at the tyrosine kinase site, activates the downstream PI3K/Akt and MAPK pathways, and regulates tumor cell proliferation, differentiation, migration and apoptosis. Trastuzumab is a humanized monoclonal antibody that binds to the Her-2 oncogene expression product P185 protein on tumor cell membranes to produce anti-cancer effects. Currently, it is mainly used for the treatment of lung cancer, gastric cancer, breast cancer, ovarian cancer and kidney cancer, etc. It has a wide spectrum of anti-tumor, high efficiency and low toxicity. Due to the emergence of tumor cell drug resistance phenomenon, with the investigation of its anti-tumor and tumor drug resistance reversal mechanism, the screening of drug-resistant target markers will help to develop combination drug regimens and provide a new way out for Her-2-positive tumor patients.

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